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# Enamelin/ameloblastin gene polymorphisms in autosomal amelogenesis imperfecta among Syrian families

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## Abstract

**Aim:** This study was undertaken to investigate whether a single G deletion within a series of seven G residues (codon 196) at the exon 9–intron 9 boundary of the enamelin gene *ENAM* and a tri-nucleotide deletion at codon 180 in exon 7 (GGA *vs* deletion) of ameloblastin gene *AMBN* could have a role in autosomal amelogenesis imperfecta among affected Syrian families.

**Methods:** A new technique – size-dependent, deletion screening – was developed to detect nucleotide deletion in *ENAM* and *AMBN* genes. Twelve Syrian families with autosomal-dominant or -recessive amelogenesis imperfecta were included.

**Results:** A homozygous/heterozygous mutation in the *ENAM* gene (152/152, 152/153) was identified in affected members of three families with autosomal-dominant amelogenesis imperfecta and one family with autosomal-recessive amelogenesis imperfecta. A heterozygous mutation (222/225) in the *AMBN* gene was identified. However, no disease causing mutations was found. The present findings provide useful information for the implication of *ENAM* gene polymorphism in autosomal-dominant/-recessive amelogenesis imperfecta.

**Conclusion:** Further investigations are required to identify other genes responsible for the various clinical phenotypes.

## Introduction

Amelogenesis imperfecta (AI) is a heterogeneous group of genetic disorders characterized by defects in tooth enamel formation in the absence of any generalized or systemic diseases.[1] AI has four main subtypes, described as hypoplastic, hypocalcified, hypomaturation and hypomaturation–hypoplastic with taurodontism.[2]

Studies presenting the epidemiology of AI have reported prevalence[3] varying from 1:700 in north Sweden to 1:12 000–14 000 in the USA.[3,4] The autosomal-dominant AI is most prevalent in the USA and Europe, while the autosomal-recessive AI is more frequent in Asia.[5]

The most crucial features associated with AI are the requirement for lifelong, complex, restorative care, and consequently, a greatly negative, psychosocial impact.[6]

Clinically, the enamel in autosomal-dominant AI (Online Mendelian Inheritance in Man [OMIM] 104500) is either hypoplastic in both primary and permanent teeth;[7] hypocalcified, with generalized, yellow–brown discoloration of the teeth;[8] or hypomineralized in both dentitions in which enamel is easily abraded, leaving an irregular surface. The teeth are softer than normal with a creamy yellow-to-white appearance.[9,10]

A wide diversity of autosomal-recessive phenotypes (OMIM 204650, 204700), including localized hypoplastic;

generalized, thin hypoplastic; hypocalcified; and hypomaturation types of AI have also been identified, and the nomenclature has been revised.[11]

Recent studies have demonstrated that enamel formation requires the expression of multiple genes that transcribe matrix proteins and proteinases necessary to control crystal growth and mineralization of forming enamel.[8] Currently, there are seven candidate genes for AI, including amelogenin, enamelin, ameloblastin, tuftelin, distal-less homeobox 3, enamelysin (matrix metalloproteinase-20), and kallikrein.[7] Amelogenin accounts for more than 90% of total enamel protein, while ameloblastin and enamelin account for approximately 5% and 2% of total protein,[4] respectively.

It has also been found that different AI phenotypes reflect the gene affected and the specific type of mutation.[1] For instance, linkage studies have mapped a local hypoplastic, autosomal-dominant AI locus to chromosome 4q11–q21 regions (OMIM 104500), where two candidate genes, enamelin[12] and ameloblastin, were identified.[13] Subsequently, a mutation in the enamelin and ameloblastin genes has been investigated in one English family,[14] and later in a Japanese family with an autosomal-dominant, hypoplastic form of AI.[1] A single G deletion within a series of seven G residues (codon 196) at the exon 9–intron 9 boundary of the enamelin gene has been identified.[1,14] More recently, a novel, dose-dependent *ENAM* gene mutation has been described, with generalized, hypoplastic AI segregating as a recessive trait, and localized enamel pitting segregating as a dominant trait.[15]

Previous work has investigated a tri-nucleotide deletion at codon 180 in exon 7 (GGA *vs* deletion) of the ameloblastin gene in Japanese patients.[16] However, no mutation in the ameloblastin gene has yet been identified in humans.[7]

To date, no studies have examined the genetic risk factors of autosomal AI among Syrian families. Therefore, this study was undertaken to assess the role of enamelin/ameloblastin gene polymorphisms in autosomal AI in Syrian children. A single G deletion within a series of seven G residues (codon 196) at the exon 9–intron 9 boundary of the *ENAM* gene and a tri-nucleotide deletion at codon 180 in exon 7 (GGA *vs* deletion) of the *AMBN* gene were investigated in Syrian families with autosomal AI. In addition, this study was based on a relatively large number of families, unlike most previous investigations, which have been focused on a small number of individuals.

## Materials and methods

This study was a family, case–control study, based on a group of children with AI (probands) and an appropriate group of controls collected from the family members of the affected children. Evidence of AI was diagnosed clinically on the Enamel Defect Index (EDI)[17] and a radiographic assessment. Controls were those who had a clinically healthy dentition and an EDI score of 0. All probands with AI who attended the Pediatric Dentistry Clinic at Damascus University, Dental Hospital, Damascus, Syria, were included in the study. Unaffected family members were used as controls. All healthy children with AI who had erupted permanent incisors and four first permanent molars present were included. Those with a systemic or syndromic condition (ectodermal dysplasia), or those having orthodontic treatment were excluded.

Ethical approval was granted by the University of Damascus, Damascus, Syria. Informed consent was obtained from parents. A further assessment was undertaken on affected individuals to the skin, hair, and fingernails to exclude associated syndromic conditions associated with AI (ectodermal dysplasia, tricho-dento-osseous syndrome).

### Collecting genetic material

*DNA extraction*

Buccal epithelial cells were collected from the cheeks and placed immediately in individual containers containing 2% sucrose. These tubes were labeled and sent for DNA analysis at the Transplantation Laboratory, Manchester Royal Infirmary, Manchester, UK. DNA extraction was performed with the EZ1 DNA tissue kit (QIAGEN, GmbH, Hilden, Germany) and a Qiagen Bio ROBOT EZ1 instrument (Magtration System 6GC; Qiagen, Germany).

*Molecular typing*

A single G deletion within a series of seven G residues (codon 196) at the exon 9–intron 9 boundary of the *ENAM* gene was determined. A tri-nucleotide deletion at codon 180 in exon 7 (GGA *vs* deletion) in the *AMBN* gene was also investigated. A total of 100 ng genomic DNA was amplified in a 20-$\mu$L final volume of polymerase chain reaction mixture containing polymerase chain reaction (PCR) grade 1× KCl buffer with an optimized $MgCl_2$ concentration, 100 $\mu$M dNTP, 6 pmol primers, 1 U *Taq* polymerase (Bioline London, UK).

Enamelin (*ENAM* gene) amplifications were performed using the forward primer (VIC-5′-CGA ACGTGG TTT TCT CCT GT-3′) and reverse primer (5′-AGG GGC GAA TGG ATT GTA A-3′). In the presence of a single G deletion, these primer sets should produce a 152-bp size product. Ameloblastin (*AMBN* gene) was also amplified by using the forward primer (FAM-5′-TTG GGT CAT ACC TCC CAA AA-3′) and reverse primer (5′-GAT GGA CCT TGT GGA TCA GC-3′). In the presence of tri-nucleotide GGA deletions, these primer sets should produce a

 

222-bp size product. The PCR reaction was carried out using GeneAmp PCR System 9700 (Applied Biosystems UK, Cheshire, UK) at the relevant annealing temperature for 35 cycles.

*Size-dependent, deletion screening*

The size-dependent, deletion screening (SDDS) technique was developed as a new method for the detection of nucleotide deletion at the Manchester Royal Infirmary Transplantation Laboratory, which uses SDDS to detect the length of fragments and distinguish between those with more than two base difference.

In this study, the amplification of the *ENAM* gene produced two fragments of 152 bp and 153 bp (with one base length difference) as a single G deletion within a series of seven G residues (codon 196) at the exon 9–intron 9 boundary. This was also the case for *AMBN*-amplified products (222 bp and 225 bp), but with a tri-nucleotide deletion at codon 180 in exon 7. A total of 10 $\mu$L fresh formamide and 0.5 $\mu$L ABI 3130 molecular size standard (ROX500; Applied Biosystems, Foster City, CA, USA) were added to 1 $\mu$L amplified *ENAM* and *AMBN* products, with fluorescent tagging, into an ABI 3130 PCR 96-well plate. The product was then denaturized at 95°C for 2 min. Data were analyzed by an ABI GeneMapper V3.7 based on fragment length differences (Applied Biosystems).

*DNA sequencing using the ABI 3130 data collection genetic analyzer*

DNA sequencing confirmed the findings by utilizing specific primers for the *AMBN* and *ENAM* genes by using the Big Dye Terminator Mix (V3.1; ABI 3130, Applied Biosystems).

## Results

Fifty patients from 12 families with AI were included in the study, 19 affected and 31 unaffected. Nine of the families segregated AI as an autosomal-recessive trait and three of the families segregated AI as an autosomal-dominant trait. A mutation in the *ENAM* gene was found in all (3/3) Syrian families with autosomal-dominant AI and in one (1/9) with autosomal-recessive AI. The pedigrees of these four families (with *ENAM* gene mutation) are presented in Figure 1. The clinical phenotypes of the affected patients in these four families are shown in Figures 2–5.

### Family 1

The affected probands of family 1 (Figure 2) showed hypocalcified enamel in both primary and permanent teeth.

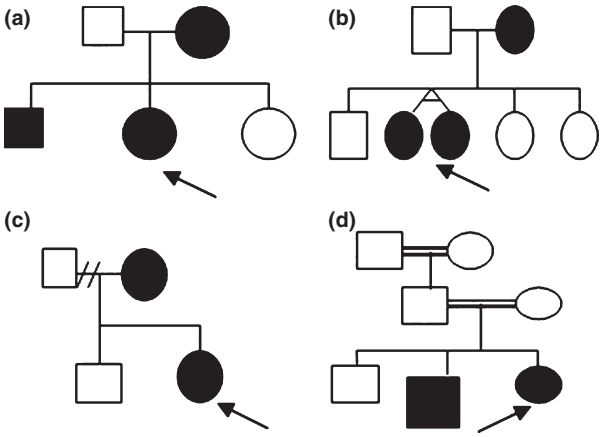


**Figure 1.** Pedigrees of the four families with amelogenesis imperfecta. Proband is indicated by arrow. (a) Family 1 (autosomal dominant). Enamelin: proband, 152; control: 153. Ameloblastin: proband: 222/225; control: 225. (b) Family 2 (autosomal dominant). Enamelin: proband, 152 or 152/153; control: 153. Ameloblastin: proband: 225; control: 225. (c) Family 3 (autosomal dominant). Enamelin: proband, 152; control: 153. Ameloblastin: proband: 222/225; control: 225. (d) Family 4 (autosomal dominant). Enamelin: proband, 152 or 152/153; control: 153. Ameloblastin: proband: 225; control: 225.

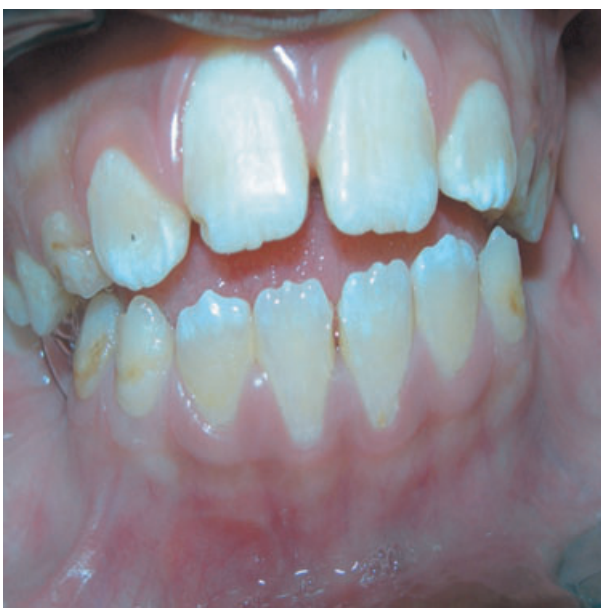

**Figure 2.** Proband in family 1 shows chalky, white enamel with generalized, hypocalcified amelogenesis imperfecta affecting primary and permanent teeth.

The findings are consistent with a diagnosis of autosomal-dominant, generalized, hypocalcified AI. In order to exclude fluorosis, a detailed history was taken, but it did not reveal excessive fluoride intake either in terms of eating

 

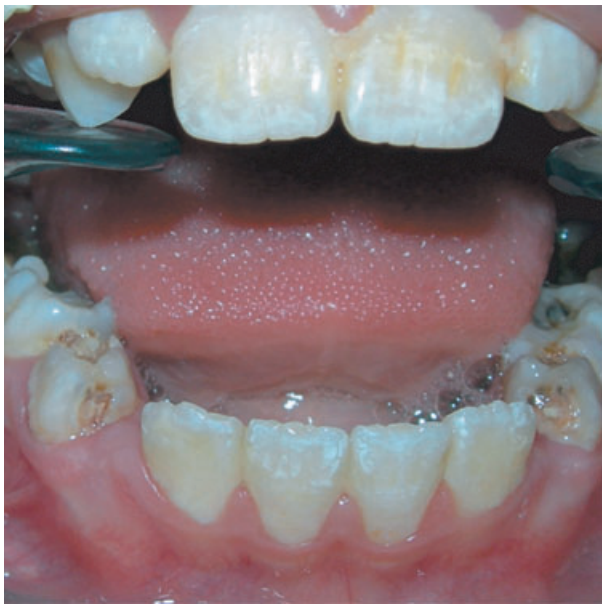

**Figure 3.** Proband in family 2 shows chalky, white enamel in a child with generalized, hypocalcified amelogenesis imperfecta affecting primary and permanent teeth.

toothpaste in childhood or in the local water supply. The proband's mother was affected, but the father and two siblings were unaffected.

### Family 2

A clinical examination of the affected twins of family 2 (Figure 3) showed a similar picture to that observed in family 1. The enamel of the buccal surfaces of the permanent teeth was chalky and hypocalcified with normal thickness. The proband's mother was affected; however, the father and three siblings were unaffected.

### Family 3

The proband of family 3 (Figure 4) showed gingival hyperplasia with rough hypoplastic, generalized, autosomal-dominant AI. The enamel was rough and had a yellowish appearance. The mother had horizontal stain lines on the posterior teeth. The upper anteriors had been crowned because of their appearance and had problems with sensitivity. The father and one sibling were unaffected.

### Family 4

Parental consanguinity was identified in family 4 (Figure 5) and intermarriage within the family was a common occurrence. The mother reported that a younger

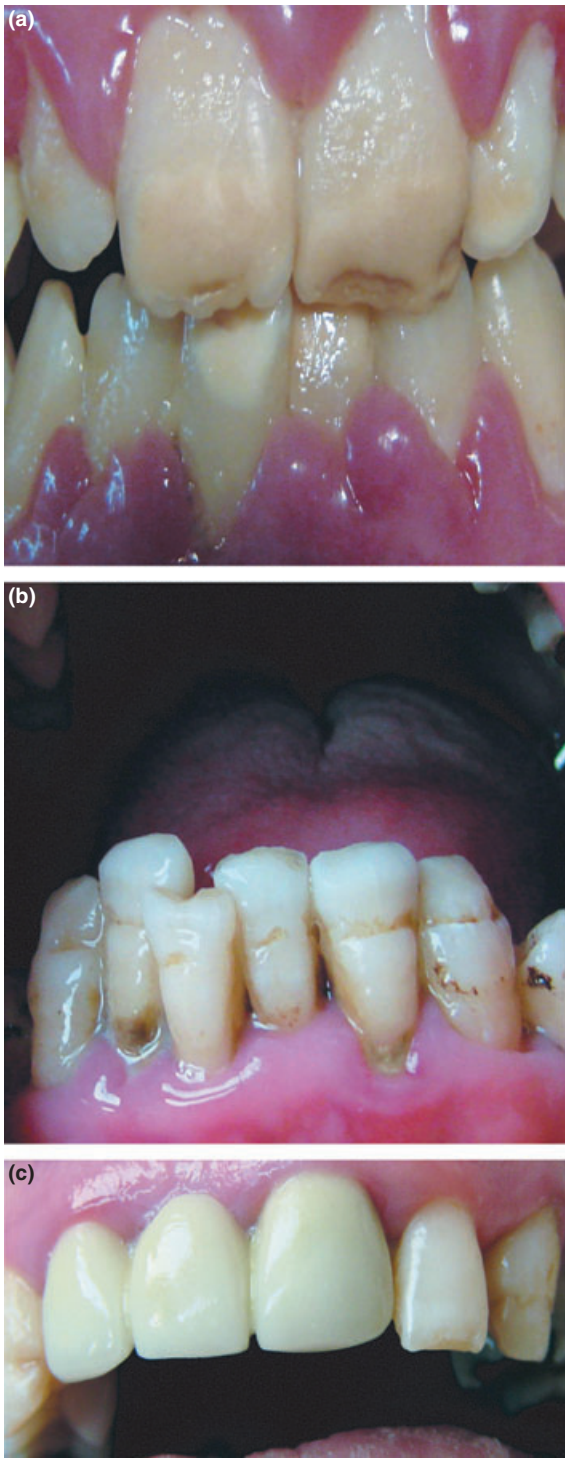


**Figure 4.** (a) Proband in family 3 (10-year-old child) shows generalized gingival hyperplasia, yellowish discoloration, and a lack of translucent enamel with rough hypoplastic, generalized, autosomal-dominant amelogenesis imperfecta (AI). (b,c) mother's teeth: local hypoplastic AI, horizontal lines in mid-third of the crowns. Mother had hypoplastic AI and received crowns placed on maxillary anterior teeth (c).

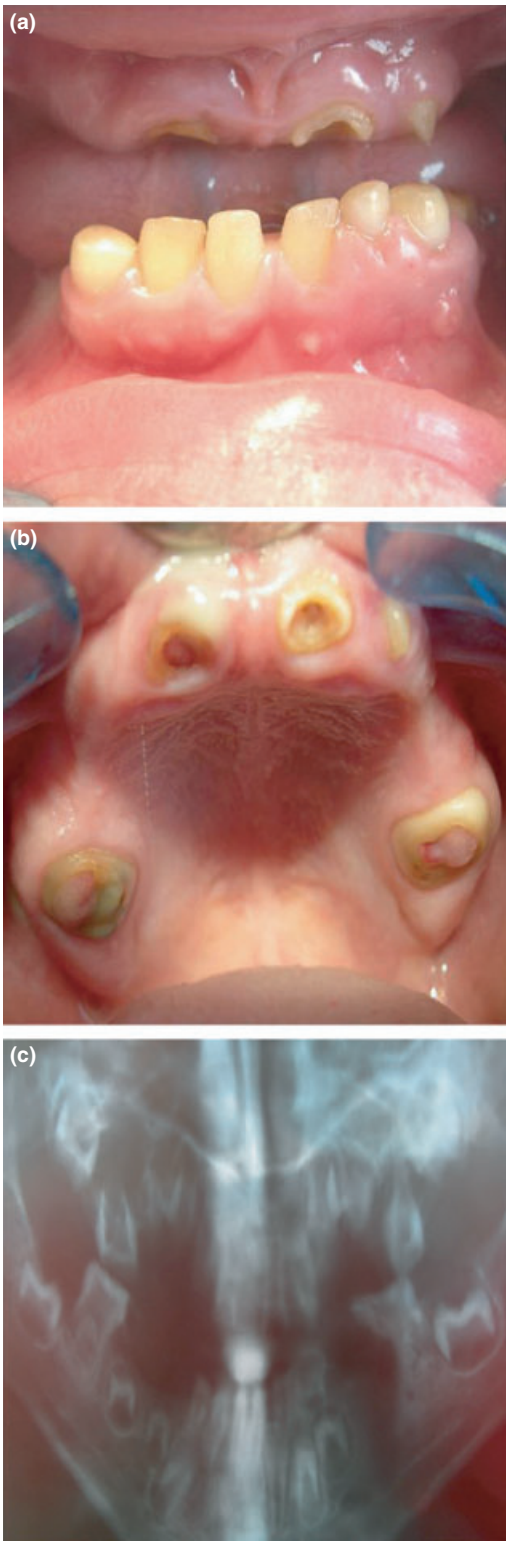


**Figure 5.** (a,b) Proband in family 4 (9-year-old child) shows thin, smooth, generalized, hypoplastic, autosomal-recessive amelogenesis imperfecta. (c) Dental panoramic radiograph showing enlarged pulps, short roots, lack of enamel on erupted and unerupted teeth, and bone loss.

male child, 2 years of age, had yellow-looking tooth. However, the proband's parents, one sibling, and paternal grandparents were unaffected.

The affected child presented with smooth, generalized, hypoplastic AI, with only a thin layer of dentin covering the pulpal chambers. Hyperplastic pulpitis was observed in all the permanent molars, and the vertical dimension was decreased.

A panoramic radiograph showed enlarged pulps; short roots; a large lesion in the left lower side, which disturbed the eruption of adjacent premolars and molars; abnormalities in teeth eruption; and generalized bone loss. The mother reported that the appearance of the primary teeth had been similar to that of the permanent dentition. These findings are consistent with smooth, generalized, hypoplastic, autosomal-recessive AI.

*ENAM* mutation results

A mutation in the *ENAM* gene was found in three Syrian families with autosomal-dominant AI and one with autosomal-recessive AI. A single G deletion at the exon 9–intron 9 boundary of the enamelin gene, which produced a 152-bp size product, was detected in probands with AI. AI-affected members in families 1 and 3 were homozygous for the mutation (152) Monozygotic twins of family 2 and probands of family 4 were determined to be heterozygous carriers of this mutation (152/153).

*AMBN* mutation results

A heterozygous mutation (222/225) of the ameloblastin gene was observed among these Syrian families; however, no mutations were found in the ameloblastin gene, as no difference was noted between affected and non-affected individuals in the distribution of ameloblastin gene polymorphism.

## Discussion

*ENAM* and *AMBN* gene mutations were investigated in three Syrian families with autosomal-dominant AI and nine families with autosomal-recessive AI. A new technique, SDDS, was utilized to detect a single G deletion within a series of seven G residues (codon 196) at the exon 9–intron 9 boundary of the *ENAM* gene and to detect a tri-nucleotide deletion at codon 180 in exon 7 (GGA *vs* deletion) in the *AMBN* gene. According to the sequencing results, we noted that the SDDS technique is a reliable way to detect two or more deletion points rather than one-point deletions.

A single G deletion within a series of seven G residues (codon 196) at the exon 9–intron 9 boundary of the *ENAM* gene was observed in three Syrian probands with autosomal-dominant AI and in one with autosomal-recessive

 

AI. However, no disease causing mutations has been found in the *AMBN* gene.

Previous linkage and mutational studies have implicated the *ENAM* gene, rather than the *AMBN* gene, in autosomal-dominant AI.[14,15] This was noted in English,[14] Japanese,[1] and Australian families.[5] The first reported human enamelin mutation was a splice donor site mutation after enamelin codon 178 (g.6395G>A), which resulted in a severe form of thin and smooth, hypoplastic AI in one English family.[14] A milder form (g.2382A>T) of AI was shown to be caused by translation termination at enamelin codon 53.[18] This milder form of AI, known clinically as autosomal-dominant, local hypoplastic AI, accounts for 27% of the autosomally inherited cases in northern Sweden. Recently, a splice donor site mutation after enamelin codon 196 (g.8344delG) was shown to cause autosomal-dominant, hypoplastic AI in a Japanese family with AI.[1] There are normally six G at the end of coding exon 7, which are followed by a seventh G at the beginning of the adjacent intron. One of these G was deleted.[1]

Interestingly, in the present study, a novel mutation in the *ENAM* gene was also identified in generalized, hypoplastic, autosomal-recessive AI, rather than in autosomal-dominant AI only.

Hart and Hart recently reported the presence of allelic *ENAM* mutations in both autosomal-dominant and -recessive, hypoplastic AI.[19]

Hart *et al.* were the first to report a novel, dose-dependent mutation of the *ENAM* gene that caused autosomal-recessive AI. They described a novel 2-bp insertion mutation that resulted in a premature stop codon in exon 10 (g.13185_131186insAG).[20] This resulted in generalized, hypoplastic AI and an open bite in homozygous individuals for the mutation and localized pitted enamel in heterozygous carriers in one Turkish family. Additionally, the same mutation in a Turkish family and another novel-insertion *ENAM* mutation (g.12946-12947insAGTCAGTCCAGTACTGTGTC) has also been identified.[15]

This new, novel *ENAM* mutation has also been introduced as a dose-dependent mutation presenting with generalized, hypoplastic AI segregating as a recessive trait and a localized enamel pitting segregating as a dominant trait.[16]

Ameloblastin is an important non-amelogenin protein that is present in the enamel prism sheath and is expressed by enamel-forming ameloblast cells. It is considered to have a critical role in the growth of enamel crystals and determination of the prismatic structure.[16] We also investigated a tri-nucleotide deletion at codon 180 in exon 7 (GGA *vs* deletion) in the *AMBN* gene, which resulted in the lack of an amino acid residue. The GGA deletions did not relate to the clinical findings in Syrian families with autosomal-dominant AI. Previous work has detected a tri-nucleotide deletion at codon 180 in exon 7 in Japanese patients. However, its linkage to disease has also not been demonstrated. Although the GGA deletions resulted in the lack of an amino acid residue, it was suggested that exons 8 and 9 could compensate for the decreased function of the ameloblastin protein, in which a mutation in exon 7 is present.[14] This might well explain our findings that no disease causing mutation was apparent in ameloblastin in Syrian families.

The present findings provide useful information on the *ENAM* gene mutation in autosomal-dominant/-recessive AI. However, it should be noted that the phenotypic characteristics of the four families with autosomal-dominant/-recessive AI showed great diversity in their clinical presentation of AI. A recent discovery in 2008 has confirmed the importance of FAM83H, which is a cellular protein expressed during pre-secretory and secretory ameloblastic stages. FAM83H mutations (OMIM 130900) have been implicated in autosomal-dominant, hypocalcified AI and appear relatively common in North America.[20–22]

Given the complexity of enamel formation, extensive investigations into other genes that are involved in enamel formation are required to explain the clinical diversity of AI.

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