



## Clinical and molecular analysis of the enamel gene *ENAM* in Colombian families with autosomal dominant amelogenesis imperfecta

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### Abstract

In this study, we analyzed the phenotype, clinical characteristics and presence of mutations in the enamel gene *ENAM* in five Colombian families with autosomal dominant amelogenesis imperfecta (ADAI). 22 individuals (15 affected and seven unaffected) belonging to five Colombian families with ADAI and eight individuals (three affected and five unaffected) belonging to three Colombian families with autosomal recessive amelogenesis imperfecta (ARAI) that served as controls for molecular alterations and inheritance patterns were studied. Clinical, radiographic and genetic evaluations were done in all individuals. Eight exons and three intron-exon boundaries were sequenced for mutation analysis. Two of the five families with ADAI had the hypoplastic phenotype, two had the hypocalcified phenotype and one had the hypomaturative phenotype. Anterior open bite and mandibular retrognathism were the most frequent skeletal abnormalities in the families with ADAI. No mutations were found. These findings suggest that ADAI in these Colombian families was unrelated to previously described mutations in the *ENAM* gene. These results also indicate that other regions not included in this investigation, such as the promoter region, introns and other genes should be considered as potential ADAI candidates.

**Key words:** amelogenesis imperfecta, *ENAM* gene, hypocalcified, hypoplastic, phenotype.

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### Introduction

Amelogenesis imperfecta (AI) is as a group of clinically heterogeneous inherited disorders that affect the quality and quantity of enamel in infant and adult dentitions; there are no systemic manifestations associated with AI (Witkop *et al.*, 1971). Affected individuals have greater dental sensitivity that predisposes them to caries and periodontal problems. These individuals also suffer from esthetic problems that lead to psychological difficulties and low self-esteem. Amelogenesis imperfecta has been classified in four categories based primarily on its phenotype: hypoplastic, hypomaturative, hypocalcified and hypomaturative-hypoplastic with taurodontism (Witkop, 1989); these categories are further subdivided into 14 subtypes based on phenotype and mode of inheritance. To date, six genes have been implicated in non-syndromic forms of AI: *AMELX* (MIM 300391), *ENAM* (MIM 606585), *MMP20* (MIM 604629), *KLK4* (MIM 603767), *FAM83H* (MIM 611927) and *WDR72* (MIM 613214) (Stephanopoulos *et al.*, 2005; Kim *et al.*, 2008; El-Sayed *et al.*, 2009). In syndromic forms

of AI, mutations in the *DLX3* (MIM190320) gene cause tricho-dento-osseous syndrome, an autosomal-dominant disorder characterized by curly hair, enamel hypoplasia and taurodontism (Stephanopoulos *et al.*, 2005; Parry *et al.*, 2009). In addition, mutations in *CNNM4* (MIM 217080) cause the Jalili syndrome characterized by the presence of cone rod dystrophy and AI (El-Sayed *et al.*, 2009).

Autosomal dominant amelogenesis imperfecta (ADAI) is the most common form of AI in the general population and has been associated primarily with a mutation in the gene *ENAM* that codes for the protein enamel (Rajpar *et al.*, 2001). Enamelin has an important role in mineralization during which it initiates the deposition of enamel through the external surface of the distal membrane of the ameloblast. In the absence of enamel the mineralization front fails and there is inadequate formation of enamel crystals leading to the deposition of fragile material that cannot withstand masticatory functions (Fukae *et al.*, 1987b).

The *ENAM* gene is located on chromosome 4q 13.3 and has 10 exons, eight of which are coding (Hu *et al.*, 2000, 2001; Hu and Yamakoshi, 2003). To date, seven exonic and three intronic mutations have been reported in this gene, with most of them being associated with ADAI

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(Kida *et al.*, 2002; Mardh *et al.*, 2002; Hart *et al.*, 2003a,b; Ozdemir *et al.*, 2005; Kim *et al.*, 2005; Gutiérrez *et al.*, 2007; Pavlic *et al.*, 2007; Kang *et al.*, 2009; Chan *et al.*, 2010) (Table 1). Some of these mutations appear to alter the regions associated with the sequence of the 32 kDa cleavage product of the *ENAM* gene and studies *in vitro* have shown that this region is important in the interaction of enamel with amelogenin; enamel interacts cooperatively with amelogenin and can regulate the assembly of amelogenin (Tanabe *et al.*, 1990). Defects in the 32 kDa polypeptide can directly influence amelogenin-enamel interactions and the formation of crystals that in turn affect the enamel phenotype (Tanabe *et al.*, 1990; Uchida *et al.*, 1991).

The hypoplastic phenotype has been reported in most of the families with ADAI, although the severity and location vary considerably, as does the occurrence of anomalies such as dental and skeletal anterior open bite, taurodontism and dental agenesis (Atasu and Eryilmaz, 1987; Pulgar *et al.*, 2001; Rajpar *et al.*, 2001). Understanding the genetic mutations involved in these alterations will be of great value in the management of these patients. Such knowledge should improve the prediction of problems associated with a given type of AI and therefore help in choosing the most appropriate treatment for each case.

The aim of this study was therefore to analyze the phenotype, clinical characteristics and presence of mutations in the *ENAM* gene in five Colombian families with ADAI and to examine the possible correlation between genotype and phenotype. For this, we analyzed eight exons and three intron-exon boundaries in the *ENAM* gene.

## Materials and Methods

This project was approved by the institutional ethics committee of the Pontificia Universidad Javeriana. The investigation was done in full accordance with current ethical principles, including the World Medical Association Declaration of Helsinki (2002).

### Population and sample

The population included five families with ADAI (15 affected individuals and 7 unaffected) and three families with autosomal recessive amelogenesis imperfecta (ARAI; three affected and five unaffected individuals) that served as controls for molecular alterations and inheritance patterns. All subjects were attended at the Faculty of Dentistry of the Pontificia Universidad Javeriana in Bogotá, Colombia.

### Clinical and radiographic analyses

The 30 participants read, understood and signed an informed consent form before undergoing clinical and radiographic examinations. The radiographic analysis included panoramic radiographs that were used to examine the dental anomalies (dental agenesis, taurodontism, root

**Table 1** - Exon and intron mutations reported in the *ENAM* gene.

Location	Mutation	Inheritance pattern	Phenotype	Possible effect of mutation	Reference
Intron 6	g.4806A > C	AD	Hypoplastic	The inclusion of 1615 bp results in the insertion of multiple stop codons before the 3 terminus of exon 6. This inclusion leads to the addition of 8-70 amino acids to the normal protein	Kim <i>et al.</i> (2005)
Intron 8	g.6395 G > A	AD	Hypoplastic soft	Results in the skipping of exon 8 following deletion of the amino acids that code for this exon	Rajpar <i>et al.</i> (2001)
Intron 9	g.8344delG	AD	Hypoplastic soft	Introduction of a premature stop codon alters the reading frame and results in a chimeric protein	Kida <i>et al.</i> (2002), Hart <i>et al.</i> (2003b), Kim <i>et al.</i> (2005), Pavlic <i>et al.</i> (2005)
Exon 5	g.2382 A > T	AD	Hypoplastic localized	The change from a lysine to a stop codon that results in a truncated protein of only 52 amino acids	Mardh <i>et al.</i> (2002)
Exon 9	c. 817 G > T	AD	Hypoplastic generalized rugged	The change from guanine to thymine alters the 32 kDa enamel polypeptide	Gutiérrez <i>et al.</i> (2007)
Exon 10	12663C > A	AD	Hypoplastic localized	Introduces a premature stop codon that produces a truncated protein	Ozdemir <i>et al.</i> (2005)
Exon 10	g.12946-12947 ins AGTCA GTACCAGTACTGTGTC	AD/AR	Hypoplastic generalized (recessive trait) or localized (dominant trait)	The insertion of 21 bp alters the reading frame and adds seven amino acids to produce a protein longer than normal	Ozdemir <i>et al.</i> (2005)
Exon 10	g.13185_13186 ins-AG	AD/AR	Hypoplastic thin generalized (recessive trait) or localized with pits (dominant trait)	A 2 bp insertion results in the introduction of a premature stop codon	Hart <i>et al.</i> (2003a), Pavlic <i>et al.</i> (2005), Chan <i>et al.</i> (2010)
Exon 10	g14917delT	AD	Hypoplastic localized.	Introduces 64 amino acids and a premature stop codon that yield a protein with a shorter C-terminal	Kang <i>et al.</i> (2009)
Exon 10	g.12573C > T; c.647C > T	AD	Hypoplastic localized.	Substitutes leucine for a phosphorylated serine (p-S216L) in the 32 kDa enamel cleavage product	Chan <i>et al.</i> (2010)

AD - autosomal dominant, AR - autosomal recessive.

dilacerations, etc.), as previously reported (Córdoba *et al.*, 2007), as well as x-ray profiles and skull laterals based on which cephalometric measurements were obtained to determine the craniofacial abnormalities in these patients (Table 2). Photographs were also taken to assist in the diagnosis and included images of the facial front, profile, in smile and incisor exposure at rest in affected and unaffected individuals.

**Phenotype and inheritance pattern**

The phenotype of AI was determined by using the classification of Witkop (1989) to determine the pattern of inheritance. Family trees were obtained using the software Cyrillic 2.1.

**DNA extraction and PCR amplification**

Samples of peripheral blood (10 mL) were obtained by venipuncture from all of the study participants. DNA was extracted by the salting out method (Miller *et al.*, 1988). Eight exons and three intron-exon boundaries of the *ENAM* gene were then amplified by the polymerase chain reaction (PCR). Because of its size, exon 10 was divided into nine fragments no larger than 600 bp. The primers used to amplify the *ENAM* gene fragments are shown in Table 3. All of the primers were designed using PRIMER 3 software.

**Sequencing**

The amplified products were purified using a Wizard DNA purification system (Promega) and direct sequencing was done in both directions to establish the presence of mutations. Sequencing was done with an automatic sequencer

**Table 2** - Reference values for measurements used in the cephalometric analysis of individuals with (n = 18) and without (n = 12) AI.

Parameter	Reference values	Source of values
< Convexity	12 ± 4	Legan
Witts PO	-1 ± 2	Legan
SN:PM	32 ± 2°	Steiner
ANB	2.5 ± 1.7	Steiner
PTM - N (II HP)	52.8 ± 4.1	Legan
Ar-PTM	37.1 ± 2.8	Legan
ENA-ENP (II HP)	57.7 ± 2.5	Legan
Co - A	99.8 ± 6.0	Mc Namara
Co - Gn	125 ± 2.0	Mc Namara
Ar - Go	52 ± 4.2	Legan
Go - Pog	83.7 ± 4.6	Legan
A I N	1 ± 2	Mc Namara
N I A (II HP)	0.0 ± 3.7	Legan
Pog - N I	0 ± 4	Mc Namara
N I Pog (II HP)	-4.3 ± 8.5	Legan
1sup: PP	106-112°	Steiner
1sup: NA	22 ± 2°	Steiner
1sup: Na	4 ± 2	Steiner
1sup: SN	103 ± 2°	Steiner
1inf: PM	85 ± 93°	Steiner
1inf: NB	25 ± 2°	Steiner
1inf: NB°	4 ± 2°	Steiner
1 sup A vertical	1-2	Mc Namara
1 inf-Pog	4-5	Mc Namara
ENA - GN	61.3 ± 3.8	Legan
ENA - Me	60.0 ± 2.9	Mc Namara

**Table 3** - Primers used for *ENAM* gene amplification.

Region	Forward primer	Reverse primer	Size (bp)
<b>Exons</b>			
3	TGAAGCTTTGCTATTCATTCA	TGTTTGGCCCTCTCAAGTGT	246
4/5	TTCACAGACAAAAATAAAAAATCAA	AACTTCTCCTCTGAGACTGAACG	292
6	TTAATGGATTCTTTGGTTGC	AAAACATGTGGATTGGTGTCTC	166
7	TGCCTTTTGGTTTGTTTGG	CTCTCCGAGGCCATTTACAG	374
8	TCTTTTCAATACCACATCACTCTGA	TGATGCACTGGTTTTGTTTCA	245
9	CGAACGTGGTTTTCTCCTGT	GGGCGAATGGATTGTAATTT	151
10A	TGGGAAACAAAGGGCAATTA	CCCAAGCAAAGAAGTTCCAC	556
10B	CATGGGGCACAGACAGAATA	GGCATCCTTCTTGACCCTAA	564
10C	AGAATTTGCCCAAAGGGATT	ATCCTCCCTTGGTTTTGATG	599
10D	CCTCAAATCAGCCAAAGGAA	GAGCTACCAGCACAGCAAGA	562
10E	AGAGAGGCTCTTGCTGTGCT	CATGGCAAATGCAGAATGTT	562
10F	GAGAGGCAACAGCAAAGACC	GCAAAAAGGAACTAACTCAGACAA	598
10G	TGTCTGAGTTAGTTTCCTTTTGC	GTCAAGAAAAATTTAGGGTACGAT	600
10H	CCACTCTCCAAATCGTACCC	GGCAAGGGACATCCAGATAA	597
<b>Introns</b>			
6	AGAGGATGGAGACAGCCTGA	TTATGACGTTTGGGTGCTGA	196
8	GGAAATGGGCTATTCCTTA	TGATGCACTGGTTTTGTTTCA	187
9	CGAACGTGGTTTTCTCCTGT	GGGCGAATGGATTGTAATTT	151

(Visible Genetics, version 2.0). Sequences were compared using reference sequence AY 167999 for *ENAM*.

## Results

### Clinical description

This study involved 30 individuals, of which 18 were affected by AI and 12 were unaffected (control group). All individuals were examined for skeletal and dental alterations. Dental agenesis, especially of the premolars, was one of the most common dental anomalies found in the families with ADAI. Pulp calcifications were present in several molars and premolars, as well as hypercementosis. Apical dilacerations were observed in several teeth. Taurodontism was seen in only one patient who was unaffected and belonged to the family with the hypomaturative phenotype.

With regard to the skeletal alterations, most of the affected and unaffected individuals belonging to the five Colombian families with ADAI had a hyperdivergent skeletal pattern and were classified as skeletal Class II. Most of the individuals with AI had a normal maxilla, whereas most of those in the control group had mandibular prognathism. Almost all individuals with ADAI and ARAI, as well as the control group, had a normal upper jaw size. The straight profile was the most frequent. Skeletal Class III, upper and lower micrognathia and an abnormal overbite were also observed, as was the presence of anterior open bite and deep bite in almost half of the affected individuals belonging to families with ADAI (Table 4).

### Phenotype and inheritance pattern

The pattern of inheritance found in 5 families with AI was autosomal dominant while in three of the families was

autosomal recessive. The phenotype in two of the families with the autosomal dominant pattern was of the hypoplastic type (localized in some members and generalized in others) with open interproximal spaces, teeth that varied in color from yellowish to brown, and vertical grooves and pits in the anterior teeth (Figures 1 and 2). Two other families with ADAI showed the hypocalcified phenotype while the remaining family had a hypomaturative phenotype. Figures 3-5 show the phenotype of one individual per family with the autosomal dominant pattern and their respective family trees. All of the families with ARAI had the hypomaturative phenotype (data not shown). Table 5 summarizes the dental and skeletal characteristics, the phenotypes and the genotypes of the five families with ADAI.

### Mutational analysis

Mutational analysis of eight exons and three intron-exon boundaries of the *ENAM* gene in eight families (five autosomal dominant families and three autosomal recessive families, the latter used as controls for molecular alterations and inheritance patterns) revealed no exonic or intronic mutations in any of the families.

## Discussion

Various studies have examined the dental and skeletal alterations associated with AI. Dental abnormalities include altered tooth eruption, dental agenesis, impacted teeth, pulpal calcifications and taurodontism (Atasu and Eryilmaz, 1987; Seow, 1993; Pulgar *et al.*, 2001; Rajpar *et al.*, 2001). In a previous study, we observed that only one individual in the five Colombian families with ADAI examined had taurodontism (Cordoba *et al.*, 2007). Pulp calcification was observed in two members of families with hypoplastic AI but not in families with other phenotypes.

**Table 4** - Skeletal alterations in patients with and without AI.

Skeletal alterations	Number of individuals showing the alteration	
	Individuals with AI	Individuals without AI
Hyperdivergency	14	4
Class II	9	6
Size of the upper jaw	Normal	Normal
Size of the lower jaw	4 Micrognathic	6 Micrognathic
Upper jaw position	3 Prognathic, 1 Retrognathic	6 Prognathic, 1 Retrognathic
Lower jaw position	7 Retrognathic	2 Prognathic, 3 Retrognathic
Profile	12 Straight, 6 Convex	6 Straight, 3 Convex
Size of the mandibular branch	3 Diminished	1 Augmented, 2 Diminished
Size of the mandibular body	1 Augmented, 4 Diminished	2 Diminished
Upper incisor inclination	6 Vestibular version, 4 Palate version	6 Vestibular version, 4 Palate version
Lower incisor inclination	6 Vestibular version, 1 Lingual version	7 Vestibular version, 1 Lingual version
Lower third	10 Augmented, 1 Diminished	3 Augmented, 2 Diminished

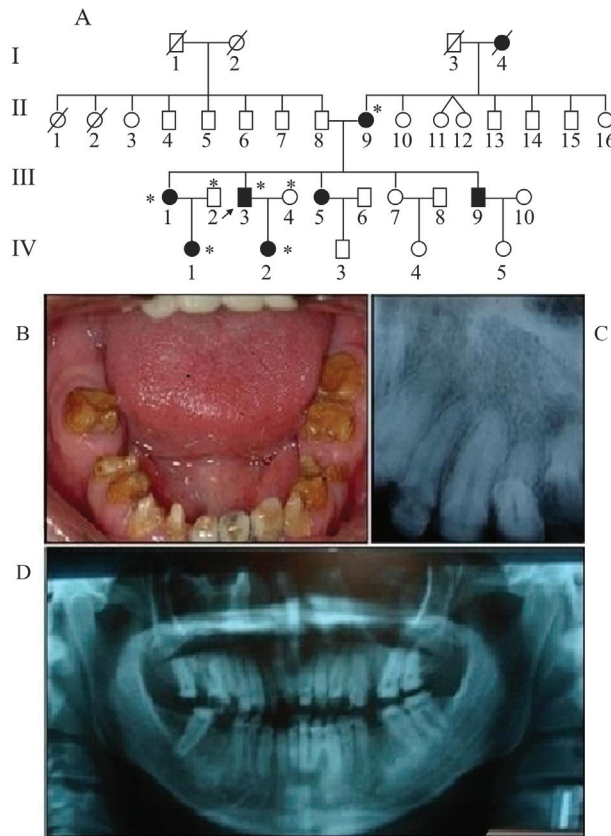
These skeletal alterations refer to 18 individuals with AI and 12 individuals without AI who ranged in age from 3 years old to > 18 years old. Hyperdivergency (related to anterior open bite) and Class II were the most frequent skeletal alterations in patients with ADAI. The three patients with ARAI had a straight profile and normal sized lower and upper jaws.



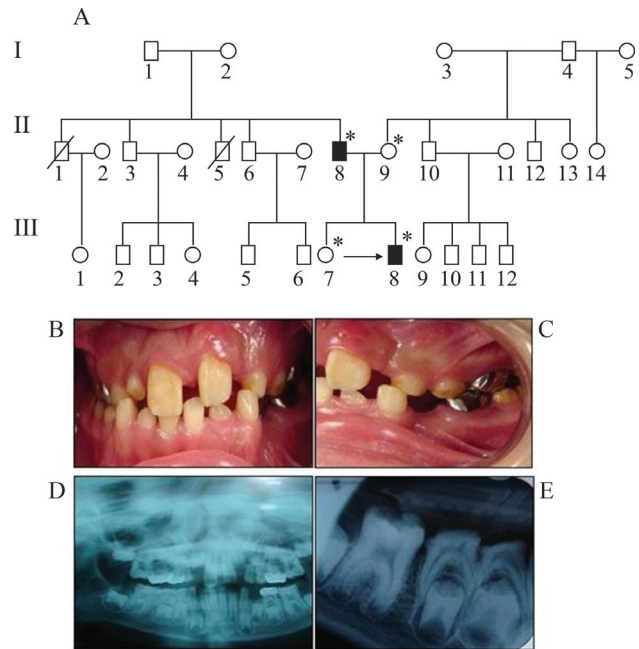
Dental agenesis associated with AI has also been reported, mainly in upper lateral incisors of hypoplastic and

hypomaturative phenotypes (Uchida *et al.*, 1991). We have also observed dental agenesis in five individuals with hypoplastic AI, but in premolars rather than incisors (Cordoba *et al.*, 2007).

In addition to dental anomalies, cephalometric studies have reported that AI increases the prevalence of craniofacial abnormalities (Persson and Sundell, 1982; Pulgar *et al.*



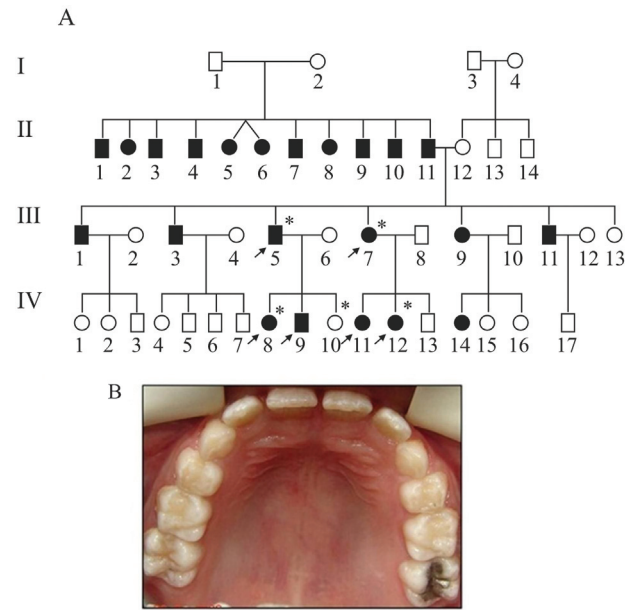
**Figure 1** - Family 1. A. The pedigree indicated autosomal dominant inheritance with eight affected members (black symbols). The asterisks indicate individuals who were analyzed clinically and whose *ENAM* gene was sequenced. Some individuals showed open anterior bite. The arrow indicates the proband III:3 patient. B. Photograph of proband III:3. The phenotype of this family was classified as generalized, rugged and severely hypoplastic with horizontal grooves. Many teeth showed caries and crown destruction while others were in the final stages of preparation for a prosthetic implant. C,D. Periapical and panorex radiographs of the same proband showing most of the teeth. Note the large areas of radiolucid enamel and the absence of several teeth.



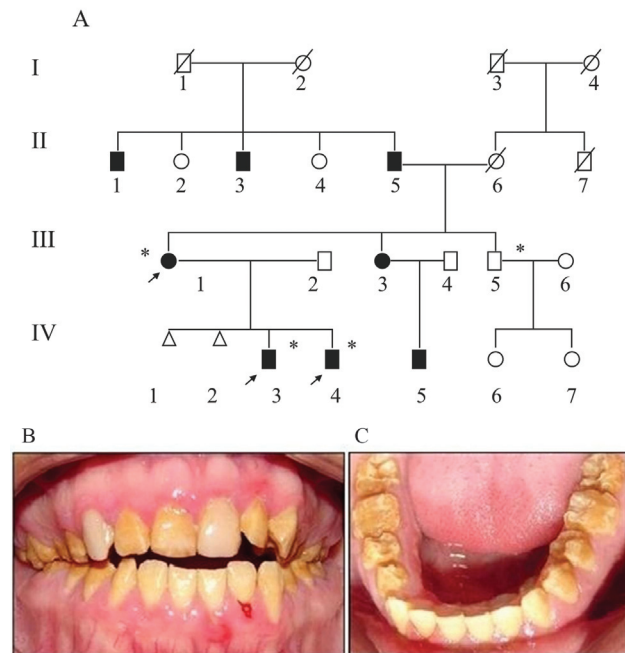
**Figure 2** - Family 2. A. The pedigree indicated autosomal dominant inheritance with two affected members (black symbols). These two cases probably represent *de novo* mutations since none of the individuals in the first generation were affected. The arrow indicates the proband patient and the asterisks indicate individuals who were analyzed clinically and whose *ENAM* gene was sequenced. B, C. Photographs of proband III:8. The phenotype was classified as localized soft hypoplastic and was observed mainly in the anterior teeth. In this patient with mixed dentition, AI was present in deciduous and permanent dentition and was more severe in the latter. D,E. Panorex and periapical radiographs of the same proband showing premolars near eruption that were radiolucid in the enamel zone (similar to dentin). The periapical radiograph shows the marked contrast between dentin and enamel in the sixth permanent lower teeth.

**Table 5** - Summary of the dental and skeletal anomalies, phenotypes and genotypes encountered in affected individuals belonging to the five families with ADAI.

Clinical characteristics			
Dental	Skeletal	Phenotype	Genotype
Pulpal calcifications, radicular dilacerations, hypercementosis, dental agenesis	Class II inferior malocclusion, inferior micrognathism, reduced mandibular size, convex profile, mandibular retrognathism, reduced mandibular body, hyperdivergency, anterior open bite	Hypoplastic generalized rugged	No mutations
Pulpal calcifications, dilacerations	Normal mandibular size	Hypoplastic soft localized	No mutations
Pulpal calcifications, dilacerations, hypercementosis, dental agenesis	Skeletal class I and class II mandibular retrognathism	Hypomaturative generalized snow capped	No mutations
Hypercementosis, dilacerations	Upper prognathism, convex profile, anterior open bite	Generalized hypocalcified	No mutations
Pulpal calcifications, dilacerations, hypercementosis, dental agenesis	Convex profile	Generalized/localized hypocalcified	No mutations



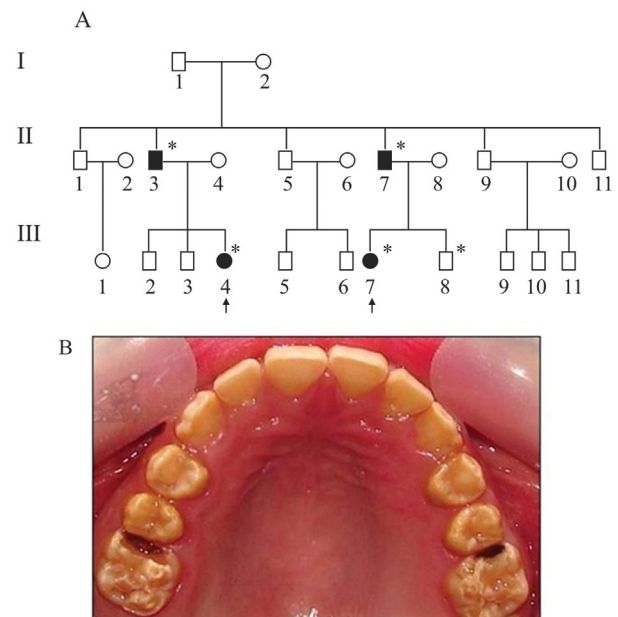
**Figure 3** - Family 3. A. The pedigree indicated autosomal dominant inheritance with 22 affected members (black symbols). The arrows show the proband patients who were attended in this study and the asterisks indicate individuals who were analyzed clinically and whose *ENAM* gene was sequenced. B. Photograph of proband IV:8. The AI phenotype in this family was classified as hypomaturative (snow capped) with characteristics similar to fluorosis. Grooves with white stains that varied in location and intensity were observed among different members of the family. These alterations were observed mainly in the cuspids.



**Figure 4** - Family 4. A. The pedigree indicated autosomal dominant inheritance with eight affected members (black symbols). The arrows show the proband patients who were attended along with their mother. The asterisks indicate individuals who were analyzed clinically and whose *ENAM* gene was sequenced. B,C Photographs of proband III:1. The phenotype was classified as generalized hypocalcified. Note the characteristic indications of wear on the occlusal surfaces and therefore high tooth sensitivity. This patient also showed anterior open bite.

*al.*, 2001). As shown here, 13 of the 18 individuals with AI had a hyperdivergent mandibular plane while seven of the 12 individuals without AI also had hyperdivergence. This retroclined mandibular plane suggests a tendency or predisposition to develop a skeletal anterior open bite. However, this condition was not unique to patients with AI since it was also observed in a significant number of individuals in the control group. Person and Sundell (1982) examined Swedish individuals with AI and found that only 38% of individuals with hypocalcified and hypoplastic phenotypes had anterior open bite and this was more common in men. Rowley *et al.* (1982) found skeletal anterior open bite in 24% of English subjects with the hypoplastic phenotype and a lower frequency in individuals with the hypocalcified phenotype. So far there have been no reports of anterior open bite in individuals with the hypomaturative phenotype. The results of the present study agreed with these authors, *i.e.*, that individuals who had anterior open bite belonged to families with hypoplastic and hypocalcified phenotypes while this skeletal abnormality was not seen in subjects with the hypomaturative phenotype.

Schulze (1957) reported anterior open bite in three families with the hypoplastic phenotype of AI. The coexistence of these two conditions may reflect a pleiotropic action of genes that produce AI and its influence craniofacial growth (Issel, 1955; Schulze, 1957). Backman *et al.* (1988) stated that the frequent association of open bite with AI was possibly related to local mechanical activity such as that as-



**Figure 5** - Family 5. A. The pedigree indicated autosomal dominant inheritance. The arrows indicate the proband patients and the asterisks indicate individuals who were analyzed clinically and whose *ENAM* gene was sequenced. B. Photograph of proband III:7. The AI phenotype in this family was classified as hypocalcified. However, AI was not generalized among members of this family but was restricted to hypocalcified areas. This individual showed no signs of wear on the occlusal surfaces, probably because of her young age.

sociated with speaking since this involves changes in temperature to which the altered enamel of these individuals is sensitive. In addition, the interposition of the tongue during speaking impedes alveolar growth and leads to anterior open bite.

Several reports have indicated an association between AI caused by mutations in the *ENAM* gene and anterior open bite (Kida *et al.*, 2002; Hart *et al.*, 2003a; Gutiérrez *et al.*, 2007; Pavlic *et al.*, 2007). Kida *et al.* (2002) described two individuals heterozygous for mutations in the *ENAM* gene who had anterior open bite in their deciduous dentition. In contrast, other studies have reported no anterior open bite in individuals with mutations in *ENAM*, while some have described anterior open bite and skeletal dysplasia in individuals homozygous for *ENAM* gene mutations but not in heterozygotes (Hart *et al.*, 2003b; Kim *et al.*, 2005; Ozdemir *et al.*, 2005). Ravassipour *et al.* (2000) suggested that the association between AI and anterior open bite may reflect the expression of multiple genes and possibly the contribution of environmental factors. Overall, these studies indicate that anterior open bite is not a dominant trait in individuals homozygous or heterozygous for *ENAM* gene mutations, but may be a trait with incomplete penetrance.

The hypoplastic phenotype is the most prevalent phenotype in autosomal dominant amelogenesis. The manifestations of this phenotype include interproximal spaces between the teeth, thin enamel that is hard to the touch, rough and irregular pits and grooves, and a color that varies from yellow to brown (Witkop and Sauk, 1971). Of the five families with ADAI studied here, two had the hypoplastic phenotype, two had the hypocalcified phenotype and one had the hypomaturative “snow capped” phenotype (Figures 1-5).

Depending on the specific mutation in the *ENAM* gene, the phenotype ranges from a hypoplastic rough, thin widely distributed enamel that is transmitted in a dominant or recessive form to a local enamel deposition that is confined to pits and is transmitted as a dominant trait (Kida *et al.*, 2002). Mardh *et al.* (2002) stated that a dominant mutation in the *ENAM* gene can also cause an intermediate phenotype (“hypoplastic located”) in which horizontal grooves and pits surround the enamel. In a previous study of a Colombian family in which a mutation involving the substitution of a guanine for cytosine (c.817G > C) was detected in exon 9 of the *ENAM* gene (Gutiérrez *et al.*, 2007) the phenotype was hypoplastic rough and was severe and widespread in all of the teeth, with vertical grooves in the canines and horizontal grooves in the front teeth; the tooth ranged from yellow to brown. The phenotype was more severe in the proband than in some members of the same family. This finding and those of Kida *et al.* (2002) indicate that the dominant mutations in this gene may be responsible for the rough phenotype with grooves and pits in all affected members. In the present study the hypoplastic phenotype

was observed in families 1 and 2 but there were no mutations in the *ENAM* gene in either of these families. Anterior open bite was seen in two affected members of family 1 and AI was more severe in permanent than in deciduous teeth in individuals with mixed dentition in this family.

The phenotype in family 2 was hypoplastic smooth localized. There was no anterior open bite in either of the affected members of this family, but the phenotype was more severe in permanent than in deciduous teeth. These findings were consistent with those of other reports (Kida *et al.*, 2002; Mardh *et al.*, 2002; Hart *et al.*, 2003a,b; Kim *et al.*, 2005; Pavlic *et al.*, 2007) in which the phenotype varied even among members of the same families with AI that had mutations in *ENAM* with an autosomal dominant inheritance pattern. The phenotype in family 1 ranged from medium severity to highly severe, indicating that although the phenotype was hypoplastic for all members of this family it was more severe in some than in others. This observation suggested that there was some dose-dependency in the phenotypic manifestations and that this may have affected permanent teeth more than deciduous teeth.

To date all mutations reported for the *ENAM* gene cause hypoplastic enamel, although the clinical and phenotypic characteristics vary according to genotype (Table 1). The reasons for these differences are not fully known and the effects of mutations in the protein are still not very well understood. The variability in clinical appearance among individuals of the same family with the same mutation may be explained by the translation of similar sized proteins that have critical functional differences (Kim *et al.*, 2005). These differences may reflect environmental influences, the pleiotropic action of mutations or the modification of gene effects (Hart *et al.*, 2003a) that can contribute to the deposition of hypoplastic enamel that is thin, has a porous mineralized surface and lacks a normal prismatic structure in some areas (Wright *et al.*, 1991; Backman, 1989). These defects in enamel quality and quantity cause discoloration, susceptibility to decay, fractures and sensitivity to temperature changes.

In most individuals of families with hypoplastic phenotype the posterior teeth in particular showed destruction of the cusps that involved decay and fractures. The teeth of all individuals with AI had a yellow to brown coloration that varied according to the severity of the phenotype (Figures 1 and 2). This finding confirmed the important protective role of enamel and the dentin-pulp complex and indicated that any alteration in the process of enamel formation could adversely affect the assembly, resistance and other its properties. Such damage reduces the resistance to decay and facilitates penetration of the tooth by bacteria and acids that cause cavities.

Our previously identified mutation in the exon 9 of the *ENAM* gene (c.817G > C) (Gutiérrez *et al.*, 2007) probably resulted in altered enamelin that led to severe hypoplastic phenotype through an autosomal dominant negative



effect. This in turn, probably caused haploinsufficiency in the gene and interfered with the proper formation of enamel, possibly by affecting crystal elongation. Pavlic *et al.* (2007) evaluated the 32 kDa product of the *ENAM* gene (which binds with high affinity to hydroxyapatite crystals in enamel through two phosphorylated serines and three glycosylated asparagines). These authors noted that this product was affected by the mutation g 13 185-13186insAG, which abolished one of the serines and all three N-terminal glycosylation sites. This alteration caused premature termination of amelogenesis and aborted crystal growth, thereby contributing to the phenotypic diversity seen among members of the same family. These observations indicate that mutations in critical regions of the *ENAM* gene can produce defective enamel.

In families with the hypoplastic phenotype, any mutation in a critical region of the 32 kDa product could theoretically lead to the inadequate formation of enamel prisms. This could account for the phenotypic variation (absence of interproximal spaces, lack of adequate enamel formation, irregularities in enamel thickness and the presence of hypoplastic areas) among members of the families studied. Animal studies have highlighted the important role of enamelin in controlling crystal nucleation and growth and have shown that enamelin is absolutely essential for proper mineralization (Hu *et al.*, 1997; Masuya *et al.*, 2005; Hu *et al.*, 2008). In this function, the 32 kDa product, which is hydrophobic and acidic, increases the acidity of enamelin and its affinity for hydroxyapatite, thereby promoting nucleation and crystal elongation (Hu *et al.*, 1997). Immunohistochemical analysis has shown a predominance of the 32 kDa product in the inner layer of prisms containing crystals and in areas of enamel matrix; this polypeptide is also responsible for interacting with amelogenin (Uchida *et al.*, 1991). In our previously reported mutation (Gutiérrez *et al.*, 2007), the change in the *ENAM* gene sequence in the family studied with AI occurred in the region coding for the 32 kDa product which is involved in the interaction between enamelin (primarily through the aminoglycosidic N-terminal) and amelogenin; possibly the mutation may disrupt the interaction of these two proteins.

Family 1 of the present study had a phenotype very similar to that of the family studied by Gutiérrez *et al.* (2007) but did not share the same *ENAM* gene mutation seen in the latter family. In contrast, the absence of shared *ENAM* mutations in family 2 compared to the family studied by Gutiérrez *et al.* (2007) was not surprising since the phenotype of family 2 was much less severe and was soft instead of rough. However, the presence of an autosomal dominant hypoplastic phenotype in family 2 suggests the presence of another mutation in the *ENAM* gene that causes this AI phenotype. Our findings generally agree with those of Kim *et al.* (2006) who reported that only six of 24 families with an autosomal dominant inheritance pattern had mutations in the *ENAM* gene. Overall, these results confirm

the genetic heterogeneity of AI and stress the need to explore other genes.

Family 3 had the hypomaturative “snow capped” phenotype that is very similar to that of individuals with fluorosis. In these patients, there was a mixture of fluorosis with other features typical of AI (Figure 3B). Small and Murray (1978) stated that in addition to fluoride, other hereditary and environmental factors have been implicated in the etiology of enamel defects. Fejerskov *et al.* (1990) proposed that the term ‘idiopathic dental fluorosis’ should be used to classify the changes associated with enamel fluorosis that show the characteristics of fluorosis but for which a significant history of the ingestion of fluoride-containing salt or water is unavailable. Some of the clinical characteristics of patients in family 3 were very similar to fluorosis while others were very typical of AI (Figure 3B). The pedigree of this family revealed a large number of individuals affected in each generation, but there was no information on the source of the water they drank (Figure 3A). Backman *et al.* (1989) reported that individuals with the hypomaturative “snow capped” phenotype with autosomal dominant inheritance had mottled white areas confined to the incisal edges of the anterior and posterior cusps of the teeth, particularly the molars. This conclusion is consistent with the clinical features observed in some members of this family (Figure 3B). Individuals III:13,IV:10,IV:15,IV:16 and IV:17 were of particular interest in diagnosing the phenotype of this family (Figure 3A) since these subjects showed no defects in their enamel even though they lived in the same area and consumed water from the same source as the rest of the family. Winter (1996) reported that 32 of 48 children he studied showed defects similar to those associated with fluorosis and suggested that a large proportion of these cases could be explained by as yet undescribed inherited abnormalities of enamel.

Finally, two families in this study showed a hypocalcified phenotype characterized by poorly mineralized enamel. The teeth were yellow to orange in color and worn on the occlusal surfaces (Figures 4B,C and 5B, which show the phenotype of one individual per family). These families had no mutation in the *ENAM* gene. These findings agree with Hart *et al.* (2003a), who found no mutations in five candidate genes, including the *ENAM* gene, in two families with ADAI and the hypocalcified phenotype.

The extensive genetic heterogeneity of AI means that further molecular studies are needed to establish the precise role played by genes in enamel formation. Such studies would also help to explain the high degree of clinical diversity, the pattern of inheritance and the genotype involved in this condition. The phenotypic and genotypic results described here for Colombian families with ADAI and for other groups and populations described in the literature, particularly in relation to the *ENAM* gene, suggest that there is a need to consider other candidate genes as possible causes of this disease. Recent studies have stressed the need



to search for the gene(s) responsible for the hypocalcified phenotype in ADAI and the *FAMH83* gene has been proposed as a candidate for this phenotype (Kim *et al.*, 2008; Urzúa *et al.*, 2012).

Although it is still unclear why certain dental and craniofacial characteristics are associated with AI, the study of these features could facilitate the development of comprehensive protocols for managing and treating AI patients. Novel therapeutic strategies involving professionals from different disciplines would need to address issues such as the functional, aesthetic and psychological factors that produce AI. Characterization of the genes and mutations that cause AI could lead to the development of effective gene-based therapies for treating this disease.

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