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Original Article

No Evidence for Association between Amelogenesis Imperfecta and Candidate Genes

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Abstract

Background: Amelogenesis imperfecta (AI) is an inherited tooth disorder. Despite the fact that up to now, several gene mutations in *MMP20, ENAM, AMELX* and *KLK4* genes have been reported to be associated with AI, many other genes suggested to be involved. The main objective of this study was to find the mutations in three major candidate genes including *MMP20, ENAM* and *KLK4* responsible for AI from three Iranian families with generalized hypoplastic phenotype in all teeth.

Methods: All exon/intron boundaries of subjected genes were amplified by polymerase chain reaction and subjected to direct sequencing.

Results: One polymorphisms was identified in KLK4 exon 2, in one family a homozygous mutation was found in the third base of codon 22 for serine (TCG>TCT), but not in other families. Although these base substitutions have been occurred in the signaling domain, they do not seem to influence the activity of KLK4 protein.

Conclusion: Our results might support the further evidence for genetic heterogeneity; at least, in some AI cases are not caused by a gene in these reported candidate genes.

Keywords: *Amelogenesis Imperfecta, KLK4, Polymorphism, Consanguineous marriages*

Introduction

Amelogenesis imperfecta (AI) is an inherited disorder and affecting tooth enamel formation (1). This disorder has been categorized into three main groups, hypoplastic, hypomaturation and hypocalcified and subdivided fourteen subtypes based on phenotype and patterns of inheritance (2). Up to now, over than 300 genes have been reported to be associated with tooth development (http://bite-it.helsinki.fi) (3). It has been well documented that the enamel formation controlled by different biomolecules including enamelin (ENAM), dentine sialophosphoprotein (DSPP) amelogenin (AMELX) ameloblastin (AMBN), tuftelin (TUFT1), and different enzymes such as kallikrein 4 (*KLK4) and* matrix metalloproteinase 20 (*MMP20*) (4, 5).
Several gene mutations such as *MMP20* (6-8), *ENAM* (8-14), *AMELX* (8) and *KLK4* (15) have been reported to be associated with the pathogenesis of AI. The prevalence of AI varies from 1 in 700 to 1 1in 14,000 in the USA (16).

In the present study we attempted to identify genetic alterations within three major candidate genes, *MMP20*, *KLK4* and *ENAM* responsible for hypoplastic AI in three Iranian families.

Material and Methods

Ascertaining family members

This study was approved by Institutional Review Board (IRB) and informed consent was obtained from all affected individuals. The affected patients with hypoplastic amelogenesis were diagnosed in the dental clinics at the Pediatric Dentistry of Tehran University in Iran. The pedigree analysis was carried out by Cyrillic 2.1 software.

Mutation Screening

In order to perform mutation screening genomic DNA was extracted using DNGPLUS kit (Cinnagene, Tehran-Iran). Five milliliters peripheral blood was collected in test tubes containing 0.5 M EDTA. DNA was examined in terms of quality and quan-

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tity using measurement of A_{260}mm and gel agarose electrophoresis, respectively, and stored at -20 °C until required.

PCR was typically performed using 0.2U *Taq* DNA polymerase (Roche, Mannheim, Germany), 10p mole of each primer, 200 µM of each dNTPs, .0.67µl of 50mM $MgCl_2$, 60ng DNA and 2.5 µl of PCR buffer in 25µl of PCR reactions. PCR amplifications of exon-intron boundaries of *ENAM, MMP20 and KLK4* genes were carried out using 18 primer pairs (Table 1). Thermocycling was performed using a Touch-down amplification program on an ABI thermocycler. The PCR conditions included an initial denaturation step for 3 min at 95 ºC, 30 sec at 95 ºC, 45 sec at 64 ºC with a 1 ºC decrease every second cycle down to 55 ºC, then 55 ºC for 14 cycles, 1 min at 72 ºC for extension, and finally 10 min at 72 ºC. PCR products were separated on 2% agarose gels and visualized with ethidium bromide. Subsequently, to determine any mutation PCR product was subjected for direct sequencing (Gene Fanavaran, Iran). Sequence data searches were performed in non-redundant nucleic and protein databases BLAST (http://www.ncbi.nlm.nih.gov/BLAST).

Results

The probands were affected with AI from two Iranian consanguineous (first-cousin) families (Fig. 1A, Fig. 2 and Fig. 3). All affected and siblings were born after a normal term pregnancy. The affected individuals represented hypoplastic amelogenesis imperfecta with yellow-brown discoloration and evidence of pathological enamel loss during wear and fracturing. Affected individuals were clinically and radiographically examined and showed no signs and symptoms of any syndromic indication. In addition, dental examination of the parents and unaffected siblings showed no evidence of any enamel defect and any syndromic signs.

PCR products from subjected regions (intron/exon junctions) of *ENAM*, *KLK4* and MMP20 genes were carried out (as described in materials and methods), subsequently subjected for direct PCR sequencing. The sequencing data for each fragment was analyzed using Chromas Version 3.1 software and search performed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST).

Analysis of sequences of affected patients could not detect significant genetic alterations in studied genes correlated with the pathogenesis of AI. In family 1, both the primary and permanent dentitions in the affected patient showed a yellow/brown discoloration (Fig. 1B).

Chromatogram of *KLK4* DNA sequencing (as shown in Figure1D) revealed a homoozygous polymorphism in probands from family 1, in which guanine changed to thymine in the third base of codon 22 in *KLK4* exon2. Despite this genetic alteration occurred in signal peptide, no amino acid substitution happened. This polymorphism can not alter restriction enzyme site.

Fig. 1C indicates the clinical and X-ray examinations of proband from consanguineous family1 and reveals structural defect in enamel and spacing in teeth. Clinical and radiographic Examinations were conducted for other probands of other families and showed hypoplastic AI (data not shown).

Table 1: List of primers used for mutation screening

PRIMER NAME	PRIMER SEQUENCES	PCR PRODUCT SIZES (BP)
	ENAM	
EN1F EN1R	5′-CTGTGCCAAGCTTTCTGACA-3′ 5′-TGTTTGGCCCTCTCAAGTGT-3′	923
EN45F EN45R	5′-CCCCATCCATTTCCATACTC-3′ 5′-TGATGGCTGGGGAAATTACT-3′	523
EN6F EN6R	5′-TCAGAAATTTTTACACTGGGAAG-3′ 5′-TGTGAGAGGATAGGGGCAAT-3′	323
EN7F EN7R	5′-GAGGATGGAGACAGCCTGAA-3' 5′-CGGGCTGAGGTTGATTATGT-3′	302
EN8F EN8R	5′-GGGAGATGTAGACTCCCAAGTTT-3′ 5′-TGATGCACTGGTTTTGTTTCA-3′	307
EN9F	5′-GATCCAGCTGAAGCCTTTGT-3′	324
	MMP20	
M1F M1R	5′-CAGGACCTGGAGGAACAACT-3′ 5′-CCAGACACCAATCTAGGTGGA-3′	201
M2F M2R	5′-CCCTGCCTTACCTGAGCAT-3′ 5′-GCCTGACGGATGGATGTAAA-3′	470
M3F M3R	5′-CCGGATTATCCCAACTGTCT-3′ 5′-ACTGTGCGAAGGAGGAGTGT-3′	472
M4F	5′-TGTCAATGCTACTCAAAATGTCC-3′	312
M5F M5R	5′-AGTTAGGAGAAGGAGATGGG-3′ 5′-CTGATGGGTCTGTGGAATG-3′	193
M6F M6R	5′-CATGTCCAGCGTGAAAGTGT-3′ 5′-GTCTGGGAGTGGAGATGAGG-3′	300
M7F M7R	5′-AGTAAGCAGTGCCCCTCTC-3′ 5′-AAACAAGGCAAGGCAAGG-3'	266
	KLK4	
K1F K1R	5′-GAGTTGAGGCAGCCTGAGAG-3′ 5′-ACAAGGAGTTGCAGGGACAC-3′	1000
K2F K2R	5′-CTGCTCCTGAACCTCTGACC-3′ 5′-ATTCCCATCCCCATCTCCTA-3′	615
K3F	5'-TGACTGCTCCTGAACCTCTG -3' 5'-CCTCGCCGTTTATGATTTG-3′	149
K4F K4R	5′-GGGGTTGAAGATGAGAATGG-3′ 5′GGCCCTGTGTGTCTCTGTCT-3′	615
K7F K7R	5′—AAACTGACCTGCCCTCCGT-3′ 5′-TGTCAGACTCGGACACGGA-3′	195

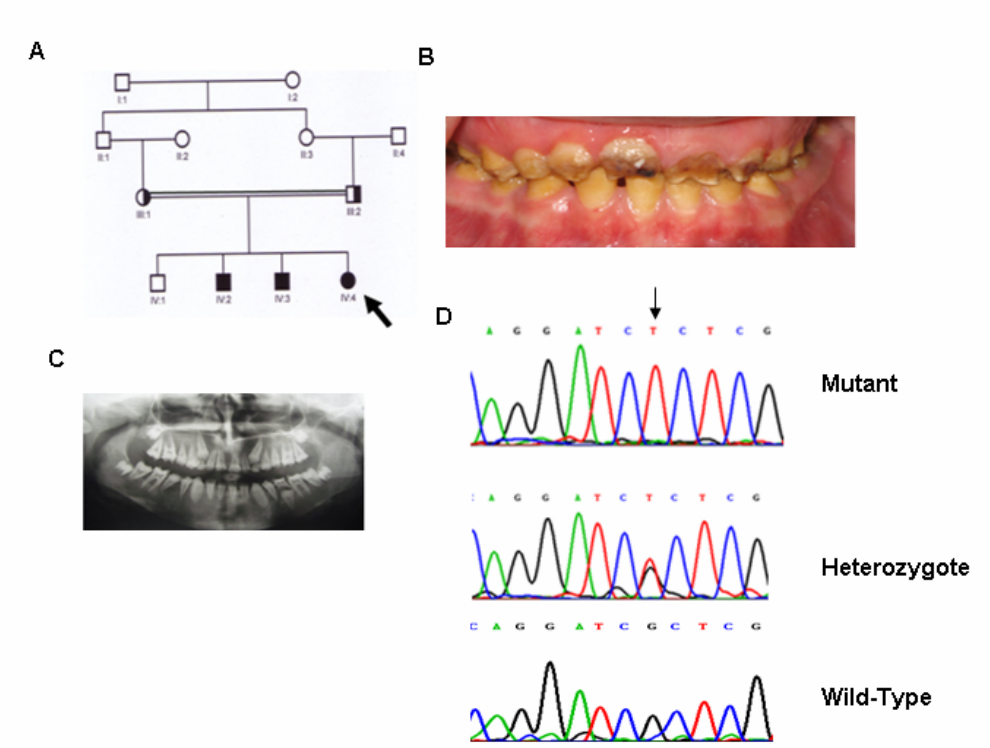

Fig. 1: Pedigree analysis, Clinical characterizations and Molecular Study of Family 1.A. The Pedigree of family 1 showed a consanguineous (first-cousin) family with an affected patient. B. Phenotype demonstrating hypoplastic AI. C. Radiographic examination indicated lack of tooth enamel in proband. D. DNA sequencing revealed heterozygous polymorphism in the codon 22 of KLK4 gene in which G →T at position 64 of mRNA. (NM_004917.3).Arrow indicates the proband.

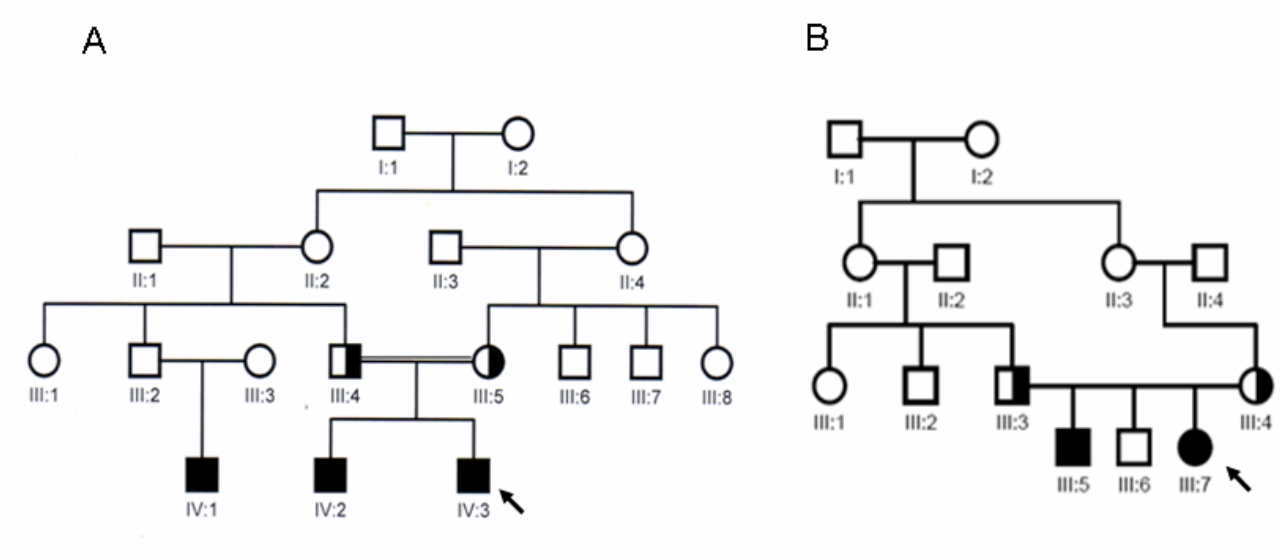

Fig. 2: Pedigree analysis of two consanguineous (first-cousin) families with affected patients suggested AI with pattern of autosomal recessive (A and B). Arrows indicate the probands.

Discussion

To date, various enamel related genes such as *ENAM, AMBN, AMELX, MMP20, KLK4* and *Amelotin* genes have been reported to be implicated in AI. Several studies have demonstrated that *MMP20* and *KLK4* proteins regulate the enamel matrix protein processing that determines enamel structure and composition. *KLK4* is a member of serine protease superfamily and its mutated forms have been reported which lead to some human autosomal recessive disorders.

We found a base substitution in codon 22 of *KLK4* in one affected patient. However, direct PCR sequencing could not detect any gene mutation in previously reported genes. In consistent with our results different studies also, failed to show any genetic defect in this disorder (8, 13, 15). In this regard, Santos et al. studied mutation detection in current candidate genes responsible for AI in two Brazilian families and they could not identify any mutation. Recently two new loci for AI have been identified (15, 17). Kim et al. (8) studied 24 families with non-syndromic enamel defects and they found only six disease-causing mutations in the AI candidate genes.

An accumulation of evidence suggested the involvement of other genes in the pathogenesis of AI. More recently, Lee et al. detected several nonsense mutations in the last exon of FAM83H (18). However, the mechanism by which FAM83H mutation acts in pathogenesis of AI reminds to be identified. Taken together, these results suggested that the other genes and/or other mechanism such as epigenetic factors could contribute to the etiology of AI. It is quit acceptable that epigenetic is one of the most important factors that act in different ways including packaging of chromatin, DNA methylation.

Although the base substitutions reported in our study occurred in signal peptide of KLK4, they do not seem to influence the modification or activity of KLK4 protein. Several lines of evidence supported that polymorphism could play a critical role in pathogenesis of human diseases. The biological function of the KLK4 polymorphisms is unknown; therefore, further studies need to be done.

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References

1. Backman B, Holm AK (1986). Amelogenesis imperfecta: prevalence and incidence in a northern Swedish county. *Community Dent Oral Epidemiol*, 14(1): 43-7.
2. Witkop CJ (1988). Amelogenesis imperfecta, dentinogenesis imperfecta and dentin dysplasia revisited: problems in classification. *J Oral Pathol,* 17(9-10): 547-53.
3. Thesleff I PS (2003). Genetics of dental defects. Encyclopedia of Human Genome. Genes & Disease John Wiley & Sons, Ltd Also online: http://elswileycom.
4. Iwasaki K, Bajenova E, Somogyi-Ganss E, Miller M, Nguyen V, Nourkeyhani H, Gao Y, Wendel M, Ganss B (2005). Amelotin-a Novel Secreted, Ameloblast-specific Protein. *J Dent Res*, 84(12):1127-32.
5. Crawford PJM, Aldred M, Bloch-Zupan A (2007). Amelogenesis imperfecta. *Orphanet J Rare Dis*, 2:17-28.
6. Papagerakis P, Lin HK, Lee KY, Hu Y, Simmer JP, Bartlett JD, Hu JC (2008). Premature stop codon in MMP20 causing amelogenesis imperfecta. *J Dent Res,* 87(1): 56-9.
7. Ozdemir D, Hart PS, Ryu OH, Choi SJ, Ozdemir-Karatas M, Firatli E, Piesco N, Hart TC (2005). MMP20 active-site mutation in hypomaturation amelogenesis imperfecta. *J Dent Res,* 84(11):1031-35.
8. Kim J-W, Simmer JP, Lin BPL, Seymen F, Bartlett JD, Hu JCC (2006). Mutational analysis of candidate genes in 24 amelo-

genesis imperfecta families. *Eur J Oral Sci,* 114 Suppl 1: 3-12.

9. Gopinath VK, Yoong TP, Yean CY, Ravichandran M (2008). Identifying polymorphism in enamelin gene in amelogenesis imperfecta (AI). *Arch Oral Biol,* 53(10): 937-40.
10. Gutierrez SJ, Chaves M, Torres DM, Briceno I (2007). Identification of a novel mutation in the enamalin gene in a family with autosomal-dominant amelogenesis imperfecta. *Arch Oral Biol,* 52(5): 503-6.
11. Ozdemir D, Hart PS, Firatli E, Aren G, Ryu OH, Hart TC (2005). Phenotype of ENAM mutations is dosage-dependent. *J Dent Res,* 84(11):1036-41.
12. Masuya H, Shimizu K, Sezutsu H, Sakuraba Y, Nagano J, Shimizu A, (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(5):575-83.
13. Hart PS, Wright JT, Savage M, Kang G, Bensen JT, Gorry MC, Hart TC (2003) Exclusion of candidate genes in two families with autosomal dominant hypocalcified amelogenesis imperfecta. *Eur J Oral Sci,* 111(4): 326-31.
14. Mardh CK, Backman B, Holmgren G, Hu JCC, 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(9): 1069-74.
15. Santos MCLG, Hart PS, Ramaswami M, Kanno CM, Hart TC, Line SRP (2007). Exclusion of known gene for enamel development in two Brazilian families with amelogenesis imperfecta. *Head Face Med,* 3(8): 1-7.
16. Witkop CJ Jr SJJ (1976). Heritable defects of enamel. In: Oral facial genetics. Stewart RE, Prescott GH, editors St Louis: CV Mosby, pp. 151-226.
17. Mendoza G, Pemberton TJ, Lee K, Scarel-Caminaga R, Mehrian-Shai R,Gonzalez-Quevedo C, Ninis V, Hartiala J, Allayee H, Snead ML, Leal SM, Line SR, Patel PI (2007). A new locus for autosomal dominant amelogenesis imperfecta on chromosome 8q24.3. *Hum Genet*, 120(5): 653-62.
18. Lee SK, Hu JC, Bartlett JD, Lee KE, Lin BP, Simmer JP, Kim JW (2008). Mutational spectrum of FAM83H: the C-terminal portion is required for tooth enamel calcification. *Hum Mutat*, 29(8): E95-E99.