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Amelogenin gene influence on enamel defects of cleft lip and palate patients

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Abstract: The aim of this study was to investigate the occurrence of mutations in the amelogenin gene (AMELX) in patients with cleft lip and palate (CLP) and enamel defects (ED). A total of 165 patients were divided into four groups: with CLP and ED (n=46), with CLP and without ED (n = 34), without CLP and with ED (n = 34), and without CLP or ED (n = 51). Genomic DNA was extracted from saliva followed by conducting a Polymerase Chain Reaction and direct DNA sequencing of exons 2 through 7 of AMELX. Mutations were found in 30% (n = 14), 35% (n = 12), 11% (n = 4) and 13% (n = 7) of the subjects from groups 1, 2, 3 and 4, respectively. Thirty seven mutations were detected and distributed throughout exons 2 (1 mutation – 2.7%), 6 (30 mutations – 81.08%) and 7 (6 mutations – 16.22%) of AMELX. No mutations were found in exons 3, 4 or 5. Of the 30 mutations found in exon 6, 43.34% (n = 13), 23.33% (n = 7), 13.33% (n = 4) and 20% (n = 6) were found in groups 1, 2, 3 and 4, respectively. c.261 C > T (rs2106416), a silent mutation, was detected in 26 subjects, and found more significantly (p = 0.003) in patients with CLP (groups 1 and 2 – 23.75%), compared with those without CLP (groups 3 and 4 – 8.23%). In the groups without ED, this silent mutation was also found more significantly (p = 0.032) among subjects with CLP (17.65% in group 2), compared with those without CLP (7.8% in group 4). In conclusion, this study suggested that AMELX may be a candidate gene for cleft lip and palate.

Keywords: Amelogenin; Dental Enamel; Cleft Lip; Cleft Palate.

Introduction

Enamel defects (ED) arise from disturbances during tooth formation, and cause an altered development or calcification of the organic matrix.[1,2] These defects in enamel may be located in a single tooth and may affect several teeth or the entire dentition. Depending on the intensity of the causative agent, their severity may range from a moderate defect to a complete failure in enamel formation.

According to the literature, there are some genes involved in the formation of dental enamel, *i.e.*: amelogenin (AMELX), enamelin (ENAM), kallikrein-4 (KLK-4), matrix metalloprotease-20 (MMP-20), ameloblastin[3] and more recently DLX3,[4] FAM83H,[5,6,7] WDR72[5] and SLC4A4.[8] AMELX encodes a member of the amelogenin family of extracellular matrix protein and has an important role in biomineralization during tooth enamel development.[9] The X-linked amelogenin gene is located on the Xp22.31-

p22.1 chromosome, and is also known as AMG, AI1E, AIH1, ALGN, AMGL and AMGX.[9]

Although the defects in enamel formation are not a public health problem, they may cause severe esthetic alterations and compromise tooth enamel structure. Severe forms may lead to early enamel loss, consequently resulting in tooth wear and impaired functioning. Additionally, the relationship between ED and tooth caries is well established. Less mineralized enamel or enamel with an irregular surface may become more susceptible to tooth caries development.[10,11,12,13,14,15]

Some studies on cleft lip and palate (CLP) subjects report a high prevalence of tooth anomalies when compared with the general population.[16,17] These enamel alterations have been frequently and mainly found in the maxillary permanent central incisors adjacent to the clefts. Although these defects are present in the primary dentition, they are more prevalent in the permanent dentition. These anomalies seem to be determined embryologically, and occur at different stages of tooth development. Reports in literature show that CLP subjects have defects in tooth enamel formation, and that the intensity seems to depend on the cleft severity.[16,17] Therefore, the aim of this study was to investigate the occurrence of mutations in the AMELX gene in patients with CLP and ED.

Methodology

The Institutional Review Board of our institution approved the protocol of this study (process #57/2010) regarding ethical issues. The parents or guardians of the children received detailed information during the pretreatment screening period, concerning the procedures involved in the study, and signed informed consent forms.

Study population

The study population was composed of 165 nonsyndromic subjects with no interfamilial relationship of gender, between the ages of 6 and 15 years, and both with and without ED, ranging from hypomineralization to hypoplasia in permanent maxillary central incisors. They were divided into four groups: Group 1 - with CLP and ED (n = 46; Figure 1A); Group 2 - with CLP and without ED (n = 34; Figure 1B); Group 3 - without CLP and with ED (n=34; Figure 1C) and Group 4 - without CLP or ED (n=51; Figure 1D).

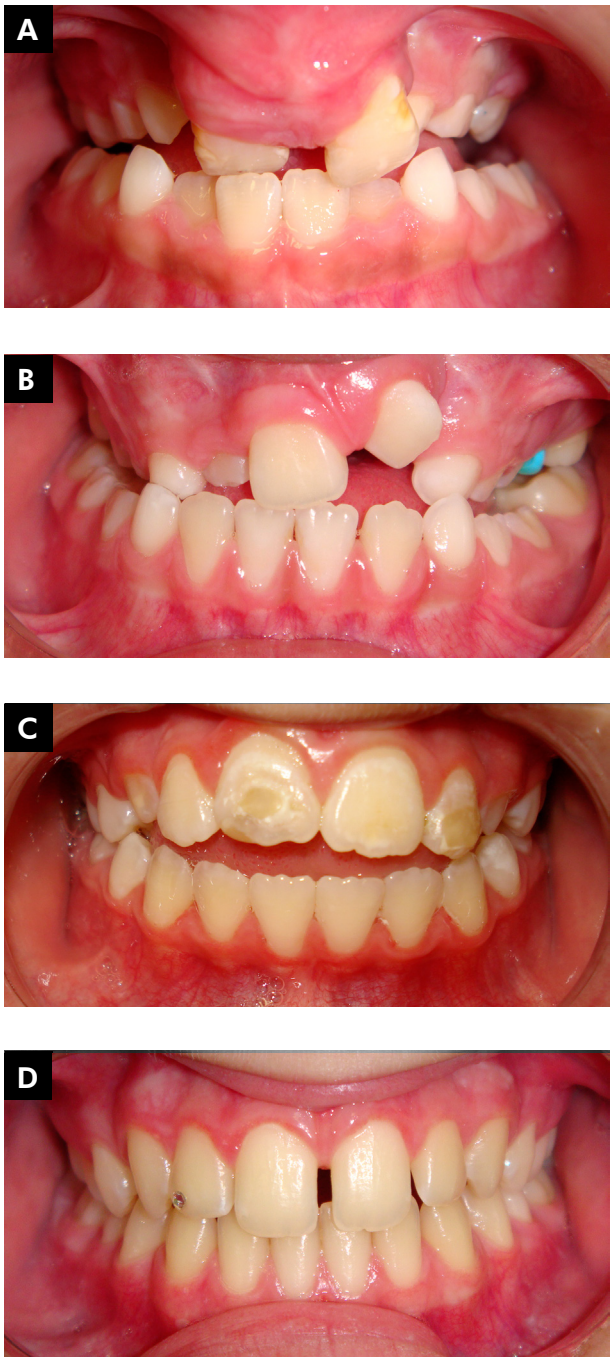


Figure 1A-D. A: Patient with complete bilateral cleft lip and palate and with enamel defect (Group 1); B: Patient with complete unilateral cleft lip and palate and without enamel defect (Group 2); C: Patient without cleft lip and palate and with enamel defect (Group 3); D: Patient without cleft lip and palate or enamel defect (Group 4).

Genomic DNA extraction, PCR and direct DNA sequencing

Saliva samples were collected from all subjects, and the genomic DNA was extracted from these samples with the InstaGene™ Matrix Kit (732-6030, Bio-Rad Laboratories, Hercules, USA), according to the manufacturer's standards and based on a previous study.[18] A Polymerase Chain Reaction (PCR) was then conducted in a thermocycler (Veriti 9902, Applied Biosystems, Carlsbad, USA), followed by direct DNA sequencing (3130xl Genetic Analyzer, 4352715, Applied Biosystems, Carlsbad, USA) of the codifier areas (exons 2, 3, 4, 5, 6 and 7 AMELX). The forward and reverse primers, as well as the PCR conditions, are listed in Table 1.

Table 1. Sequence of forward and reverse primers designed for exons 2, 3, 4,5, 6 and 7 of AMELX and PCR conditions

Exon	Primer (5' – 3')	bp	AT
2	F: AGATTATGTGTGTTTTATGGAGCA	233	62°C
	R: CCCTAATTTCACCAACTATGAGC		
3	F: TCCTTTAATGTGAACAATTGCAT	250	57°C
	R: TCTGGGATAAAGAATCAACACA		
4-5	F: TGAAGAATGTGTGTGATGGATG	367	60°C
	R: TCCCATTAATGTCTGCATGTG		
6	F: CCATAATGGCAAAGAAAACAC	599	59°C
	R: TGGTTGTCGGAGACCTTAGAA		
7	F: TGACAAAACTGAAGCCAGACAT	237	61°C
	R:TGCATTTTATTGTCTGCTAATGG		

F: forward primer; R: reverse primer; bp: base pairs; AT: annealing temperature

Analysis of the sequences obtained

The sequences obtained were analyzed by SeqScape Software® 2.6 (Applied Biosystems, Carlsbad, USA). Mutations found in sequences using the forward primer were confirmed by the sequencing using the reverse primer.

Each variation of the nucleotide sequence identified in the sequencing was described using the den Dunnen and Antonarakis[19] nomenclature system. In order to find the variations, the bases were numbered as of the first methionine (ATG) of the protein resulting from this gene.

A search was performed at Blast[20] and dbSNP[21] databases to determine whether the alterations found represented polymorphisms. Specific programs were used, such as Ensembl,[22] to check if mutations found in the present study had been previously cataloged.

The power test was used to determine the number of patients, and a minimum of 30 patients per group was established. A statistical power of 80% and 95% of confidence were used. Data were submitted to statistical analysis using the Fisher's exact test. Statistical significance was established at 5%. Statistical analysis was performed with STATISTICA (version 11.0, StatSoft Inc., Tulsa, USA).

Results

In relation to the different groups, mutations were found in 30% (n = 14), 35% (n = 12), 11% (n = 4) and 13% (n = 7) of the subjects from groups 1, 2, 3 and 4, respectively.

Thirty seven mutations were detected and distributed throughout exons 2 (1 mutation – 2.7%), 6 (30 mutations – 81.08%) and 7 (6 mutations – 16.22%) of AMELX. No mutations were found in exons 3, 4 and 5.

Of the 30 mutations found in exon 6, 43.34% (n = 13), 23.33% (n = 7), 13.33% (n = 4) and 20% (n = 6) were found in groups 1, 2, 3 and 4, respectively. c.261C>T (rs2106416), which is a silent mutation, was detected in 26 subjects, and significantly more were found (p = 0.003) in patients with CLP (groups 1 and 2 – 23.75%), compared with those without CLP (groups 3 and 4 – 8.23%). In the groups without ED, this silent mutation was also found more significantly (p = 0.032) among subjects with CLP (17.65% in group 2), compared with those without CLP (7.8% in group 4).

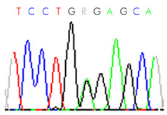
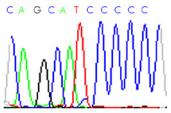
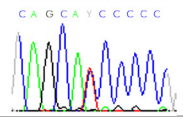
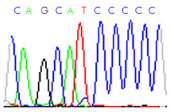
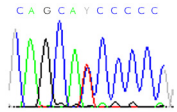
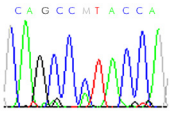
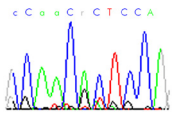
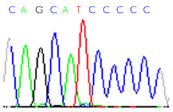
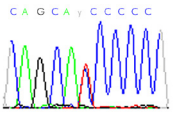
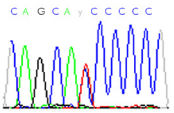
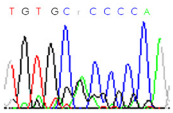
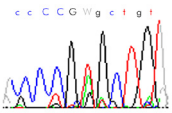
Aside from this single nucleotide polymorphism (SNP), five other mutations that lead to an amino acid substitution (Table 2) were found, one for exon 2 (c.34G>R) and four for exon 6 (c.245T>W, c.362A>G, c.420C>M and c.482C>R).

Table 2 illustrates the mutations found in the present study.

Discussion

Enamel development involves the expression of multiple genes needed to control the complex process of mineralization. Mutations in enamel proteins and protease genes have been associated with ED.[3,23,24,25,26] The cause of ED could be a

Table 2. Distribution of the mutations found in the four groups studied

Group	N	Exon	Mutation	Electropherogram	Amino acid
1	1	2	c.34G>R (A/G) (hetero)		p.12G>R (Gly>Arg)*
	7	6	c.261C>T (homo)		p.87H>H (His>His)
	6	6	c.261C>Y (C/T) (hetero)		p.87H>H (His>His)
	(14)				
2	2	6	c.261C>T (homo)		p.87H>H (His>His)
	4	6	c.261C>Y (C/T) (hetero)		p.87H>H (His>His)
	1	6	c.420C>M (A/C) (hetero)		p.140P>P (Pro>Pro)
	5	7	off transcript		---
	(12)				
3	1	6	c.362A>R (A/G) (hetero)		p.121H>R (His>Arg)*
	1	6	c.261C>T (homo)		p.87H>H (His>His)
	2	6	c.261C>Y (C/T) (hetero)		p.87H>H (His>His)
	(4)				
4	4	6	c.261C>Y (C/T) (hetero)		p.87H>H (His>His)
	1	6	c.482 A>R (A/G) (hetero)		p.161H>R (His>Arg)*
	1	6	c.245T>W (A/T) (hetero)		p.82V>E (Val>Glu)*
	1	7	off transcript		---
	(7)				
Total	37				

n=number; ()=total per group; homo=homozygous mutation; hetero=heterozygous mutation; * means amino acid substitution

genetic disorder linked to specific and nonspecific diseases during odontogenesis with the mineral metabolism, especially calcium phosphate. Some researchers suggest that CLP could be related to these enamel alterations, in which case a direct relationship would exist between defect appearance and cleft presence.[16,17] Considering that ED could be related to different mutations located in several genes involved in tooth enamel formation, and that the amelogenin gene forms the skeleton for the mineralization and the formation of the enamel crystals,[3] the investigation of genes that transcribe the main proteins and proteases of enamel is essential to gaining a better understanding of these alterations. AMELX is composed of seven exons and six introns, and alternative splicing results in three different transcripts, according to Ensembl.[22] In the present study, the exons participating in the transcription of AMELX were sequenced (exons 2-7).

Previous studies have revealed that AMELX is associated with amelogenesis imperfecta (AI),[27,28] caused by a mutation in the genes critical to normal enamel formation.[27] A mutated AMELX produces an altered amelogenin polypeptide resulting in AI.[29] A study[3] evaluated AMELX in mouse models to gain an understanding of the pathogenesis of AI. The authors asserted that ED is poorly understood and that many studies regarding genes in human tooth development are significantly limited.[3] Thus, since AMELX encodes a protein that plays a key role in the organization and structure of the enamel, it is the key player for organizing and structuring this highly mineralized tissue.[30]

Another study reported two SNPs in the AMELX coding sequences in humans. rs2106416 is a C>T silent substitution in amino acid 87 (His>His), It is located in exon 6, and was also found in the present study (Table 2). The other SNP, rs6639060, is also a C>T silent substitution in amino acid 152 (Leu>Leu),[30] and was not detected in the present study. Nonetheless, it is known that the central region of AMELX (exon 6), although highly variable in mammalian evolution, is highly preserved in humans.[30]

Association and comparison among groups are pertinent in the present study. The mutations found were distributed in all groups studied. Our results suggest that the presence of CLP significantly increases the frequency of mutations in AMELX, as compared with the presence of ED, since 70.2% of the mutations were found in CLP groups (groups 1 and 2), whereas 29.7% were found in ED groups (groups 3 and 4). CLP may be involved in a broader dysmorphic spectrum of anomalies[17] – 81.08% of all mutations were found in exon 6 of AMELX. A single mutation common to all groups was c.261C>T mutation, SNP rs2106416, located in region NM_006125.2 of chromosome X, representing 70.2% of all mutations detected. This SNP changes the C ancestral allele into the T mutant allele. In 1,000 genomes allele frequencies, the T mutant allele is present in 17% of all populations, whereas in the American population it is present in 12%.[26] In our study, this mutant allele was found in 15.7% of the sample (26 SNPs in 165 individuals; Table 2).

This is the first report demonstrating that AMELX may be a candidate gene for CLP. Further studies are necessary to investigate mutations and polymorphisms, including SNPs, in the codifying and splicing of candidate gene areas for defects in enamel formation. This will contribute to elucidating the consequences of these mutations in the proteins, as regards tooth enamel development, and determining why CLP is more related to mutations than ED is.

Conclusion

In conclusion, the present study suggested that AMELX may be a candidate gene for cleft lip and palate.

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