BJOS
Brazilian Journal of Oral Sciences

Original Article

Volume 23
2024
e241330

ENAM gene polymorphisms associated with dental anomalies in individuals with cleft lip and palate

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Editor: Altair A. Del Bel Cury

Received: Oct 27, 2022
Accepted: May 15, 2023

Aim: This study aimed to investigate the occurrence of enamelin gene (ENAM) single nucleotide polymorphisms (SNP) and ENAM polymorphism association with dental anomalies (DA) in individuals with unilateral or bilateral cleft lip and palate (CLP). **Methods:** Saliva samples were collected from 147 individuals aged between 6 and 15 years-old, both genders, and divided into 4 groups: Group 1 (G1) - CLP and DA; Group 2 (G2) - CLP without DA; Group 3 (G3) - without CLP with DA; Group 4 (G4) - without CLP and DA. The genomic DNA was extracted from saliva samples and the following ENAM SNPs markers were genotyped: rs3796703, rs3796704, rs3796705, rs7671281, rs2609428, and rs35951442. Fisher exact and Pearson's Chi-square tests statistically analyzed the results (α=5%). **Results:** Individuals without CLP with DA (Group 3 - 19.2%) showed statistically higher prevalence of SNP rs2609428 heterozygotes (p=0.006) than individuals with CLP and DA (Group 1 - 0%). Individuals without CLP (10%) exhibited statistically higher prevalence of mutated heterozygotes/homozygous (p=0.028) than in individuals with CLP (1.3%). **Conclusion:** SNP rs2609428 marker of ENAM gene may be associated with dental anomalies in individuals without cleft lip and palate.

Keywords: Polymorphism, single nucleotide. Cleft lip. Cleft palate. Extracellular matrix proteins.

Introduction

Many genes play a role in craniofacial development and polymorphism may be associated with cleft lip and palate (CLP)[1]. The lack of more detailed definition in phenotype partially explains difficulty in genetic studies on CLP[2]. The accurate classification of affected individuals is crucial in complex genetic traits, in which phenotype can be highly variable. CLP phenotype is complex due to many combinations as cleft side and extension or a variety of associated subclinical markers[2]. Epidemiological data suggests individuals with CLP exhibit higher prevalence of caries and dental anomalies especially related to the tooth enamel structure than those without CLP[3], suggesting the presence of dental anomalies in individuals with CLP could be an additional clinical marker due to a common genetic etiology[4].

Dental anomalies (DA) may affect the enamel at different degrees. The expression of multiple genes plays a role in controlling the complex process of enamel mineralization[5]. Single Nucleotide Polymorphism (SNP), e.g., the alteration of a single base in DNA sequence, is the most common type of genetic variation among people. SNPs can act as biological markers, aiding in searching genes associated with diseases[6,7]. The occurrence of SNP in either the gene or regulatory region close to the gene may play a more direct role in disease by affecting the gene function. Some of these genetic differences, however, proved to be very important in the study of human health[6,7]. Researchers found SNPs may help predict the response of an individual to certain drugs, susceptibility to environmental factors such as toxins, and risk of developing specific diseases[6,7]. SNPs can also be used to control inheritance of genes for specific diseases in families[6,7].

The literature reports that individuals with CLP show tooth enamel formation defects[8], with intensity depending on the cleft severity[9]. Genetic disorder associated with specific and non-specific diseases during tooth development are related to dental anomalies, by interfering with mineral metabolism, mainly calcium phosphate[10]. Researchers suggest CLP is directly associated with the presence of enamel changes[8,9,11]. This study aimed to investigate the occurrence of ENAM SNPs and their association with dental anomalies (DA) in individuals with unilateral or bilateral cleft lip and palate (CLP).

Materials and Methods

This study was approved by the Institutional Review Board (protocol CAAE: 00911012.3.0000.5441). The parents or caretakers were consulted and informed about the research and were invited to sign in an informed consent form, on behalf of their children.

Inclusion criteria comprised individuals aged between 6 and 15 years, both genders, with and without CLP, with and without enamel alterations in the maxillary permanent central incisors, regularly registered in the institution. Dental anomalies ranged from enamel hypomineralization to enamel hypoplasia. Enamel hypoplasia is a quantitative defect, while hypomineralisation is a qualitative defect characterized by abnormal enamel translucency. Exclusion criteria were individu-

als with associated syndromes or malformations; teeth with amelogenesis imperfecta; teeth with restorations, appliances, or orthodontic accessories; and teeth with fluorosis.

Individuals with unilateral or bilateral CLP and individuals without CLP were divided into the following groups: Group 1 - individuals with CLP and DA (n=43); Group 2 - individuals with CLP without DA (34); Group 3 - individuals without CLP with DA (n=26); Group 4 - individuals without CLP and DA - control group (n=44).

Non-stimulated saliva samples of the selected individuals were collected and stored in a freezer at -20ºC. The tubes containing saliva samples were thawed at room temperature and homogenized in vortex (AP56, Brazil). DNA extraction from saliva samples was performed with QIAamp DNA Mini Kit (Qiagen, South San Francisco, CA, USA) according to the manufacturer's recommendations[12].

Genotyping of SNPs in the ENAM gene

The different genotypes of ENAM gene were evaluated using Real-Time PCR System (Applied Biosystems, Waltham, MA, USA).

For determination of genotypes of SNPs rs3796703, rs3796704, rs3796705, rs7671281, rs2609428, rs35951442, ENAM produced and validated gene tests (Applied Biosystems, Waltham, MA, USA) were respectively used with the following catalog numbers: C__27517802_10, C__25766207_10, C__27488875_10, C__25763290_10, C__16047590_30, C__15929426_10. The selection of tests considered the location in the gene (exonic regions), type of mutation (missense), and frequency within the American population. The tests consisted of the presence of four oligonucleotides: forward primer, from position 5' to position 3' of the DNA; reverse primer, from position 3' to position 5' of the DNA; and two probes, from position 5' to position 3' of the DNA.

The forward and reverse primers flanked the DNA region with the polymorphism, which was amplified by PCR technique. The probes annealed exactly in the polymorphism. One probe had the correct bases and was marked with fluorophore VIC, while the other probe was marked with fluorophore FAM and virtually had the same sequence of bases, except for one base that characterized the polymorphism. After the end of the experiment, the amplification of DNA fragments marked with fluorophore VIC occurred, indicating the ancestral homozygous individual for the given gene. DNA fragments marked only with the fluorophore FAM were also amplified indicated the mutated homozygous (or polymorphic) individual. Finally, the amplification of DNA fragments of both probes indicated a heterozygous individual. The characteristics of the SNPs studied are described in Table 1.

Table 1. Characteristics of SNPs of ENAM gene studied for genotyping identification

SNP	Nitrogenous base	Probe	Allele
rs7671281	C	VIC	ancestral
	T	FAM	mutated

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Continuation

rs2609428	C	VIC	ancestral
	T	FAM	mutated
rs3796705	A	VIC	ancestral
	G	FAM	mutated
rs3796703	C	VIC	ancestral
	T	FAM	mutated
rs3796704	A	VIC	ancestral
	G	FAM	mutated
rs35951442	C	VIC	ancestral
	T	FAM	mutated

All statistical analyses were performed by Statistica software (version 11.0, StatSoft Inc, Tulsa, United States). The results were statistically analyzed by Fisher exact and Pearson's Chi-square tests (α=5%).

Results

SNP rs3796703 replaces C allele (ancestral) with T allele (mutated) in position 70643597 of chromosome 4. In the American population, the ancestral allele is 99% homozygous and 1% heterozygous. In Group 1, 2% of individuals were heterozygous, *i.e.,* they had an ancestral allele and one mutated allele. In Groups 2, 3, and 4, 100% of individuals exhibited only the ancestral allele.

SNP rs3796704 replaces C allele (ancestral) with T allele (mutated) in position 70643714 of chromosome 4. In the American population, the ancestral allele is 81% homozygous and, 18% heterozygous, and only 1% has both mutant alleles (A/A). In Groups 1, 2, 3, and 4, respectively, 19%, 21%, 19%, and 18% of individuals were heterozygous and 81%, 79%, 81%, and 82% were homozygous for the ancestral allele. All studied groups showed 100% of individuals with only the ancestral allele.

SNP rs7671281 replaces C allele (ancestral) with T allele (mutated) in position 70643369 of chromosome 4. In the American population, 80% were homozygous for the mutated allele, 19% were heterozygous, and only 1% is homozygous for the allele. In the present study, 81%, 76%, 73%, and 84% of individuals in Groups 1, 2, 3, and 4, respectively, were homozygous for the mutated allele (T/T), while 19%, 24%, 27%, and 16%, respectively, had a mutated allele and an ancestral allele (heterozygous).

SNP rs2609428 replaces C allele (ancestral) with T allele (mutated) in position 70643152 of chromosome 4. In the American population, 97% of genomes were homozygous for the ancestral allele and 3% have a mutated allele, *i.e.*, heterozygous (C/T). In Group 1, 100% of individuals showed two ancestral alleles (ancestral homozygous), while 3%, 19%, and 5% of individuals in groups 2, 3 and 4, respectively, had an ancestral allele and one mutated allele (heterozygous); 97%, 81%, and 95% respectively had the two alleles. The incidence of heterozygotes in the SNP rs2609428 was

statistically higher (p=0.006) in the group without CLP with DA (Group 3, n=5; 19.2%) than in the group with CLP with DA (Group 1, n=0). The prevalence of mutated heterozygotes/homozygous in groups without CLP (Groups 3 and 4, n=7; 10%) was higher (p=0.028) than in groups with CLP (Groups 1 and 2, n=1; 1.3%).

The SNP rs35951442 replaces C allele (ancestral) with T allele (mutated) in position 70644703 of chromosome 4. In the general population, 100% of genomes are homozygous for the ancestral allele (C/C). In this study, 100% of individuals of the four studied groups were homozygous for the ancestral allele.

The frequency values of alleles in the American and in the general population were based on ENSEMBL[13]. The results of genotyping of SNPs rs3796703, rs3796704, rs3796705, rs7671281, rs2609428, and rs35951442 in the ENAM gene were described in Tables 2, 3, and 4.

Table 2. Summary of genotyping results for detection of SNPs rs3796703, rs3796704, rs3796705, rs7671281, rs2609428, and rs35951442 in ENAM gene

Groups	n	rs3796703			rs3796704			rs3796705			rs7671281			rs2609428			rs35951442		
		AH	HT	MH	AH	HT	MH	AH	HT	MH	AH	HT	MH	AH	HT	MH	AH	HT	MH
1	43	42	1	0	35	8	0	43	0	0	0	8	35	43	0	0	43	0	0
2	34	34	0	0	27	7	0	34	0	0	0	8	26	33	1	0	34	0	0
3	26	26	0	0	21	5	0	26	0	0	0	7	19	21	5	0	26	0	0
4	44	44	0	0	36	8	0	44	0	0	0	7	37	42	2	0	44	0	0
TOTAL	147	146	1	0	119	28	0	147	0	0	0	30	117	139	8	0	147	0	0

Group 1 = with CLP and DA; Group 2 = with CLP and without DA; Group 3 = without CLP and with DA; Group 4 = without CLP and DA; AH = ancestral homozygote; HT = heterozygote; MH = mutated homozygote.

Table 3. Frequency of occurrence of the mutated allele of SNP rs2609428 in ENAM gene of the studied group.

Groups	n	AH	HT	MH
1	43	43 (100%)	**0 (0%) ***	0 (0%)
2	34	33 (97.1%)	1 (2.9%)	0 (0%)
3	26	21 (80.8%)	**5 (19.2%) ***	0 (0%)
4	44	42 (95.5%)	2 (4.5%)	0 (0%)
TOTAL	147	139 (94.6%)	8 (5.4%)	0 (0%)

Group 1 = with CLP and DA; Group 2 = with CLP and without DA; Group 3 = without CLP and with DA; Group 4 = without CLP and DA; AH = ancestral homozygote; HT = heterozygote; MH = mutated homozygote; *p=0.006 (Chi square test).

Table 4. Grouping of genotyping results for the detection of SNP rs2609428 in ENAM gene according to the presence of CLP

Groups	n	AH	HT + MH
1 + 2	77	76 (98,7%)	**1 (1.3%) ***
3 + 4	70	63 (90%)	**7 (10%) ***
TOTAL	147	139 (94.6%)	8 (5.4%)

Groups 1 + 2 = with CLP; Groups 3 + 4 = without CLP; AH = ancestral homozygote; HT = heterozygote; MH = mutated homozygote; *p=0.028 (Fisher's Exact Test).

Discussion

All stages of dental enamel formation (pre-secretion, secretion, transition, and maturation) must be well coordinated. This coordination includes not only components of the extracellular matrix secreted by ameloblasts, but also other genetic factors that influence on the function of ameloblasts[14]. Thus, the cause of enamel developmental defects may be or may be not genetically[15] associated to diseases during tooth development interfering with calcium phosphate metabolism. Some researchers suggest that cleft lip and palate can be involved in a broader dysmorphic spectrum of anomalies and is related with the presence of DA, with a direct relation between the appearance of the defect and the presence of cleft[12,16,17]. Considering that DA may be related to different alterations located in many genes involved in dental enamel formation, the study of genes that transcribe the main enamel proteins and proteases, as ENAM, is essential for better understanding these alterations, as proposed by this study.

The enamelin gene, ENAM (OMIM 606585), is located on chromosome 4q13.3 and is also known as ADAI, AI1C, and AIH2. This gene has 14 exons, but only nine participate in the four different transcripts[18]. Enamelin is produced by ameloblasts during the secretory stage concentrating near the Tomes process[19] and is essential for the formation and growth of enamel crystals[20,21]. Enamelin is present in small amounts, as a series of products cleaved to generate multiple polypeptides participating in the enucleation, extension, and regularization of the enamel crystals[22]. The enamelin gene encodes the largest (200kDa) and least abundant (3 - 5%) protein among the top three proteins of the extracellular matrix in enamel development[23]. The ENAM gene was mapped on chromosome 4 and only 15Kb differs ENAM from the ameloblastin gene, suggesting that this region contains a set of enamel protein-coding genes[24]. Enamelin acts as a support and adhesion molecule in the differentiation of ameloblasts[25].

Mutations in ENAM gene can result in type 1B and 1C amelogenesis imperfecta (AI) (OMIM 104500 AI1B and OMIM 204650 AI1C)[14]. AI is a clinically and genetically heterogeneous group of hereditary defects of the dental enamel[26]. Amelogenesis occurs due to the genetic regulation of secretion, organization, and processing of dental enamel extracellular matrix development[27]. The mutations in ENAM gene can cause enamel hypoplasia[28], but the severity of enamel defects is sometimes highly variable, even among individuals with the same mutation[14]. To this date, no clear explanation exists to support either the penetrance absence or the extremely moderate phenotype in an individual. Other genetic factors, such as not yet identified cis or trans elements, could regulate and influence the expression and function of protein enamelin. More genetic and functional studies are necessary to understand the dynamics and variable nature of enamel formation[14].

The SNPs in ENAM gene were selected considering the location in the gene where they necessarily should be inserted (exonic regions), the type of mutation (necessarily missense), the frequency within the population, and the availability in trials produced and validated for genotyping by a well-established company. Six SNPs

were selected for this study, all of them located at exon 9 of ENAM gene, which is the largest exon of the gene, including 2,841 nucleotides. According to the results, no statistically significant differences were found in mutant homozygous and/or heterozygous frequency of SNPs rs3796703, rs3796704, rs3796705, rs7671281, and rs35951442 in ENAM gene between the study groups. The incidence of heterozygotes in the SNP rs2609428 was higher (p=0.006) in the group without CLP with DA (Group 3) than in the group with CLP with DA (Group 1). The prevalence of mutated heterozygotes/homozygous in the groups without CLP (groups 3 and 4) was higher (p=0.028) than the groups with CLP (Groups 1 and 2). Based on these results, we suggested that the SNP rs2609428 in ENAM gene may be associated with DA in the absence of CLP. It is known that mutations in the ENAM gene are related to AI, but the literature lacks studies that correlates mutations or polymorphisms in the ENAM gene with the DA and CLP.

Another study showed that the SNP rs3796704 is associated with MIH (molar-incisor hypomineralization)[29]. Researchers investigated the genotype-phenotype correlation between mutant mice and wild AI controls caused by mutations in the AMELX and ENAM genes[29]. Daubert et al.[30] (2016) reported the first direct evidence of ENAM gene role in the adaptive evolution of human species and Gerreth et al.[31] (2016) demonstrated that variant in ENAM gene is a strong candidate gene to caries susceptibility in primary teeth. This was the first study in humans associating ENAM gene, CLP, and DA. Further studies using the method proposed by this research are necessary to investigate mutations and polymorphisms, including SNPs in coding and splicing areas of candidate genes to the formation of enamel defects, to elucidate the consequences of these changes in the final protein.

The understanding of the molecular bases of genetically complex disorders as more common diseases will enable the diagnosis of the disease susceptibility prior to its onset in individuals at risk, aiming at starting intervention strategies. The application of molecular biology techniques aims at the early detection and diagnosis of the disease. The professionals will soon be able to prevent diseases and promote health effectively through these diagnostic techniques. The identification of molecular targets of environmental agents will help understanding the disease process and developing appropriate treatment strategies. In such cases, understanding the cause of the disease will have a key role in pre-symptomatic testing to identify and determine effective treatment strategies for individuals at risk. This information could contribute to a better understanding of the mechanism involved in dental anomalies, which will be useful in preventive and curative therapies. The prediction is that treatment will be more personalized and adopted based on the individual's genome.

Conclusion

Based on these results, we suggest that SNP rs2609428 marker of ENAM gene may be associated with dental anomalies in individuals without cleft lip and palate.

Acknowledgements

The authors would like to acknowledge the financial support of Sao Paulo Research Foundation (FAPESP; FVO grant #2011/21190-7, TMO grant #2009/12233-4, and CFS grant #2008/08927-8).

Conflict of interests

None.

Data availability

Datasets related to this article will be available upon request to the corresponding author.

Authors contribution

Fernanda Veronese Oliveira – have made substantial contributions to conception and design, acquisition of data, the manuscript's findings, analysis and interpretation of data. And have been involved in drafting the manuscript or revising it critically for important intellectual content., and have revised and approved the final version of the manuscript.

Carlos Ferreira Santos – Contributed substantially to the conception and design of the study, the acquisition of data, the manuscript's findings, and the analysis and interpretation, have revised and approved the final version of the manuscript.

Thiago José Dionísio - Contributed substantially to the conception and design of the study, the acquisition of data, the manuscript's findings, and the analysis and interpretation, have revised and approved the final version of the manuscript.

Lucimara Teixeira Neves - agree to be accountable for all aspects of the work as the manuscript's findings, in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved, have revised, and approved the final version of the manuscript.

Gisele da Silva Dalben - the manuscript's findings, drafted or provided critical revision of the article. Provided final approval of the version to publish. Agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Eloá Cristina Passucci Ambrosio - the manuscript's findings, drafted or provided critical revision of the article. Provided final approval of the version to publish. Agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Paula Karine Jorge - the manuscript's findings, drafted or provided critical revision of the article. Provided final approval of the version to publish. Agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Maria Aparecida Andrade Moreira Machado - the manuscript's findings, drafted or provided critical revision of the article. Provided final approval of the version to publish. Agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Thais Marchini Oliveira - the manuscript's findings, drafted or provided critical revision of the article. Provided final approval of the version to publish. Agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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