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Original Article

Polymorphisms in genes involved in enamel development are associated with dental fluorosis

Erika Calvano Küchler[a,1], Patricia Nivoloni Tannure[b,f,1], Daniela Silva Barroso de Oliveira[a], Senda Charone[c], Paulo Nelson-Filho[a], Raquel Assed Bezerra da Silva[a], Marcelo de Castro Costa[d], Leonardo Santos Antunes[e,f], Mônica Diuana Calasans Maia[f], Lívia Azeredo Alves Antunes[e,f,*]

[a] Department of Pediatric Dentistry, School of Dentistry of Ribeirão Preto, University of São Paulo, Ribeirão Preto, São Paulo, Brazil
[b] School of Dentistry, Veiga de Almeida University, Rio de Janeiro-RJ, Brazil
[c] Department of Biological Sciences, Bauru School of Dentistry, University of São Paulo, Bauru, São Paulo, Brazil
[d] Department of Pediatric Dentistry and Orthodontics, School of Dentistry, Federal University of Rio de Janeiro, Rio de Janeiro-RJ, Brazil
[e] Department of Specific Formation, School of Dentistry, Fluminense Federal University, Nova Friburgo, Rio de Janeiro, Brazil
[f] Postgraduate Program in Dentistry, Fluminense Federal University, Niterói, Brazil

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ABSTRACT

Objective: To evaluate the association between polymorphisms in DLX1, DLX2, MMP13, TIMP1 and TIMP2 genes with dental fluorosis (DF) phenotype.
Design: Four hundred and eighty one subjects (108 with DF and 373 DF free) from 6 to 18 years of age were recruited. This population lived in Rio de Janeiro, a city with fluoridation of public water supplies. DF was assessed using the Dean's index modified. Only erupted permanent teeth were assessed. Genetic polymorphisms in *DLX1, DLX2, MMP13, TIMP1* and *TIMP2* were analyzed by real-time PCR from genomic DNA. Association between DF, genotype, and allele distribution were evaluated using chi-square and logistic regression analyses with an alpha level of 5%.
Results: DF was more prevalent in Afro-descendants than in Caucasians ($p = 0.08$; OR = 1.83; CI 95% = 1.18–2.82). Logistic regression analysis adjusted by the ethnicity demonstrated a statistical difference for *TIMP1* genotype ($p = 0.033$; OR = 2.93, 95%CI, 1.09–7.90). When only the severer cases of DF were analyzed, polymorphisms in *DLX1* and *DLX2* were associated with DF ($p < 0.05$).
Conclusion: Our results provided evidence that polymorphisms in *TIMP1, DLX1* and *DLX2* genes may be associated with DF phenotypes.

1. Introduction

Dental fluorosis (DF) occurs as a result of excess fluoride ingestion during enamel formation (Bronckers, Lyaruu, & DenBesten, 2009; DenBesten & Li, 2011). Many of the changes caused by excess fluoride are related to cell, matrix, and mineral interactions while enamel is being formed (Aoba & Fejerskov, 2002; Bronckers et al., 2009). During the early maturation stage, the relative quantity of protein is increased in fluorosed enamel in a dose-related manner (DenBesten, 1986). This appears to result from a delay in the removal of protein during enamel maturation (DenBesten et al., 2002; DenBesten, 1986).

Genetic factors influence DF susceptibility in mice and humans. Two mice strains have been used to identify loci associated with DF susceptibility (Everett et al., 2009; Everett, Yin, Yan, & Zou, 2011). The "susceptible" strain has a severe development of DF, while the "resistant" strain has a minimum development of DF (Everett et al., 2002). Studies in humans with endemic DF confirm the genetic influence in DF etiology (Ba et al., 2009; Ba et al., 2011; Huang et al., 2008; Jiao, Mu, Wang, An, & Jiang, 2013; Jiang, Mu, Wang, Yan, & Jiao, 2015; Wen et al., 2012; Zhang et al., 2013).

Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) are involved with tissue remodeling

* Corresponding author at: Fluminense Federal University, Rua Doutor Silvio Henrique Braune, 22 – Centro, 28625-650, Nova Friburgo, Rio de Janeiro, Brazil.
E-mail addresses: erikacalvano@gmail.com (E.C. Küchler), pntannure@gmail.com (P.N. Tannure), barrosodaniela@hotmail.com (D.S.B.d. Oliveira), sendacharone@yahoo.com.br (S. Charone), nelson@forp.usp.br (P. Nelson-Filho), pttpo2009@yahoo.com.br (M.d.C. Costa), leonardoantunes@id.uff.br (L.S. Antunes), monicacalasansmaia@gmail.com (M.D. Calasans Maia), liviaazeredo@gmail.com (L.A.A. Antunes).
[1] These authors contributed equal to this work.

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and extracellular matrix degradation. MMPs and TIMPs participate in the enamel formation, and regulate biomineralization by controlling the proteoglycan turnover (Hannas, Pereira, Granjeiro, & Tjäderhane, 2007). *MMP13* has been previously associated with caries risk (Tannure et al., 2012).

Distal-less homologs, the DLX genes, are expressed during initial stages of the odontogenesis, and enamel formation. DLX have a participation in establishment of dental epithelial functional organization and the control of enamel morphogenesis via regulation of amelogenin expression (Lézot et al., 2008; Zhao, Stock, Buchanan, & Weiss, 2000).

Based on these evidences, polymorphisms in genes expressed during enamel formation may be involved in the DF etiology. Therefore, the aim of this study was to investigate the association between polymorphisms in *DLX1, DLX2, MMP13*, *TIMP1* and *TIMP2* genes with DF phenotype.

2. Materials and methods

The local Human Ethics Committee (113/09) approved this study. Informed consent was obtained from all participating individuals or parents/legal guardians.

2.1. Type of study and sampling

This cross-sectional eligible unrelated healthy subjects from 6 to 18 years of age recruited at the Pediatric Dental Clinics, Federal University of Rio de Janeiro. This population lived in Rio de Janeiro, a city with fluoridation of public water supplies. The subjects were divided in groups according presence or absence of DF. The current analysis used data from early-erupting permanent teeth. For the mixed dentition, only erupted permanent teeth were assessed.

2.2. Sample characterization

The following data were obtained: age, gender, dentition, ethicinity. The ethnicity definition was ascertained based on self-reported information. The institution where the subjects were recruited is located in the Southeast of Brazil, the most densely heterogeneous populated and industrialized region of the country.

2.3. Determination of dental fluorosis phenotype

Trained examiners conducted dental examinations. The kappa scores on intra- and inter-examiner were between good to excellent; weighted kappa scores were 1.00 for intra-examiner reliability and 0.89 for inter-examiner reliability. Subjects were seated in a dental chair, and the examiner used a probe and dental mirror according to the criteria recommended by the World Health Organization guidelines (WHO, 2013). DF was assessed using the Dean's index modified (Rozier, 1994) with the examination and a score was registered. This index allows the classification of DF into three degrees: mild (very mild and mild), moderate, and severe. The questionable degree was excluded.

2.4. DNA samples and genotyping

Genomic DNA for molecular analysis was extracted from buccal cells based on the reported method (Küchler et al., 2012). Genetic polymorphisms in *DLX1, DLX2, MMP13, TIMP1* and *TIMP2* were genotyped by real time polymerase chain reactions (Real Time PCR) using the Taqman assay (Agilent Technologies, Stratgene Mx3005P) (Shen, Abdullah, & Wang, 2009). The selected genes are expressed in at least one stage of the enamel development (http://bite-it.helsinki.fi/). The characteristics of the studied polymorphisms are presented in Table 1.

2.5. Statistical analysis

The data were analyzed using the Epi Info 3.5.7. The *t* test, chi-square test and odds ratio calculations were used to compare age, ethnicity, gender, between DF group and DF free group.

Chi-square or Fisher's exact tests and odds ratio, at a level of significance of 0.05, were used to compare allele and genotype distributions between 'All DF' and 'DF free' groups and between 'Moderate plus severe DF' and 'DF free'. For TIMP1, gender was used as a covariant in the model because is a gene in a sex chromosome.

Logistic regression analysis was also implemented for all polymorphisms analysed using ethnicity as covariate in order to test the possibility of ethnicity background influence. A level of significance of 0.10 was used for multivariate analysis.

Hardy-Weinberg equilibrium was evaluated using the chi-square test within each polymorphism.

3. Results

From 618 subjects evaluated, 481 subjects were included in this study (108 subjects with DF and 373 DF free subjects). The dropout due to questionable degree of DF was 8. Table 2 summarizes the characteristics of the studied population. There were no significant differences in age and gender between the groups ($p > 0.05$). DF was more prevalent in Afro-descendants than in Caucasians ($p = 0.008$; OR = 1.83; CI 95% = 1.18–2.82). Mild DF affected 85 subjects (78.7%), moderate DF affected 18 subjects (16.6%) and the severe DF affected 5 subjects (4.7%).

Table 3 presents the genotypes frequency distribution of the genes among the groups. We were not able to identify any statistical difference of genotype and allele distribution between 'All DF' and 'DF free' groups ($p > 0.05$); however, a borderline association was observed between *TIMP1* and genotype distribution ($p = 0.073$). When only the severer cases of DF (Mild DF plus Severe DF) were analyzed, genotype distribution was different in *DLX1* gene, in which GG genotype was less frequent in the DF free group ($p = 0.048$). In *DLX2* gene, genotype and allele distributions were different among the groups ($p = 0.022$ and $p = 0.013$; respectively).

Table 4 demonstrated the results of the logistic regression analysis adjusted by the ethnicity, statistical difference was

Table 1
Candidate studied genes and polymorphisms.

Gene	Polymorphism	*Locus*	Location in the gene	Base Change	Alteration	MAF
DLX1	rs788173	2q31.1	UTR	A/**G**	unknown	0.403
DLX2	rs743605	17q24.1	UTR	A/**G**	upstream variant	0.316
MMP13	rs2252070	11q22.3	promoter	**A**/G	up regulation[a]	0.364
TIMP1	rs4898	Xp11.23	Intron	**C**/T	unknown	0.469
TIMP2	rs7501477	17q25	promoter	**G**/T	down regulation[a]	0.153

Note: Bold form indicates ancestral allele.MAF means Minor Allele Frequency obtained from databases: http://www.ncbi.nlm.nih.gov.
[a] Change in transcription.