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Genes involved in the enamel development are associated with calcium and phosphorous level in saliva

Erika Calvano Kuchler[1], **Giovana Daniela Pecharki**[2], **Myrella Lescio Castro**[3], **Junia Ramos**[3], **Fernando Barbosa Jr**[4], **João Armando Brancher**[5], **Alexandre Rezende Vieira**[6], **Raquel Fernanda Gerlach**[3], and **Paula Cristina Trevilatto**[7]

[1]Department of Pediatric Dentistry, Ribeirao Preto Dental School, USP - University of Sao Paulo, Ribeirao Preto, SP, Brazil

[2]Department of Community Health, Federal University of Parana, Curitiba, Brazil

[3]Department of Morphology, Physiology and Basic Pathology, Ribeirao Preto Dental School, USP - University of Sao Paulo, Ribeirao Preto, SP, Brazil

[4]Department of Clinical, Toxicological and Bromatological Analysis, Faculty of Pharmacy of Ribeirao Preto, University of Sao Paulo (FCFRP/USP), Ribeirao Preto, SP, Brazil

[5]Department of Dentistry, Universidade Positivo, Curitiba, Brazil

[6]Department of Oral Biology, University of Pittsburgh, Pittsburgh, PA

[7]Center for Health and Biological Sciences, Pontifícia Universidade Católica do Paraná (PUCPR), Curitiba, Brazil

Abstract

Saliva components play a crucial role in the integrity of the dental enamel and in the caries susceptibility. The saliva characteristics are controlled by many factors, including genetic factors. Therefore, this study aimed to evaluate the association between the genetic variations in genes expressed in enamel development with calcium and phosphorous levels in saliva. We collected 276 unrelated 12-year-old children from private and public schools. Saliva was collected for DNA extraction from oral cells and for measurement of calcium and phosphorous. Inductively Coupled Plasma-Mass Spectrometry determined calcium and phosphorous levels in whole saliva. Fifteen genetic variations in 9 genes were analyzed. Genotype was determined by real time polymerase chain reactions. Data were analyzed using Plink with an alpha of 5%. Genetic variations in *AMELX*, *AMNB* and *ESRRB* were associated calcium level in saliva ($p<0.05$). A borderline association was observed in *ENAM* allele distribution showed with phosphate level in saliva

Corresponding Author: Paula Cristina Trevilatto, Center for Health and Biological Sciences, Pontifícia Universidade Católica do Paraná (PUCPR), Curitiba, Brazil. paula.trevilatto@pucpr.br.

The authors have no conflicts of interest to declare.

Role of all authors

JAB, PCT and GPD performed activities related to subject recruitment, phenotype definitions, and biological sample collection. ARV and ECK performed the genotypes. MLC, FB, JR and RFG evaluated the calcium and phosphate levels. ARV, PCT and RFG conceptualized and designed the study. ECK analyzed and interpreted the data, and wrote the paper. All authors critically revised the manuscript.

(p=0.049). In conclusion, our results is the first to report that genetic variations contribute to calcium and phosphorous levels in saliva

Keywords

Enamel; polymorphism; saliva

Introduction

Dental enamel is the hardest substance in the human body and contains the highest percentage of minerals. The mineralization of the dental enamel is a complex process involving the sequential passage of mineral ions across cell and fluid barriers, in which several key genes are involved in the enamel formation stages, mediated by the ameloblasts [Lacruz et al., 2012].

It is well known that, in the oral cavity, a high concentration of calcium phosphate salts is dissolved in saliva [Burstein et al., 1979]. The saliva components play a crucial role in the integrity of the dental enamel as well as in caries susceptibility, since saliva needs to be supersaturated in relation to calcium and phosphate, otherwise enamel apatite will dissolve. Thus, saliva helps in the enamel stability and protection [Hay et la., 1986; Larsen and Pearce, 2003].

Calcium and phosphate levels in saliva are controlled by many factors, including host factors. Genes involved in the enamel development are candidates for altered levels of calcium and phosphorous in saliva. Therefore, the present study aimed to evaluate genetic variations in genes expressed in enamel development and their association with calcium and phosphorous levels in saliva.

Methods

Studied population and clinical examination

The Human Ethics Committee of the PUC-PR and the Pittsburgh Institutional Review Board approved this study. Informed written consent was obtained from the parents and age appropriate assent document were used for all children.

Two hundred seventy–six biological unrelated children, with no syndrome and/or systemic illness, were included. They were 12-year-old, both sex students from private and public schools of Curitiba, PR, Brazil. This population was previously described [Brancher et al., 2011].

Saliva collection measurements

Salivary flow was evaluated by means of stimulated saliva collection. The method used was that of mechanical masticatory stimulation, using a piece of sterile rubber tourniquet of a standardized size (1.5 cm), masticated continuously by the patient for 6 min. Saliva produced during the first minute of stimulation was discarded. During the following 5 min, the patient expelled saliva into a sterilized universal collecting recipient that had been

previously weighed using Marte_analytical scales, model AL 500 (São Paulo, SP, Brazil). Stimulated salivary flow rate was evaluated by means of the gravimetric method and expressed in millilitre per minute (Banderas-Tarabay et al., 1997). Immediately following saliva collection, the salivary pH was assessed using a QUIMIS_Q400BD pocket pH meter (direct electrode) (QUIMIS, Diadema, SP, Brazil).

Calcium and phosphorous levels in whole saliva were determined at the Laboratory of Metals Toxicology, University of São Paulo in Ribeirão Preto (Brazil), by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS).

The remaining saliva was used for genomic DNA extraction from buccal cells for genotyping analyses. The extraction was performed according to an established protocol [Aidar and Line, 2007].

Phenotypes determination

The phenotype determination of calcium and phosphorous was based in their values. Calcium and phosphorous were categorized as ‘low’ and ‘high’ level based on the medium value of each variable. Subjects with salivary calcium levels over the median value (9.84 micrograms/ml) were included in the ‘high salivary calcium group’. Subjects with salivary phosphorous levels over the median value (126.81 micrograms/ml) were included in the ‘high salivary phosphorous group’.

Caries phenotype was diagnosed using a modified World Health Organization guidelines recommended for oral health surveys (WHO). Teeth lost to trauma or primary teeth lost to exfoliation were not included in the final DMFT scores. Carious lesions were recorded as present when a break in enamel was apparent on visual inspection. All the examiners carried out the clinical examination after being calibrated by an experienced specialist [Brancher et al., 2011]. The DMFT score ranged between 0 to 9 and 140 children were caries free.

Genotyping

Fifteen genetic variations were selected within 9 candidate genes. The selected genes were expressed during enamel formation and/or in the salivary gland. The studied genetic variations are described in table 1. Genotype was performed by polymerase chain-reactions with the Taqman method with the real-time PCR system ABI PRISM® 7900HT Sequence Detection System (Foster City, CA, USA). Probes were supplied by Applied Biosystems (Foster City, CA, USA).

Statistical analyses

Calcium and phosphorous values were analyzed as continuous variables and as dicothomous variables ‘low’ (n=138) and ‘high’ (n=138). Differences in genotype and allele frequencies were tested for all phenotypes. A genotype analysis was also performed in a recessive model. The differences between ‘low’ and ‘high’ groups were tested using chi-square or Fisher’s exact tests were used with an established alpha of 5%. The t-test was used to compare the difference between the means. Hardy-Weinberg equilibrium was evaluated using the chi-square test within each SNP.

Results

All markers studied were in Hardy-Weinberg equilibrium (data not shown). Mean calcium level was 11.34 (±6.77); while the median (Q1–Q3) were 9.84 (6.77–13.65). Mean phosphate level was 133.49 (±36.94); while the median (Q1–Q3) were 126.81 (109.32–149.47). Gender, ethnicity, age, oral hygiene habits, dietary factors and caries experience were not associated with calcium and phosphorous level in saliva (table 2). The population characteristics and distribution between low and high calcium and phosphorous groups are presented in table 2.

The group with low phosphorous level had an increased salivary flow (p=0.012). The group with high calcium level presented a more acid salivary pH (p=0.023). Phosphorous level exhibited a borderline association with pH (p=0.056).

Table 3 demonstrated the genotype and the allele distribution between low and high calcium level. Allele distribution in *AMELX* was statistically different between groups (p=0.021). For *AMBN*, both, genotype and allele distribution were statistically different between the high and low calcium groups (p=0.025 and p<0.001).

Two polymorphisms in *ESRRB* (rs745011 and rs6574293) were also associated with calcium level (table 3). Also, in a recessive model, the polymorphism rs1077430 in *ESRRB* analysis demonstrated that AA genotype was associated with low calcium level (OR=2.3, 95% CI=1.1–5.1; p=0.036).

Table 4 demonstrated the genotype and the allele distribution between low and high phosphorous level. Phosphorous level was associated only with the allele distribution of the genetic variant in *ENAM* (p=0.049).

Discussion

Intraoral mineralization capacity has been a matter of scientific interest for decades. To the best of our knowledge, this is the first study that aimed to evaluate the role of the genetic background in the calcium and phosphorous levels in saliva. We evaluated genes involved in enamel development due the relationship that exists between the dental enamel and these components in saliva and caries susceptibility. To understand their relationship, it is important to know that the mature dental enamel is a crystalline structure containing up to 96% hydroxyapatite (HAP, $Ca_{10}(PO_4)_6(OH)_2$) by weight and with water and protein accounting for the other 4% [Mann and Dickinson, 2006].

Here, we found that genetic variations in genes involved in enamel development are associated with the calcium and phosphorous levels in saliva. Some of our previous studies investigated the association between these genes and caries susceptibility and enamel alteration [Deeley et al., 2008; Tannure et al., 2012; Shimizu et al., 2012; Jeremias et al., 2013; Abbasoğlu et al., 2015; Bayram et al., 2015]. *TUFT1* and *TUIP11* were associated with variations in enamel microhardness [Shimizu et al., 2012]. *ENAM* were associated with enamel hypoplasia [Jeremias et al., 2013]. *AMELX, TFIP11, ENAM, TUFT1, KLK4, AMBN* and *MMP20* also observed an association between some of these genes with caries

susceptibility in primary and permanent dentition [Deeley et al., 2008; Tannure et al., 2012; Shimizu et al., 2012 Jeremias et al., 2013; Abbasoğlu et al., 2015; Bayram et al., 2015].

Mature enamel contains enamel protein-specific peptides that can be recovered from mature contemporary teeth [Porto et al., 2006; Porto et al., 2011a], as well as from ancient teeth [Porto et al., 2011b] and fluorotic teeth [Porto et al. 2016]. Dissolution of the whole enamel allowed the identification of enamel protein-specific peptides [Castiblanco et al., 2015]. Superficial enamel etching with diluted hydrochoric acid and refinement of the recovery technique allowed the successful recovery of sex-specific peptides (dimorphic AMELX peptides) without destruction of the tooth crown [Stewart et al., 2016]. Peptides derived from AMELX splicing variants were also found, as well as AMBN, and ENAM peptides [Stewart et al., 2016]. It is very likely that in the near future the genetic basis of enamel formation can be associates with the differences either in aminoacid sequence or peptide concentration in the mature enamel.

Our results demonstrated that calcium level was associated with genetic variations in *AMELX, AMNB* and *ESRRB*. AMELX and AMNB are involved in the enamel mineralization. Mutations in both these genes are responsible for the *Amelogenesis Imperfecta* phenotype (OMIN), which support their link with enamel alterations as well as in the enamel mineralization. Also, *AMNB* gene is located in the calcium-binding phosphoprotein gene cluster in chromosome 4.

Two genetic variations evaluated in *ESRRB* were associated with calcium level in saliva. Our previous work aimed to explore the role of ESRRB in the oral tissues and found the association of this gene with caries susceptibility as well as its expression in the salivary gland and in the enamel development [Weber et al., 2014]. *ESRRB* is expressed in enamel development and could have a role in the calcium composition in the enamel. However, it is also possible that the association between *ESRRB* and calcium level in saliva is through the salivary gland function. Although we demonstrated that this gene is expressed in salivary gland [Weber et al., 2014], its role in salivary function is still unknown.

ESRRB is an estrogen-related receptor gene and presents similarities with the estrogen receptor. It is likely that this gene also participates in calcium balance. Calcium is a necessary component for the maintenance of healthy teeth. Calcium as well as phosphorous concentrations in saliva and plaque plays a key role influencing the tooth demineralization and remineralization processes. This process has a strong correlation with caries experience.

It is important to emphasize that other factors might be involved in calcium and/or phosphorous levels in saliva, however, in order to minimize our bias, the sample that we used in this work did not include children with systemic illnesses. In addition the groups with low and high calcium and phosphorous levels did not present statistical difference between caries experience and other environmental factors.

Briefly, to the best of our knowledge, this is the first study to evaluate the association between with calcium and phosphate levels in saliva. More studies should be performed to evaluate the role of other genes in saliva composition as well as in oral health.

Conclusion

Genetic variations in *AMELX, AMNB* and *ESRRB* are associated with calcium level in saliva, while genetic variation in *ENAM i*s associated with phosphorous in saliva.

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Table 1

Description of the studied genetic variations

Gene symbol	Gene name	Variant	Location	Base change	Average heterozygosity ± standard error	MAF
AMELX	*Amelogenin*	rs946252	Xp22.2	A/**G**	0.420±0.183	0.300
AMBN	*Ameloblastin*	rs4694075	4q13.3	C/**T**	0.499+0.022	0.478
AQP1	*Aquaporin 1*	rs17159702	7p14.3	C/**T**	0.493+0.057	0.443
AQP8	*Aquaporin 8*	rs2287798[a]	16p12.1	C/**G**	0.485+0.085	0.414
ENAM	*Enamelin*	rs12640848	4q13.3	**A**/G	0.459±0.137	0.357
ESRRB	*Estrogen-related receptor beta*	rs745011	14q24.3	C/**T**	0.486±0.082	0.417
		rs6574293		A/**G**	0.225±0.249	0.129
		rs1676303		C/**T**	0.326±0.238	0.205
		rs10132091		C/**T**	0.500±0.014	0.486
		rs2860216		C/**T**	0.422±0.182	0.302
		rs1997532		C/**T**	0.494±0.054	0.446
		rs4903399		**C**/T	0.293±0.246	0.178
		rs1077430		**A**/G	0.473±0.113	0.384
TFIP11	*Tuftelin interacting protein 11*	rs5997096	22q12.1	C/**T**	0.498±0.030	0.470
TUFT1	*Tuftelin*	rs4970957	1q21.3	**A**/G	0.366±0.222	0.241

Obtained from databases: http://www.ncbi.nlm.nih.gov; http://genome.ucsc.edu. MAF: Minor allele frequency; bold form indicates mutant allele.

Table 2

Characteristics of the studied subjects

Characteristics	Calcium		p value	Phosphorous		p value
	Frequency n(%)			Frequency n (%)		
	low (n = 138)	high (n = 138)		low (n = 138)	high (n = 138)	
Gender						
Male	76 (55.1)	66 (47.8)	0.139#	70 (50.7)	72 (52.2)	0.405#
Female	62 (44.9)	72 (52.2)		68 (49.3)	66 (47.8)	
Ethnicity						
Caucasian	126 (91.3)	127 (92.0)	0.415#	124 (89.9)	129 (93.5)	0.192#
Afro-descendents	12 (8.7)	11 (8.0)		14 (10.1)	9 (6.5)	
Age (SD)	12.0 (0.48)	11.9 (0.50)	0.800*	11.9 (0.53)	11.9 (0.45)	0.808*
Tooth brush frequency						
Once a day	73 (52.9)	69 (51.1)	0.384#	74 (53.6)	68 (50.4)	0.296#
Two or more times a day	65 (47.1)	66 (48.9)		64 (46.4)	67 (49.6)	
Use of dental floss						
No	48 (34.8)	43 (31.2)	0.627#	51 (37.0)	40 (29.0)	0.134#
Sometimes	66 (47.8)	74 (53.6)		70 (50.7)	28 (20.3)	
Yes	24 (17.4)	21 (15.2)		17 (12.3)	70 (50.7)	
Ingestion of sweeties between meals						
No	13 (9.4)	12 (9.6)	0.484#	15 (0.9)	12 (8.1)	0.281#
Yes	125 (90.6)	126 (90.4)		123 (89.1)	126 (91.9)	
DMFT/dmft index (SD)	1.31 (2.04)	1.50 (1.77)	0.432*	1.36 (2.10)	1.45 (1.70)	0.683*

#Chi-square test;

*t test.

Table 3

Genotype and allele distribution between calcium groups

Gene	rs#	Frequency		p value genotype	p value allele
		Low level dd/dD/DD[§]	High level dd/dD/DD[§]		
AMELX	rs946252	23/12/96	31/21/82	0.095	**0.021**
AMBN	rs4694075	42/65/26	25/73/39	**0.025**	<**0.001**
AQP1	rs17159702	19/54/52	13/57/43	0.482	0.986
AQP8	rs2287798	27/61/47	27/52/55	0.511	0.473
ENAM	rs12640848	22/15/55	14/23/64	0.154	0.214
ESRRB	rs745011	32/44/39	14/60/45	**0.007**	**0.028**
	rs6574293	4/24/100	0/15/114	**0.030**	**0.008**
	rs1676303	3/30/86	2/25/83	0.837	0.551
	rs10132091	22/66/44	22/68/42	0.962	0.944
	rs2860216	19/44/48	11/55/48	0.190	0.482
	rs1997532	6/28/36	8/23/36	0.701	0.922
	rs4903399	3/32/73	9/36/72	0.236	0.069
	rs1077430	21/51/54	10/64/51	0.065	0.438
TFIP11	rs5997096	26/74/33	24/75/36	0.903	0.398
TUFT1	rs4970957	2/53/74	5/42/82	0.226	0.307

[§]Uppercase letters denote the wild allele. Bold form indicates statistical difference.

Table 4

Genotype and allele distribution between phosphorus groups

Gene	rs#	Frequency		p value genotype	p value allele
		Low level dd/dD/DD[§]	High level dd/dD/DD[§]		
AMELX	rs946252	22/20/88	32/13/90	0.195	0.454
AMBN	rs4694075	29/73/33	36/65/34	0.540	0.605
AQP1	rs17159702	16/53/47	16/58/48	0.958	0.978
AQP8	rs2287798	24/61/49	30/52/53	0.463	0.968
ENAM	rs12640848	20/20/50	16/18/69	0.256	**0.049**
ESRRB	rs745011	27/47/45	19/57/39	0.257	0.771
	rs6574293	2/19/106	2/20/108	0.995	0.981
	rs1676303	2/31/83	3/24/86	0.575	0.844
	rs10132091	22/66/41	18/68/45	0.437	0.277
	rs2860216	20/44/50	10/55/46	0.096	0.497
	rs1997532	7/24/40	7/27/32	0.642	0.432
	rs4903399	8/33/66	4/35/79	0.363	0.210
	rs1077430	15/48/57	16/67/48	0.176	0.163
TFIP11	rs5997096	31/69/32	19/80/37	0.135	0.147
TUFT1	rs4970957	1/47/80	6/48/76	0.159	0.273

[§]Uppercase letters denote the wild allele; Bold form indicates statistical difference.