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Genes Expressed in Dental Enamel Development Are Associated with Molar-Incisor Hypomineralization

Fabiano Jeremias[1], **Mine Koruyucu**[3], **Erika C. Küchler**[4], **Merve Bayram**[3], **Elif B. Tuna**[3], **Kathleen Deeley**[4], **Ricardo A. Pierri**[1], **Juliana F. Souza**[1], **Camila M.B. Fragelli**[1], **Marco A.B. Paschoal**[1], **Koray Gencay**[3], **Figen Seymen**[3], **Raquel M.S. Caminaga**[2], **Lourdes dos Santos-Pinto**[1], and **Alexandre R. Vieira**[4,5]

[1]Department of Pediatric Dentistry School of Dentistry of Araraquara, São Paulo State University (UNESP), Araraquara, SP, Brazil

[2]Department of Morphology, School of Dentistry of Araraquara, São Paulo State University (UNESP), Araraquara, SP, Brazil

[3]Department of Pedodontics, Faculty of Dentistry, Istanbul University, Istanbul, Turkey

[4]Department of Oral Biology School of Dental Medicine, University of Pittsburgh, Pittsburgh, PA, USA

[5]Department of Pediatric Dentistry, School of Dental Medicine, University of Pittsburgh, Pittsburgh, PA, USA

Abstract

Genetic disturbances during dental development influence variation of number and shape of the dentition. In this study, we tested if genetic variation in enamel formation genes is associated with molar-incisor hypomineralization (MIH), also taking into consideration caries experience. DNA samples from 163 cases with MIH and 82 unaffected controls from Turkey, and 71 cases with MIH and 89 unaffected controls from Brazil were studied. Eleven markers in five genes [ameloblastin (*AMBN*), amelogenin (*AMELX*), enamelin (*ENAM*), tuftelin (*TUFT1*), and tuftelin-interacting protein 11 (*TFIP11*)] were genotyped by the TaqMan method. Chi-square was used to compare allele and genotype frequencies between cases with MIH and controls. In the Brazilian data, distinct caries experience within the MIH group was also tested for association with genetic variation in enamel formation genes. The *ENAM* rs3796704 marker was associated with MIH in both populations (Brazil: p=0.03; OR=0.28; 95% C.I.=0.06–1.0; Turkey: p=1.22e–012; OR=17.36; 95% C.I.=5.98–56.78). Associations between *TFIP11* (p=0.02), *ENAM* (p=0.00001), and *AMELX* (p=0.01) could be seen with caries independent of having MIH or genomic DNA copies of *Streptococcus mutans* detected by real time PCR in the Brazilian sample. Several genes involved in enamel formation appear to contribute to MIH.

Corresponding Author: Alexandre R. Vieira, 614 Salk Hall, Dept. Oral Biology, School of Dental Medicine, University of Pittsburgh, 3501 Terrace St., Pittsburgh, PA, 15261, USA, Phone # 412-383-8972; FAX # 412-624-3080; arv11@pitt.edu.

Keywords

Dental Enamel; Amelogenesis; Dental Enamel Hypoplasia; Dental Caries; Dental Caries Susceptibility

Introduction

The dental enamel is the most mineralized tissue of the human body, characterized by a highly complex developmental process (1). This process involves ameloblasts, which are known by their higher sensitive (2,3). The amelogenesis phase of the enamel development is under strict genetic control, and the size, shape, shade, caries susceptibility (4), and even enamel microhardness (5) can be affected by genetic variation. Different kinds of enamel defects may occur depending on the stage of development affected. The dental defect known as enamel hypoplasia is the result of the reduction of enamel thickness, which occurs during the secretory phase of amelogenesis. When ameloblasts are affected in the late amelogenesis stage of mineralization or maturation, a defect in the enamel translucence can occur. These defects are called enamel hypomineralization (6,7). A common pattern of enamel hypomineralization affects molars and incisors. This dental defect is called the molar incisor hypomineralization (MIH). Clinically, it presents asymmetric severity with demarcated opacities that vary in color shade from white to yellow/brownish, with sharp demarcation between the affected and sound enamel (8).

The first report of MIH dates from the late 70′s (9). In 2001, this defect was given a new name (MIH) with the definition of a “systemic hypomineralization” that affects one or more permanent first molars with or without permanent incisor involvement (10). The main characteristic of teeth with MIH is porous enamel that can be easily damaged due to masticatory forces. This can result in exposed dentinal tissues that may facilitate the development of carious lesions hence MIH is associated with caries (8, 11–17).

Children with MIH could present more intense dental sensitivity due to temperature variations (18). This is the result of the combination of the chronic pulp inflammation and innervation of the region right under the hypomineralized area (19). Consequently, children with MIH may have hampered anesthetic action, which can affect their behavior.

There is no conclusive data in relation to the etiology of MIH (17). Considering that the entire enamel formation process is under genetic control[4], it is reasonable to hypothesize that genetic variations could be associated with alterations in the amelogenesis. Genetic mutations have been associated with different kinds of amelogenesis imperfecta (20). Also, susceptibility to caries has been associated with genetic variation (5,21–29). Therefore, we hypothesize that variation in genes involved in the enamel formation contributes to increased MIH experience in humans.

Subjects and Methods

Subject Screening and Sample Collection

Two cohorts, one from Brazil and the second from Turkey, were studied. Biologically unrelated subjects were recruited as approved by the São Paulo State University (#45/10), Istanbul University (2006/2508), and University of Pittsburgh (PRO0710045 and PRO12080056) Institutional Review Boards and informed consent/assent was obtained from all participants and their parents. Eligible individuals were enrolled in the Pedodontics Clinics of São Paulo State University and Istanbul University and in daycare facilities in Araraquara, Brazil (n=160) and Istanbul, Turkey (n=245). The exclusion criteria included

having evidence of a syndrome, fluorosis, or use of a fixed appliance. Calibrated examiners carried out the clinical examination. In Brazil, F.J. was calibrated by L.S-P. In Turkey, E.B.T. calibrated M.B. Exam calibrations were performed according to the following protocol: First, the calibrator presented to the examiner the criteria for MIH detection, showing pictures of several situations to be observed in the exam and discussing each of these situations in a session that lasted one to two hours. Next, the calibrator and examiner(s) examined 10 to 20 subjects and discussed each case. In Brazil, the intraexaminer agreement was assessed by a second clinical examination in 10% of the sample after two weeks, with a kappa of 1.0. In Turkey, E.B.T. and M.Y. pre-screened subjects, and M.B. performed the full exam. The MIH diagnosis was performed according to the EAPD (European Association of Paediatric Dentistry) criteria (12). Cases were defined as a subject with MIH phenotype, while controls were defined as subjects with no evidence of MIH (including no evidence of fluorosis). Clinical examinations were done with the use of a flashlight and mouth mirror. In addition, in the Brazilian site, caries experience data (DMFT/dmft) was collected according to established protocols. In brief, visible lesions in dentin, as well as visible active lesions in enamel (white spots) and failed restorations with decayed tissue were scored as decayed. An explorer was gently used for assessing the smoothness of tooth surfaces. Gauze was used to dry and clean teeth prior to exam. Artificial light and a dental operatory were used for all evaluations. White spot lesions were distinguished from developmental enamel defects simply on clinical grounds based on the association of the lesions with areas of mature plaque and location on the tooth (i.e., white lesion appearing to be slightly supragingival, either associated with slight gingival inflammation or healthy gingival tissue, combined with the aspect of the lesion when dry (white chalk looking versus dry). No radiographs were available. Exam calibrations were performed according to the following protocol: First, the calibrator (L.S-P.) presented to the examiner (F.J.) the criteria for caries detection, showing pictures of several situations to be observed in the exam (sound and decayed tooth surfaces, filled teeth with and without secondary lesions, missing teeth due to caries or due to other reasons) and discussed each of these situations in a session that lasted one to two hours. Next, the calibrator and examiner(s) examined 10 to 20 subjects and discussed each case. Drinking water in the Istanbul region is not artificially fluoridated. Demographical characteristics of the enrolled subjects are presented in Table 1.

Unstimulated saliva samples were obtained from all participants (subjects were asked to spit) and they were stored in Oragene DNA Self-Collection kits (DNA Genotek Inc.) at room temperature until being processed. No centrifugation was performed in the saliva samples. No plaque samples were collected. DNA was extracted according to the manufacturer's instructions.

Genotyping

Eleven single-nucleotide polymorphism (SNP) markers were selected in genes involved in enamel formation (Table 2) and genotyped using predesigned TaqMan™ genotyping assays (30). For all TaqMan assays, DNA amplification was carried out in 3.0μl volume containing 1.0 μl of 2.0 ng/μl of DNA and 2.0 μl of primers and TaqMan master mix. The amplifications were made in a GeneAmp® PCR System 9700 (Perkin Elmer Applied Biosystems, Foster City, California, USA) and fluorescent signals detected at a 7900 HT Sequence Detection System (Applied Biosystems, Foster City, California, USA).

Presence of Genomic DNA of Streptococcus mutans

To generate data on *Streptoccocus mutans* colonization in the Brazilian cohort that had caries data, we interrogated DNA samples extracted from human saliva for evidence of copies of genomic DNA of the bacteria. Real-time PCR was performed by the use of the

ABI PRISM 7900HT Fast Real-Time PCR System (Applied Biosystems). Each reaction tube contained 10 ul of reaction mixture, including 1 X SYBR Green PCR buffer, 0.025 U/ul of AmpliTaqGold DNA polymerase, 0.01 U/ul of AmpErase UNG (uracil N-glycosylase), 1.2 mM of each of the dNTPs, 3mM MgCL2 (SYBR Green PCR Core Reagents, Applied Biosystems), 2 ul of human DNA extracted from saliva samples and 0.8 mM of each primer specific to *Streptococcus mutans* (forward 5'AGCCATGCGCAATCAACAGGTT3' and reverse 5'CGCAACGCGAACATCTTGATCAG3'). The specificity of these primers was tested previously (31) and *Streptococcus mutans* genomic DNA (*Streptococcus mutans* ATCC® 25175) was included in all reactions as positive control and standard DNA curves were generated. The cycling conditions were 2 minutes at 50°C for uracil *N-glycosylase* (this treatment prevents carryover cross-contamination by digesting uracil-containing fragments generated prior the PCR assay), 10 minutes at 95°C for activation of AmpliTaqGold, 40 cycles of 15 seconds at 95°C for denaturation and 1 minute at 68°C for annealing and extension. Values of the threshold cycle (Ct) above zero were considered positive for *Streptococcus mutans* infection based on the successful amplification of the target sequence. These values were correlated with DMFT scores and alpha of 0.05 was considered statistically significant.

Statistical Analysis

The χ^2, Fisher's exact, and Student *t* tests were performed to compare gender and age frequencies and to test for deviations in the allele and genotype distributions between the groups. Deviations from Hardy-Weinberg equilibrium were also examined also using the χ^2 test (tested both the MIH and control groups). Comparisons of allele and genotype frequencies between cases and controls were performed taking into consideration MIH detection, as well as considering MIH severity (mild versus severe). In mild cases there are demarcated enamel opacities without enamel breakdown. In severe cases there are demarcated enamel opacities with breakdown, caries, spontaneous hypersensitivity and strong aesthetic concerns (32). The risk associated with individual alleles or genotypes was calculated as the odds ratio (OR) with 95% confidence intervals (C.I.). A probability level of $p<0.0045$ was considered to indicate statistical significance in the analysis by marker (0.05/11) or $p<0.002$ for the analysis by each marker based on severity of MIH (0.05/22). Finally, we performed logistic regression modeling to test the association between genetic variation and caries experience, adjusting the analysis by *Streptococcus mutans* colonization status and MIH status. Adjusting the analysis by MIH status is important since our previous data suggest MIH is associated with greater caries experience in this population (33).

Results

All genotypes were in Hardy-Weinberg equilibrium (data not shown). Supplemental Tables 1 and 2 summarize the results of the allele and genotype frequency comparisons in the Brazilian population. Considering the comparison between the total number of cases (MIH) and controls (no MIH), borderline results were found for *ENAM* rs3796704 (p=0.02 for the allele frequency, and p=0.06 for the genotype frequency). Individuals carrying the A allele appear to be protected against MIH development (OR=0.42; 95% C.I.=0.2–0.47). When MIH severity was considered in the analysis, statistically significant difference in allele frequency was observed for the *AMBN* rs4694075 marker (p= 0.003; OR=0.46; 95% C.I.=0.26–0.8), with the T allele suggesting a protective role. The *ENAM* rs3796704 was also associated with MIH severity (OR=0.28; 95% C.I.=0.06–1.0), since the G allele was more frequent in the severe MIH subgroup (p=0.03) in comparison with the no MIH subgroup. A suggestive overrepresentation of the TT genotype of the *TFIP11* rs5997096 marker was seen in cases with severe MIH diagnosis (p=0.01) when compared with cases

with mild diagnosis. On the other hand, the TT genotype of the *TFIP11* rs134136 was overrepresented in cases with mild MIH diagnosis (p=0.02).

In regards to the Turkish population, the comparison between cases and controls showed association with MIH for the *TUFT1* rs2337360, rs4970957, rs3790506 markers, as well as the *AMBN* rs4694075, *ENAM* rs3796704, and *TFIP11* rs5997096, considering both the allele and genotype distributions (Supplemental Table 3). Most significant associations with protection against the development of MIH were found for the *TUFT1* rs4970957 (p=3.287e–023; OR=0.12; 95% C.I.=0.08–0.19), and rs3790506 (p=1.64e–0.11; OR=0.19; 95% C.I.=0.11–0.33) markers and *TFIP11* rs5997096 (p=0.00027; OR=0.49; 95% C.I.=0.49–0.74). The most significant result was for *ENAM* rs3796704, since it was observed that individuals who carry the G allele were 17 times more likely to be affected by MIH than those who carry the A allele (p=1.22e–012; OR=17.36; 95% C.I.=5.98–56.78). When MIH severity was considered in the Turkish population (Supplemental Table 4), suggestive associations were noted for allele and genotype frequencies for the following SNPs: *TUFT1* rs4970957 ($p<0.001$) and rs3790506 ($p<0.01$), *ENAM* rs3796704 ($p<0.01$), and *TFIP11* rs5997096 ($p<0.05$).

We also analyzed MIH based on caries experience (DMFT) scores in the Brazilian cohort. The DFMT mean was 5.04 ± 3.12, however we did not find any differences in the level of colonization of *Streptococcus mutans* based on having detectable copies of *Streptococcus mutans* genomic DNA in caries free individuals versus individuals with caries experience. Table 3 shows the allele and genotype frequencies of the polymorphisms investigated in MIH patients related to caries experience (individuals with DMFT up to 3 versus individuals with DMFT 4 or higher). Suggestive associations were found for three markers: *TFIP11* rs134136 (p=0.01; OR=1.78; 95% C.I.=1.08–2.94 for allele frequency), and *AMELX* rs946252 (p=0.001; OR=0.16; 95% C.I.=0.09–0.27 for allele frequency) and rs17878486 (p=0.03; OR=0.53; 95% C.I.=0.29–0.98 for allele frequency and p=0.03 for genotype frequency). Table 4 summarizes the results of the regression analysis adjusted by, age, *Streptococcus mutans* and MIH statuses. Associations between the same markers in *TFIP11* (p=0.02) and *AMELX* (p=0.01) could be seen with caries independent of having MIH or genomic DNA copies of *Streptococcus mutans* detected by real time PCR. In addition, *ENAM* was associated with caries when specific genetic models were considered in the analysis.

Discussion

Considering that there is no conclusive data in relation to the etiology of MIH (17), to the best of our knowledge, this is the first study to evaluate the possible association between MIH and variants in genes related to enamel formation. An important discussion point is that there is still some debate if MIH is a truly recognizable distinct condition. Enamel defects restricted to incisors and molars can be considered purely environmental in origin (i.e., infection) or mistaken by under ascertained cases of amelogenesis imperfecta, particularly in the instance that children at ages when the only permanent teeth erupted are the incisors and molars. In our study, some subjects are as young as 6 years of age with the case group ranging to 20 years. It is possible to argue that some younger subjects may have amelogenesis imperfecta. Mutations in *AMELX* and *ENAM*, two genes which markers were included in our study, can lead to amelogenesis imperfecta. Most cases of amelogenesis imperfecta are caused by mutations in *ENAM*, which can be inherited as autosomal dominant or recessive. About 5% of cases are X-linked recessive forms of the condition linked to mutations in *AMELX* (33). Estimates of the prevalence of amelogenesis imperfecta range from 1 in 700 in northern Sweden to 1 in 14,000 in the US (34). Hence, although we may have one or two cases of amelogenesis imperfecta in our sample, we do

not expect them to greatly impact the association results of common variants in these genes. But even more importantly, while inactivating mutations of these genes lead to full blown amelogenesis imperfecta, hypomorphic variants (i.e., common SNPs in the population) could play a role during enamel formation and lead to mild forms of defects that affect just portions of the dentitions and are less severe. Also, the limitation related to the age of the subjects can affect the diagnosis of older individuals. In adult subjects, developmental enamel defects may have been masked by restorations and remineralizing agents, so the accuracy of diagnosis of presence/absence of enamel defects may be questioned. The consequence of this potential bias is including controls with undetected enamel defects, which would implicate in possibly missing true associations. If on one hand, this is a concern, on the other hand, it may suggest that the associations found in this study are indeed due to true biological relationships, since differences between cases and controls were significant enough to overcome the chance some controls have been misclassified as such.

The morphologic appearance of ameloblasts differs at each successive stage of the enamel development (presecretory, secretory, transitional, and maturation) with corresponding marked changes in the expression of several well-characterized genes (35–38). During the progression from the earlier to the later stages of enamel maturation a dynamic process takes place with cellular, biochemical, genetic, and epigenetic changes in the developing tissue. It is noted that high rates of mineral acquisition, associated fluctuations in extracellular pH, and resorption of extracellular enamel proteins occur. During maturation stage, ameloblasts change from having a tall, thin, and highly polarized organization (characteristic of the secretory stage), to having a low columnar and widened morphology. These results indicate that ameloblasts undergo widespread molecular changes during the maturation stage of amelogenesis (39). It has been suggested that the enamel defect observed in MIH occurs due to ameloblasts sensitization during the maturation phase of dental enamel, leading to hypomineralized enamel (10).

The present study suggested trends for association between hypomineralized enamel and genetic variation in enamel formation genes. It was possible to note that some variants indicated a trend for association with susceptibility to MIH, while others showed a trend in the other direction, suggesting a protective effect against the development of MIH. Interestingly, for both independent populations investigated, no association was found in regards to *AMELX*, a fundamental gene that secretes the main protein of dental enamel, the amelogenin, during the secretion stage of amelogenesis. This can suggest that the enamel defect in MIH seems not to be directly related with the function of amelogenin during the secretion stage of amelogenesis.

Amelx, *Enam*, and *Ambn* have their expression levels decreased by the late maturation stage of amelogenesis (39). However, these changes are subtler for *Ambn*. This finding is in agreement with reports that *Ambn* is expressed during the maturation stage (35). Although no gross morphological changes have been described between early and late maturation of the enamel organ, a number of ultrastructural changes (vacuolation patterns and lysosomal content) occurs. Moreover, inferred physiological activities, such as those possibly derived from differences in pH and differences in the rates of mineral acquisition described earlier between these two stages (40,41), are probably related to differences in gene regulation (42). We found a trend for association between variation in *AMBN* and MIH in both cohorts, which may suggest that variation in the regulation of *AMBN* is a mechanism that leads to MIH.

Tuftelin has been suggested to play an important role during the development and mineralization of enamel, but its precise function is still unclear. It is also expressed in

several non-mineralizing soft tissues, suggesting that Tuftelin has a universal function and/or a multifunctional role (43). Tuftelin-interacting protein 11 (TFIP11) was first identified in a yeast two-hybrid screening as a protein interacting with tuftelin. The ubiquitous expression of *TFIP11* suggested that it might have other functions in non-dental tissues (44). We found evidence for a trend of association between genetic variation of *TUFT1* and *TFIP11* and MIH, which suggest these genes are potentially involved in individual predisposition to MIH.

We also evaluated the association between genetic markers in enamel formation genes and caries, based on MIH. Since we do not had radiographs to review, there is the potential that some carious lesions went undetected, however this is a limitation for the whole sample and there is no reason to believe cases and controls would have been differently affected by this limitation. Cases of MIH have their caries experience suggestively associated with variants in *AMELX*, *ENAM*, and *TFIP11*. The association between *AMELX* or *ENAM* and caries has been reported in hypomineralized-free enamel (5,22,24). Genetic changes leading to abnormal protein function or decreased amounts of protein could lead to some degree of disorganization of the enamel prisms that increases the individual's susceptibility to caries (5). The association with *TFIP11* is intriguing since previous attempts to detect this association have failed (21,22,24). *TFIP11* was associated with early stages of enamel demineralization and fluoride-mediated remineralization (5), which suggest that genetic analyses of caries should take into consideration more detailed description of the disease status.

Our study has some obvious limitations. Cases and controls were not perfectly matched. There were slightly more females than males among the cases (although this difference was not statistically significant). Caries experience (DMFT scores) tends to increase with age; however, we do not think the age difference between cases and controls greatly impacted our results since controls were older than cases in the Brazilian population studied. Finally, population substructure may have gone undetected. In Istanbul, 75% of the population is Turkish, and Kurds, Armenians, Jews, and Greeks represent the remainder (24). In the Brazilian group from Araraquara, the population is fully interbred. Brazilians form one of the most heterogeneous populations in the world, resulting from interethnic crosses between Europeans (mainly Portuguese and Italians), Africans (brought as slaves), and autochthonous Amerindians (45).

Our study provides rationale to expand this work to other genes that are suggested to be involved in hypomineralized/hypocalcified amelogenesis imperfecta. These genes include *KLK4* (46), *MMP20* (47), *WDR72* (48), and *FAM83H* (49). Similar to the amelogenesis imperfecta causing genes *ENAM* and *AMELX*, the hypothesis is that genetic variation in these genes that do not have such dramatic consequences as full-blown amelogenesis imperfecta may cause localized form of enamel. hypomineralization.

In summary, we provide new insight into the development of hypomineralized enamel defects. Considering that the maturation period of tooth enamel that is commonly affected by MIH (first permanent molars and permanent incisors) corresponds to the last trimester of pregnancy to the third year of a child's life, it is possible that genetic variation may somehow interact with environmental factors. Further studies are needed to assess this potential interaction as well as patterns of penetrance of MIH.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Table 1

Demographical characteristics.

	Brazil (*n*=160)			Turkey (*n*=245)			Both Populations (*n*=405)		
	MIH	**No MIH**	***p*-value**	**MIH**	**No MIH**	***p*-value**	**MIH**	**No MIH**	**p-value**
Mean age in years (Standard Deviation)	13(7.6)	38(6.8)	0.00001	9.7(1.5)	5.9(0.11)	<0.00001	10.5(4.5)	22.8(16.9)	<0.0001
Gender									
Male	37(52.1)	38(42.7)	0.24	78(65.0)	42(35.0)	0.61	115(59.0)	80(41.0)	0.63
Female	34(47.9)	51(57.3)		85(68.0)	40(32.0)		119(56.7)	91(43.3)	

Characteristics of MIH Sample

MIH Severity	Brazil		Turkey		Both Populations	
	n	**%**	***n***	**%**	***n***	**%**
Mild	42	59.1	19	11.7	61	26
Severe	29	40.9	144	88.3	173	74

Table 2

Markers studied.

Gene	Locus	Marker public ID	Base pair change
Ameloblastin (AMBN)	4q21	rs496502	G/T
		rs4694075	C/T
Amelogenin (AMELX)	Xp22.31-p22.1	rs17878486	C/T
		rs946252	C/T
Enamelin (ENAM)	4q13.3	rs3796704	A/G
		rs12640848	A/G
Tuftelin (TUFT1)	1q21	rs3790506	A/G
		rs233736	A/G
		rs4970957	A/G
Tuftelin interacting protein 11 (TFIP11)	22q12.1	rs134136	C/T
		rs5997096	C/T

Table 3

Summary of the allele and genotype frequency comparisons related to caries experience in Brazilians with MIH.

Subjects	Alleles (%)		*p*-value	OR (95%C.I.)	Genotypes (%)			*p*-value
TUFT1								
rs2337360	**A**	**G**			**AA**	**AG**	**GG**	
Low	49(31.4)	107(65.6)	0.81	1.06(0.64–1.76)	6(7.7)	37(47.4)	35(44.9)	0.79
High	49(32.7)	101(67.3)			8(10.7)	33(44.0)	34(45.3)	
rs4970957	**A**	**G**			**AA**	**AG**	**GG**	
Low	124(75.6)	40(24.4)	0.99	1.00(0.58–1.72)	50(61.0)	24(29.3)	8(9.8)	0.56
High	118(75.6)	38(24.4)			45(57.7)	28(35.9)	5(6.4)	
rs3790506	**A**	**G**			**AA**	**AG**	**GG**	
Low	51(31.1)	113(68.9)	0.49	0.84(0.51–1.4)	8(9.8)	35(42.7)	39(47.6)	0.72
High	43(27.6)	113(72.4)			7(9.0)	29(37.2)	42(53.8)	
AMBN								
rs4694075	**C**	**T**			**CC**	**CT**	**TT**	
Low	79(48.2)	85(51.8)	0.43	1.19(0.75–1.89)	16(19.5)	47(57.3)	19(23.2)	0.27
High	82(52.6)	74(47.4)			23(29.5)	36(46.2)	19(24.4)	
rs496502	**T**	**G**			**GG**	**GT**	**TT**	
Low	121(76.6)	37(23.4)	0.52	1.19(0.67–2.12)	48(60.8)	25(31.6)	6(7.6)	0.62
High	121(79.6)	31(20.4)			48(63.2)	25(32.9)	3(3.9)	
ENAM								
rs12640848	**A**	**G**			**AA**	**AG**	**GG**	
Low	83(50.7)	81(49.3)	0.78	1.07(0.66–1.73)	21(25.6)	41(50.0)	20(24.4)	0.4
High	70(52.2)	64(47.3)			13(16.9)	44(57.1)	20(26.0)	
rs3796704	**A**	**G**			**AA**	**AG**	**GG**	
Low	21(13.6)	133(86.4)	0.76	0.9(0.44–1.85)	1(1.3)	19(24.7)	57(74.0)	0.13
High	19(12.5)	133(87.5)			4(5.3)	11(14.5)	61(80.3)	
TFIP11								
rs5997096	**C**	**T**			**CC**	**CT**	**TT**	
Low	61(38.1)	99(61.9)	0.16	1.39(0.86–2.23)	9(11.3)	43(53.8)	28(35.0)	0.07
High	70(46.1)	82(53.9)			19(25.0)	32(42.1)	25(32.9)	

Subjects	**Alleles (%)**		***p*-value**	**OR (95%C.I.)**	**Genotypes (%)**			***p*-value**
rs134136	**T**	**C**			**CC**	**CT**	**TT**	
Low	68(50.7)	66(49.3)	0.01	1.78(1.08–2.94)	30(36.6)	38(46.3)	14(17.1)	0.17
High	101(64.8)	55(36.2)			38(48.7)	25(32.1)	15(19.2)	
AMELX								
rs946252	**T**	**C**			**CC**	**CT**	**TT**	
Low	139(85.8)	23(14.2)	0.001	0.16(0.09–0.27)	63(77.8)	13(16.0)	5(6.2)	0.99
High	134(85.9)	22(14.1)			61(78.2)	12(15.4)	5(6.4)	
rs17878486	**C**	**T**			**CC**	**CT**	**TT**	
Low	41(25.0)	123(75.0)	0.03	0.53(0.29–0.98)	15(18.3)	11(13.4)	56(68.3)	0.03
High	23(15.1)	129(84.9)			4(5.3)	15(19.7)	57(75.0)	

Note: OR (95% C.I.)= Odds ratios; 95% confidence intervals

Table 4

Summary of the regression analysis of the association between caries and genetic variation in enamel formation genes adjusted by age, MIH, and *Streptococcus mutans* statuses.

Gene/Marker	Genotypes	Multivariate analysis		Test/model	Genotypes	P-value
		P-value	OR (95%C.I.)			
AMELX						
rs946252	CC	Reference	Reference	Genotype	CC/CT/TT	0.72
	CT	0.88	0.93 (0.39–2.22)	Dominant Model	CC+CTvsTT	0.51
	TT	0.97	1.02 (0.28–3.73)	Recessive Model	CCvsCT+TT	0.70
rs17878486	CC	Reference	Reference	Genotype	CC/CT/TT	**0.04**
	CT	**0.01**	5.38(1.38–20.98)	Dominant Model	CC+CTvsTT	0.35
	TT	**0.02**	3.91(1.21–12.55)	Recessive Model	CCvsCT+TT	**0.01**
TFIP11						
rs5997096	CC	Reference	Reference	Genotype	CC/CT/TT	0.07
	CT	**0.03**	0.34(0.13–0.88)	Dominant Model	CC+CTvsTT	0.78
	TT	0.07	0.41(0.41–1.09)	Recessive Model	CCvsCT+TT	**0.02**
rs134136	CC	Reference	Reference	Genotype	CC/CT/TT	0.38
	CT	0.07	0.52(0.26–1.05)	Dominant Model	CC+CTvsTT	0.37
	TT	0.69	0.84(0.35–2.01)	Recessive Model	CCvsCT+TT	0.17
AMBN						
rs4694075	CC	Reference	Reference	Genotype	CC/CT/TT	0.78
	CT	0.11	0.53(0.25–1.16)	Dominant Model	CC+CTvsTT	0.63
	TT	0.42	0.69(0.28–1.7)	Recessive Model	CCvsCT+TT	0.75
rs496502	GG	Reference	Reference	Genotype	GG/GT/TT	0.80
	GT	0.17	0.60(0.29–1.2)	Dominant Model	GG+GTvsTT	0.51
	TT	0.85	0.86(0.19–3.88)	Recessive Model	GGvsGT+TT	0.82
ENAM						
rs12640848	AA	Reference	Reference	Genotype	AA/AG/GG	**0.05**
	AG	0.19	1.73(0.76–3.96)	Dominant Model	AA+AGvsGG	**0.01**
	GG	0.32	1.6(0.62–4.11)	Recessive Model	AAvsAG+GG	0.43
rs3796704	AA	Reference	Reference	Genotype	AA/AG/GG	**0.01**
	AG	0.1	1.15(0.01–1.5)	Dominant Model	AA+AGvsGG	**0.02**
	GG	0.25	0.27(0.02–2.58)	Recessive Model	AAvsAG+GG	<**0.00001**
TUFT1						
rs3790506	AA	Reference	Reference	Genotype	AA/AG/GG	0.54
	AG	0.87	0.91(0.29–2.84)	Dominant Model	AA+AGvsGG	0.76
	GG	0.78	1.17(0.38–3.59)	Recessive Model	AAvsAG+GG	0.36
rs233736	AA	Reference	Reference	Genotype	AA/AG/GG	0.23
	AG	0.49	0.66(0.2–2.12)	Dominant Model	AA+AGvsGG	0.19
	GG	0.57	0.71(0.22–2.32)	Recessive Model	AAvsAG+GG	0.15

Gene/Marker	Genotypes	Multivariate analysis		Test/model	Genotypes	P-value
		P-value	OR (95%C.I.)			
rs4970957	AA	Reference	Reference	Genotype	AA/AG/GG	0.50
	AG	0.45	1.02(0.53–1.95)	Dominant Model	AA+AGvsGG	0.54
	GG	0.5	0.27(0.66–2.57)	Recessive Model	AAvsAG+GG	0.47

Bold font indicates p-values lower than 0.05.