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Early Childhood Caries is Associated with Genetic Variants in Enamel Formation and Immune Response Genes

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Abstract

Early childhood caries (ECC) is a chronic, infectious disease that affects the primary dentition of young children. It is the result of unequal contributions of risk factors and protective factors that influence the disease. The aim of this study was to assess genetic and environmental factors that may contribute to ECC. Two hundred fifty-nine unrelated children were evaluated using a cross-sectional design. Data on oral habits were obtained through a questionnaire and caries experience data were collected by clinical examination. Twenty-three markers in ten genes were studied. Genotyping of the selected polymorphisms was carried out by real-time PCR. Regression analyses were performed comparing individuals with and without caries experience. Of 259 subjects, 123 were caries-free. The genotype TT in *ALOX15* (rs7217186) was a risk factor for ECC whereas the genotypes GG in *ENAM* (rs1264848), AG and GG in *KLK4* (rs198968), CT in *LTF* (rs4547741), and GG in *TUFT1* (rs3790506) were protective for EEC. In conclusion, environmental factors and gene-interactions can act as protective or risk factors for early childhood caries. These factors together contribute to the presence and severity of the disease.

INTRODUCTION

Early childhood caries (ECC) is defined as “the presence of one or more decayed (noncavitated or cavitated lesions), missing (due to caries), or filled tooth surfaces” in any primary tooth in a 71-month or younger child [Drury et al., 1999]. ECC represents one of the major diseases that impact on children’s health and remains a public health problem in many

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communities. It results from a chronic imbalance between multiple risk factors and protective factors [American Association of Pediatric Dentistry, 2008].

It is well established that environmental factors such as diet, oral hygiene, other oral habits, and socio-economic factors are risk or protective factors for caries [Levy et al., 2003; Ferreira et al., 2007; Menghini et al., 2008; Tannure et al., 2012a]. However, the factors related to the host are under genetic control, and environmental factors can overcome the genetic component of this complex disease. Our more recent studies continue to demonstrate that genetic variation in the host is associated with caries experience, and these variations can play a role in caries etiology as risk or as protective factors [Patir et al., 2008; Deeley et al, 2008; Vieira et al., 2008; Ozturk et al., 2010; Shaffer et al., 2011; Tannure et al., 2012c and b; Shimizu et al., 2012; Wang et al., 2012; Briseño-Ruiz et al., 2013; Shimizu T et al., 2013]. But one criticism of these studies is the incomplete information of environmental factors to include as covariates in the genetics analysis. In this study, we evaluated the association between genes involved in enamel formation and genes involved in immune response and their interaction with environmental factors in ECC experience.

SUBJECTS AND METHODS

The Human Ethics Committee of Marmara University, Turkey 2011 and the University of Pittsburgh Institutional Review Board approved this study. Informed consent was obtained from all parents/legal guardians.

Healthy unrelated children with no chronic illnesses from 2 to 5 years of age who had no systemic fluoride consumption were enrolled in this cross-sectional study. All children sought dental treatment at the Pediatric Dental Clinics of Marmara University during the period of 2011 to 2012, and all parents/caregivers answered a questionnaire about the child's diet and oral hygiene habits.

Determination of Caries Experience

The examiner (Z.A.) carried out the clinical examination after being trained by an experienced specialist (A.M.K.) in pediatric dentistry. Caries was diagnosed by visual examination and was recorded if there was definite visual evidence of a breach in the enamel with or without extension into dentin. Visible presence of white spot lesions due to enamel demineralization was also recorded. 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's guidelines. Caries experience was assessed using the dmft and dmfs indexes for each individual. Calculations excluded teeth lost to trauma or primary teeth lost to exfoliation.

Subjects were classified according to caries experience level. They were categorized into two groups: caries free (children with dmft=0) and children with caries experience (dmft≥1).

DNA Samples and Genotyping

Genomic DNA was extracted from buccal cells using a QIAmp DNA isolation protocol. Twenty-four markers in ten genes (seven involved in enamel formation and three involved

in immune response) were included in this study (table 1). Genotyping was performed by polymerase chain reactions using the Taqman method [Ranade et al., 2001] with an ABI PRISM® 7900HT Sequence Detection System (Foster City, CA, USA). Pre-designed probes were supplied by Applied Biosystems (Foster City, CA, USA). Markers were chosen based on previous association with caries experience, allele frequency, position on the gene, and linkage disequilibrium relationships to maximize information content.

Statistical Analysis

Data was subsequently processed and analyzed using the Epi Info 3.3.2 statistical software package (http://www.cdc.gov/epiinfo). Student's *t* test was used to assess mean differences, and chi-square or Fisher's exact tests were used to find the difference in frequencies between caries free and children with caries experience. Logistic regression analysis of each genetic marker was performed. The environmental factors identified as possible modifiers for ECC experience were included as covariates during the multivariate analyses to detect gene-environment interactions. The established alpha was 5%, and Hardy-Weinberg equilibrium was evaluated by chi-square test with one degree of freedom within each marker.

RESULTS

Of the 259 children included in this study, 123 (47.5%) were caries free and 136 (52.5%) were children with caries experience. The mean age was 4.6 years (standard deviation 0.61). Caries free children (4.14 years old, standard deviation 0.9) were younger than children with caries experience (4.45 years old, standard deviation 0.32) (p=0.0001). Among the affected children the dmft varied from 2 to 19 and mean dmft was 5.16 (standard deviation 5.5). In this group the dmfs varied from 2 to 62 and the mean dmfs was 10.44 (standard deviation 13.17). All children with caries experience had a carious lesion in at least one posterior tooth, and almost all children had additional lesions in an anterior tooth (130; 95.6%). Demographic data and environmental risk factors for ECC are summarized in table 2. Two environmental factors were associated with ECC in this population. Brushing the child's teeth for the first time after the window of infectivity (19 to 31 months of age, Caufield et all, 1993) was a risk factor for ECC (OR=1.33; CI 95% 0.67–2.65). The frequency of sugar and/or acidic drink consumption each day increased the risk for ECC almost three times.

The environmental factors for ECC identified and described above were included in the multivariate analyses in order to identify gene-environment interactions. The results of the univariate and multivariate analyses of the association of genotypes with ECC are presented in table 3. The genotype TT in *ALOX15* (rs7217186) was a risk factor for ECC in the multivariate analysis. The genotype GG in *ENAM* (rs1264848) was a protective factor for ECC in the multivariate analysis. The genotypes AG and GG in *KLK4* (rs198968) were associated as protective factors with ECC in the multivariate analysis. The genotype CT in *LTF* (rs4547741) was a protective factor for EEC in the univariate and in the multivariate analyses. Finally, the genotype GG in *TUFT1* (rs3790506) was a protective factor for ECC in the univariate and in the multivariate analyses.

DISCUSSION

Although it is well established that multiple factors contribute to an individual's risk for caries, not many studies evaluated the interactions between environmental factors and genetic factors. The genome wide scan of caries experience in the primary dentition (Shaffer et al., 2011) included children 3–12 years of age and included analysis of genetic association based on having sufficient or deficient home fluoride exposure but no statistically significant association were found despite some borderline suggestive results. To the best of our knowledge this is the first work to look for interactions between genetic variants and environmental factors in ECC. It is not difficult to propose that genetic mechanisms that modulate the enamel development and the immune response are involved with ECC experience and are influenced by factors such as oral hygiene, diet, and possibly other environmental factors.

It is important to emphasize that both of the groups analyzed here had a similar lifestyle and were dependent on the same health service. Among all self-reported environmental factors analyzed here, only the frequency of sugar and/or acid drink consumption, and time of the first tooth brushing were associated with ECC. Also, more caries free children were among 2 and 3 year olds. For this reason, these factors were included as covariates in the multivariate analysis. Multivariate analyses are useful to elucidate the interactions of environmental factors and genetic variants influencing a given trait [Leboyer et al., 1998].

We studied genes involved in enamel development [ameloblastin *(AMBN)*, amelogenin *(AMELX*), enamelin *(ENAM)*, kallikrein 4 *(KLK4)*, matrix metalloproteinase 20 *(MMP20)*, tuftelin *(TUFT1)*, and tuftelin interacting protein 11 *(TFIP11)*] and genes related to the immune response of the host [beta-defensin 1 *(DEFB1)* and lactoferrin *(LTF)*]. Arachidonate 15-lipoxygenase *(ALOX15)* was associated with bone mineralization [Vilella et al., 2009], and it is plausible this gene is involved in the formation of the hard structures of teeth. This gene has also been related to inflammatory response [Kelavkar and Badr, 1999]. Based on the complex and multifactorial nature of caries, it was not surprising that we found associations between some of these genes and ECC.

Dental enamel is a highly mineralized tissue with 85% of its volume occupied by hydroxyapatite crystals. This structure is rigorously controlled in ameloblasts through the interaction of a number of organic matrix molecules such as ENAM, AMELX, AMBN, TUFT1, and TFIP11. ENAM is the largest protein in the enamel matrix during development and comprises approximately 5% of total enamel matrix protein [Pavlic et al, 2007]. In our results, the multivariate analyses demonstrated that the GG in *ENAM* (rs1264848) was protective for ECC. Our previous study also demonstrated the association of this gene with caries experience in Turkish children when the presence of *Streptococcus mutans* was modeled with the T allele of rs3796704 [Patir et al., 2008]. Another study from our group demonstrated that the mechanism *ENAM* is possibly involved with caries is by contributing to an enamel surface more susceptible to demineralization [Shimizu et al., 2012].

We also found that the GG genotype in *TUFT1* rs3790506 was protective for EEC in the univariate and multivariate analyses. Previous studies also found an association between this

gene and caries experience in children [Slayton et al., 2005; Patir et al., 2008; Shimizu et al., 2012] and in adults [Deeley et al., 2008; Shimizu et al., 2012]. Slayton et al. [2005] suggested that two polymorphisms in *TUFT1* interacted with the presence of *Streptococcus mutans* and explained 27% of the variability of caries experience in children from Iowa, USA. In Patir et al. [2008] the CT genotype of *TUFT1* rs3790506 was overrepresented in cases with dmft scores higher than 5. Shimizu et al. [2012] showed that the G allele of TUFT1 rs4970957 was overrepresented in populations both from Argentina and Brazil. In Guatemala, Deeley et al. [2008] showed that *TUFT1* rs2337360 genotype distribution was different depending if individuals had DMFT scores 2 or lower versus 3, 4, 5, or 6 and higher. Similarly to *ENAM*, the mechanism *TUFT1* may predispose to caries is by forming an enamel structure more susceptible to demineralization [Shimizu et al., 2012].

Mutations in *MMP20* and *KLK4* have been previously implicated in amelogenesis imperfecta [Ozdemir et al., 2005]. Our hypothesis is that common genetic variations of these genes may be involved in subclinical changes of the enamel and, as a consequence, be involved in differences in caries experience. In the study presented here, the AG and GG genotypes in *KLK4* (rs198968) protective for ECC. We found no evidence of association between EEC and *MMP20*.

Regarding our findings related with the immune response of the host, we did not find an association with *DEFB1*, a gene that we previously associated with caries in adults [Ozturk et al 2010]. However, we found that a polymorphism in *LTF* is associated with ECC. The reason *DEFB1* was not associated with ECC and *LTF* was can be explained by the age of children affected by ECC and differences in microbiota of ECC. *LTF* is a glycoprotein that is present in various secretory fluids, including saliva, and has been previously associated with caries [Azevedo et al., 2010; Brancher et al., 2011]. It is one of the components of the immune system and has antimicrobial activity, particularly in human infants. *LTF* has an important role against *Candida albicans* [Viejo-Díaz et al., 2004] and interestingly, *Candida albicans* is an important component of dental biofilm associated with ECC [Yang et al., 2012]. Since *Candida albicans* produces, and is also very tolerant of, acids it has the potential to induce or exacerbate carious lesions [Klinke et al., 2011].

In spite of all that is known about preventing ECC, there are still children who appear to be more susceptible and those who are extremely resistant, regardless of the environmental risk factors to which they are exposed. In summary, our results suggest that genetic variation in genes involved in enamel formation and genes involved in immune response may contribute to ECC, and that susceptibility is the result from gene-environment interactions.

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Table 1

Genes and markers included in the study.

Genetic Marker	Gene	Chromosome	Base Change	Minor Allele Frequency
rs2619112	*ALOX15*	17	A/G	0.447
rs7217186	*ALOX15*	17	C/T	0.472
rs4694075	*AMBN*	4	C/T	0.479
rs34538475	*AMBN*	4	G/T	0.187
rs17878486	*AMELX*	X	C/T	0.111
rs946252	*AMELX*	X	A/G	0.300
rs11362	*DEFB1*	8	A/G	0.405
rs1800972	*DEFB1*	8	C/G	0.154
rs12640848	*ENAM*	4	A/G	0.357
rs3796704	*ENAM*	4	A/G	0.120
rs2235091	*KLK4*	19	C/T	0.340
rs198968	*KLK4*	19	A/G	0.313
rs2269436	*LTF*	3	A/G	0.103
rs743658	*LTF*	3	A/G	0.103
rs4547741	*LTF*	3	C/T	0.059
rs17078878	*LTF*	3	A/C	0.146
rs1784418	*MMP20*	11	A/G	0.407
rs5997096	*TFIP11*	22	C/T	0.468
rs134136	*TFIP11*	22	C/T	0.337
rs7526319	*TUFT1*	1	C/T	0.338
rs4970957	*TUFT1*	1	A/G	0.240
rs3828054	*TUFT1*	1	C/T	0.105
rs3790506	*TUFT1*	1	C/T	0.248
rs2337360	*TUFT1*	1	A/G	0.250

Table 2

Demographic characteristics and environmental risk factors for ECC in the studied population.

Variables	Total Children (n=259)	Caries Experience* (n=136)	Caries Free (n=123)	Odds Ratio 95% Confidence Interval	p-value
Sex (%)					
Male	129(49.8)	70(51.5)	59(48.0)	0.89(0.53–1.41)	0.573
Female	130(50.2)	66(48.5)	64(52.0)	Reference	
Age (%)					
2 years old	6(2.3)	0(0.0)	6(4.9)	-	**0.0001**
3 years old	34(13.1)	9(6.6)	25(20.3)	0.08(0.03–0.17)	
4 years old	74(28.6)	36(26.5)	38(30.9)	0.56(0.31–0.99)	
5 years old	145(56.0)	91(66.9)	54(43.9)	Reference	
Mean birth Weight (SD)	3228(599.2)	3202(627.0)	3257(568.1)	-	0.463
Milk bottle (%)					
Yes	185(71.4)	100(73.5)	85(69.1)	Reference	
No	74(28.6)	36(26.5)	38(30.9)	0.80(0.46–1.38)	0.431
Milk bottle usage duration					
Time in month- mean (SD)	22.1(11.22)	22.5(10.4)	21.7(12.12)	1.0(0,98–1.02)	0.635
Milk ingredient (%)					
No milk consumption	70(27.0)	35(25.7)	35(28.5)	-	-
No sugar	71(27.4)	34(25.0)	37(30.1)	0.76(0.42–1.38)	0.439
With sugar	118(45.6)	67(49.3)	51(41.5)	Reference	
Milk consumption before sleeping (%)					
Yes	171(66.0)	95(69.9)	76(61.8)	0.69(0.41–1.16)	0.171
No	88 (34.0)	41(30.1)	47(38.2)	Reference	
Snack consumption					

Variables	Total Children (n=259)	Caries Experience* (n=136)	Caries Free (n=123)	Odds Ratio 95% Confidence Interval	p-value
Number per day- mean (SD)	2.50(1.10)	2.59(1.11)	2.39(1.08)	-	0.150
Sugar and/or acidic drink consumption (%)					
Never	90(34.7)	36(26.5)	54(43.9)	-	**0.001**
Occasional	123(47.5)	14(10.3)	15(12.2)	1.40(0.60–3.24)	
Once a day	17(6.6)	80(58.8)	43(35.0)	2.69(1.59–4.89)	
Twice or more a day	29(11.2)	6(4.4)	11(8.9)	0.81(0.27–2.41)	
First time brushing teeth (%)					
Before the window of infectivity	6(2.3)	0(0.0)	6(4.87)	-	**0.023**
During the window of infectivity	206(79.5)	109(80.1)	97(78.9)	Reference	
After the window of infectivity	40(15.4)	24(17.6)	16(13.0)	1.33(0.67–2.65)	
Did not know	7(2.8)	3(2.3)	4(3.23)	-	
Tooth brushing before sleeping (%)					
Everyday	97(37.5)	51(37.5)	46(37.4)	Reference	0.995
Sometimes	138(53.3)	73(53.7)	65(52.8)	0.90(0,34–2.36)	
Never	20(7.7)	10(7.4)	10(8.1)	0.89(0.34–2.27)	
Did not know	4(1.5)	2(1.4)	2(1.7)	-	
Tooth brushing frequency (%)					
No brushing	11(4.2)	6(4.4)	5(4.1)	Reference	0.671
2 or 3 per week	18(6.9)	7(5.1)	11(8.9)	0.90(0.25–3.19)	
One per day	138(53.3)	75(55.1)	63(51.2)	1.71(0.61–4.81)	
Twice per day	92(35.5)	48(35.3)	44(35.8)	0.91(0.54–1.55)	

Note: P-values below 0.05 are considered statistically significant.

*Caries lesions were defined as definite breakdown of enamel with or without an extension to dentin and visible white spot lesions due to demineralization of enamel.

Table 3

Univariate and multivariate analyses of the genotypes.

Gene	Genetic Marker	Genotype	Univariate analysis		Multivariate analysis	
			P-value	OR (95%CI)	P-value	OR (95%CI)
ALOX15	rs2619112	AA	Reference	-	Reference	-
		AG	0.864	0.95(0.50–1.79)	0.869	1.06(0.54–2.09)
		GG	0.521	0.79(0.39–1.61)	0.886	0.95(0.44–2.02)
	rs7217186	CC	Reference	-	Reference	-
		CT	0.368	0.63(0.23–1.72)	0.239	0.52(0.18–1.54)
		TT	0.061	2.57(0.96–6.92)	**0.050**	2.97(1.00–8.86)
AMBN	rs4694075	CC	Reference	-	Reference	-
		CT	0.151	1.99(0.78–5.08)	0.265	1.74(0.66–4.61)
		TT	0.153	0.51(0.21–1.28)	0.258	0.58(0.22–1.49)
	rs34538475	GG	Reference	-	Reference	-
		GT	0.606	0.84(0.43–1.64)	0.439	0.75(0.36–1.57)
		TT	0.170	0.42(0.12–1.45)	0.186	0.41(0.11–1.54)
AMELX	rs17878486	CC	Reference	-	Reference	-
		CT	0.823	1.10(0.46–2.65)	0.931	1.04(0.41–2.64)
		TT	0.506	1.28(0.62–2.67)	0.534	1.28(0.59–2.77)
	rs946252	CC	Reference	-	Reference	-
		CT	0.189	1.54(0.80–2.96)	0.188	1.59(0.79–3.18)
		TT	0.172	1.59(0.81–3.13)	0.383	1.37(0.67–2.78)
DEFB1	rs11362	CC	Reference	-	Reference	-
		CT	0.956	0.98(0.56–1.74)	0.818	0.93(0.51–1.71)
		TT	0.934	0.97(0.49–1.92)	0.667	0.85(0.41–1.77)
	rs1800972	CC	Reference	-	Reference	-
		CG	0.432	1.89(0.39–9.22)	0.287	2.57(0.45–14.7)

Gene	Genetic Marker	Genotype	Univariate analysis		Multivariate analysis	
			P-value	OR (95%CI)	P-value	OR (95%CI)
		GG	0.701	1.35(0.29–6.18)	0.700	1.39(0.26–7.34)
ENAM	rs12640848	AA	Reference	-	Reference	-
		AG	0.221	0.65(0.32–1.30)	0.200	0.61(0.29–1.29)
		GG	0.100	0.53(0.25–1.13)	**0.032**	0.41(0.18–0.92)
	rs3796704	AG	Reference	-	Reference	-
		CT	0.963	-	0.962	-
		GG	0.247	0.63(0.29–1.37)	0.217	0.58(0.25–1.37)
KLK4	rs2235091	AA	Reference	-	Reference	-
		AG	0.531	1.58(0.38–6.55)	0.518	1.65(0.36–7.57)
		GG	0.401	1.78(0.46–6.88)	0.467	1.70(0.40–7.18)
	rs198968	AA	Reference	-	Reference	-
		AG	0.265	0.43(0.09–1.90)	**0.037**	0.15(0.03–0.89)
		GG	0.275	0.45(0.11–1.87)	**0.040**	0.17(0.03–0.92)
LTF	rs2269436	f	Reference	-	Reference	-
		AG	0.787	1.12(0.50–2.50)	0.521	1.34(0.55–3.26)
		GG	0.396	2.68(0.27–26.2)	0.627	1.77(0.18–17.5)
	rs743658	AA	Reference	-	Reference	-
		AG	0.438	0.38(0.03–4.24)	0.769	0.69(0.06–7.8)
		GG	0.403	0.37 (0.03–3.69)	0.657	0.59(0.06–5.89)
	rs4547741	CC	Reference	-	Reference	-
		CT	**0.036**	0.47(0.23–0.95)	**0.038**	0.44(0.21–0.96)
		TT	0.427	0.38(0.03–4.21)	0.257	0.24(0.02–2.79)
	rs17078878	AA	Reference	-	Reference	-
		AC	0.382	0.35(0.03–3.67)	0.676	0.60(0.06–6.53)
		CC	0.441	0.41(0.04–3.99)	0.669	0.61(0.06–6.01)

Gene	Genetic Marker	Genotype	Univariate analysis		Multivariate analysis	
			P-value	OR (95%CI)	P-value	OR (95%CI)
MMP20	rs1784418	CC	Reference	-	Reference	-
		CT	0.484	1.25(0.66–2.37)	0.598	1.2(0.61–2.39)
		TT	0.919	1.04(0.52–2.05)	0.947	1.02(0.49–2.12)
TFIP11	rs5997096	CC	Reference	-	Reference	-
		CT	0.414	0.75(0.37–1.51)	0.242	0.64(0.31–1.35)
		TT	0.683	1.19(0.52–2.68)	0.950	1.03(0.44–2.39)
	rs134136	CC	Reference	-	Reference	-
		CT	0.568	1.18(0.67–2.06)	0.185	1.58(0.80–3.11)
		TT	0.383	1.39(0.66–2.9)	0.860	1.06(0.58–1.91)
TUFT1	rs7526319	CC	Reference	-	Reference	-
		CT	0.255	1.38(0.79–2.40)	0.344	1.34(0.73–2.43)
		TT	0.473	1.32(0.62–2.85)	0.465	1.36(0.60–3.09)
	rs4970957	AA	Reference	-	Reference	-
		AG	0.577	0.85(0.48–1.50)	0.952	0.98(0.53–1.79)
		GG	0.358	0.64(0.25–1.63)	0.249	0.57(0.22–1.48)
	rs3828054	AA	Reference	-	Reference	-
		AG	0.649	1.17(0.59–2.29)	0.866	1.06(0.52–2.17)
		GG	0.579	0.51(0.05–5.66)	0.511	0.44(0.04–5.06)
	rs3790506	AA	Reference	-	Reference	-
		AG	0.881	0.93(0.34–2.53)	0.442	0.64(0.21–1.98)
		GG	**0.039**	0.34(0.12–0.94)	**0.014**	0.23(0.07–0.74)

Note: The analyses were adjusted for age, sugar and/or acid drink consumption and time of the first time of teeth brushing. OR(95%C.I.)=Odds ratios; 95% confidence intervals. Bold forms indicated p≤0.05. The markers rs17878486, rs946252, rs3796704, rs2337360 were not in Hardy-Weinberg equilibrium and were not further tested.