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Genetic and Environmental Factors Associated with Dental Caries in Children: The Iowa Fluoride Study

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Key Words
Association analysis · Candidate gene · Children · Dental caries · Genetics

Abstract
Dental caries remains the most common chronic childhood disease. Despite strong evidence of genetic components, there have been few studies of candidate genes and caries. In this analysis we tried to assess genetic and environmental factors contributing to childhood caries in the Iowa Fluoride Study. Environmental factors (age, sex, race, tooth-brushing frequencies and water fluoride level) and three dental caries scores (d\textsubscript{2}f\textsubscript{s}-total, d\textsubscript{2}f\textsubscript{s}-pit/fissure, and d\textsubscript{2}f\textsubscript{s}-smooth surface) were assessed in 575 unrelated children (mean age 5.2 years). Regression analyses were applied to assess environmental correlates. The Family-Based Association Test was used to test genetic associations for 23 single nucleotide polymorphism (SNP) markers in 333 Caucasian parent-child trios. We evaluated the associations between caries status and the level of both single and multiple SNPs (haplotype) respectively. Permutation procedure was performed for correction of inflated type I errors due to multiple testing. Age, tooth-brushing frequency and water fluoride level were significantly correlated to at least one carious score. Caries on pit and fissure surfaces was substantially higher than on smooth surfaces (61 vs. 39%). SNPs in three genes (DSPP, KLK4 and AQP5) showed consistent associations with protection against caries. Of note, KLK4 and AQP5 were also highlighted by subsequent haplotype analysis. Our results support the concept that genes can modify the susceptibility of caries in children. Replication analysis in independent cohorts is highly needed in order to verify the validity of our findings.

Dental caries, a multifactorial complex disease [Shuler, 2001], remains the most common chronic disease of childhood. Although a decline in dental caries rates in the United States and other industrialized nations in the primary dentition was found until the mid-1980s, later reports have suggested that this decline has slowed or even reversed in the United States and elsewhere [Burt, 1994; Downer, 1994]. This has also been supported by recent NHANES surveillance summaries on oral health...
data [2005]. Further, billions of dollars are spent for the treatment of dental caries every year and the costs keep increasing. The etiology of dental caries involves a complex interplay of environmental and genetic factors [Hunter, 1988]. Epidemiological studies have tried for many years to understand fully the mechanisms of this disease, with the eventual goal of prevention. Thus, identifying the underlying genetic and environmental risk factors is a crucial step toward that goal.

Pioneering twin studies investigating the heritability (i.e. proportion of variation due to genes) of dental caries in children have clearly supported the key role of genetics in tooth decay [Liu et al., 1998; Bretz et al., 2006]. Caries heritability estimates for children based on twins range from 64 to 85%. In our recent heritability study based on larger families [Wang et al., 2010], heritability of dental caries in the primary dentition was >50%. Also, we found that genes affecting caries might be different for the primary and permanent teeth. These findings have reinforced the importance of studying genetic components of dental caries in primary teeth separately.

Numerous efforts on gene mapping have been made so far to identify specific genetic loci contributing to caries susceptibility [Werneck et al., 2010]. Results from previous studies have highlighted features involved in processes related to development of caries in the following four categories: (1) Salivary composition and flow: Saliva contains components that can directly kill cariogenic bacteria. It is also rich in calcium and phosphates, which are actively involved in the tooth enamel remineralization process. The physical flow of saliva helps to dislodge pathogens (viruses, bacteria, and yeast) from teeth and mucosal surfaces. Saliva can also cause microbes to clump together so that they can be swallowed before they become firmly attached [Stookey, 2008]. (2) Tooth morphology: Tooth morphology refers to the number and shape of cusps, ridges, grooves, and even the tooth size as a whole. Malposition of the teeth, deep anatomy grooves, and areas of retention due to the natural morphology of the tooth structure can cause difficulties in tooth-brushing and fluoride penetration, and thus be considered as caries risk factors, especially for pit and fissure caries [Guzman-Armstrong, 2005]. (3) Dietary and taste preferences: In general, there are multiple potential effects of diet. Diet can influence the amount and type of plaque formation and debris and the presence of relative numbers of cariogenic microorganisms on tooth surfaces. The interactions of the cariogenic potential of foods (e.g. sucrose), the frequency of eating and the physical state (or type) of the diet all can affect individually or jointly the carious process [Wendell et al., 2010]. On the other hand, taste preference affects dietary habits. Dietary choices often are guided by how foods taste. For example, children especially love sweets and dislike bitter tastes and eat more of the foods they like best [Drewnowski, 1997]. (4) Enamel and dentin formation: dental caries itself is a destructive process causing decalcification of the tooth enamel and leading to continued destruction of enamel and dentin, and eventually to cavitation of the tooth. Demineralization of enamel leads to caries and in contrast, enamel remineralization can help prevent caries. Thus, genes regulating enamel/dentin formation make significant contributions to caries experience. There are two plausible theories which explain how enamel genes may associate with caries lesion development: they interact with oral bacteria (such as Streptococcus mutans) to affect caries susceptibility [Pati et al., 2008] and/or they enhance enamel thickness and enamel fluoride concentration to protect the teeth against caries [Keene et al., 1980].

Besides genetics, environmental determinants also play a crucial role in caries susceptibility in children [Verrips et al., 1992; Lukacs and Largaespada, 2006]. Demographic parameters, including age, sex and ethnicity, are important predictors for caries. In general, older children have more caries because their teeth are exposed to the environment and at risk longer; girls are typically found to exhibit higher caries prevalence rates than boys, mostly due to earlier eruption of their teeth and food preferences [Ferraro and Vieira, 2010]. Moreover, caries prevalence was observed to vary among different racial groups, with non-Hispanic whites having the lowest caries prevalence and severity [Beltrán-Aguilar et al., 2005]. However, this could be partly due to different genetic factors as well. In addition, other environmental factors such as tooth-brushing behaviors, water fluoride levels and family education level are acknowledged to be protective factors for primary teeth [Levy et al., 2003; Ferreira et al., 2007; Menghini et al., 2008].

Taken together, genetic and environmental factors play crucial roles in susceptibility and resistance to this complex disease, and thus both should be considered in any study of dental caries. Considering the higher caries prevalence and evidence for a stronger genetic influence in primary versus permanent dentitions, conducting a genetic epidemiology study concerning caries in a younger age cohort is particularly meaningful. Hence, the purpose of the current study was to assess genetic and environmental determinants of primary tooth caries in the Iowa Fluoride Study cohort.
Subjects and Methods

Study Sample

The recruitment of the Iowa Fluoride Study cohort has been described previously [Levy et al., 2003]. 575 unrelated children (300 girls and 275 boys) and their parents were included in our analyses. Most children were aged 4–7 years, with two outliers (age 2.8 and 7.6 years, respectively). The mean and standard deviation of age was 5.2 ± 0.4 years. Of these children, 95% were from Caucasian families, 2% of African American and 3% from other racial/ethnic groups.

Phenotype

Phenotype data on dental caries were assessed on children, but not their parents. A trained and calibrated dentist performed a caries examination on each participant. Procedures and criteria have been described in detail previously [Warren et al., 2006]. Briefly, the assessment included dental caries status for each surface on all erupted primary teeth, i.e. sound, decayed (d) or filled (f), and examinations were conducted with portable equipment (chair, headlight and the Denlite System). Examinations were primarily visual, with explorer use to confirm caries found. Radiographs were not taken. On primary molars, pit and fissure decay and fillings were distinguished from smooth surface ones. The missing (m) component was not included in our dental caries scores due to possible misidentification of exfoliated primary and unerupted permanent teeth in children. Also, although scored separately, we did not include non-cavitated (d1, white-spot/incipient) lesions. Finally, we included the following three dental caries scores in our genetic analysis as phenotypes: (1) total number of tooth surfaces with frank cavitated or filled caries experience (d2fs-total), (2) pit and fissure surfaces with caries experience (d2fs-pit/fissure), and (3) caries experience of all other tooth surfaces (d2fs-smooth surface). These scores were dichotomized in the downstream analyses as cases (children with scores >1) and controls (scores = 0). The phenotypic correlations among the above three caries scores were moderately high ($r^2 = 0.5–0.8$).

Genotype

We genotyped 23 single nucleotide polymorphisms (SNPs) from 7 candidate genes for 333 Caucasian parent-child trios (Table 1). Genes involved in tooth development, enamel mineralization,
tion and tooth morphology, and salivary buffering were selected for analysis, with the premise that some of the genes involved in these basic biological processes may also play a role in caries susceptibility/resistance. Genotyping was performed using TaqMan assays, amplified in a 384-well thermocycler and semi-automatically analyzed on an Applied Biosystems (ABI) 7900 DNA Analyzer. TaqMan assays were selected using an ABI SNP browser that displays haplotype block structure characterized in the four HapMap populations (http://www.hapmap.org). In addition to HapMap, we also used the following public databases to identify SNPs: http://www.ncbi.nlm.nih.gov/SNP/ and http://bioinfo.weizmann.ac.il/cards/index.shtml. SNPs were chosen based on a balance between availability characteristics of the SNP and knowledge about LD block structure from the HapMap Project. To optimize genotyping information, SNPs were selected from different haplotype blocks. Priority was given to functional and non-synonymous SNPs. PCR primer sets were purchased from ABI. PCR primer conditions were obtained from the ABI website (http://snp.ims.u-tokyo.ac.jp). Selected SNPs were genotyped, along with no template controls, two positive control reference individuals and several internal replicates in order to determine genotyping error rates. The inheritance pattern of each marker was checked using PedCheck [O’Connell and Weeks, 1998]. Tripads with unresolved Mendelian inconsistencies were removed from the analysis. All 23 SNPs were consistent with Hardy-Weinberg equilibrium and the minor allele frequencies (MAF) ≥0.04 (MAF of 21 SNPs between 0.12 and 0.46; MAF for 2 SNPs at ENAM gene = 0.04). Further, when compared to HapMap (latest release #28) CEU (samples with Northern and Western European ancestry) data, the MAF of all SNPs were comparable to the reference population except for SNP rs1728679 from SP1 gene (minor allele C with MAF = 0.41 in our data; minor allele T with MAF = 0.16 in HapMap CEU).

Environmental Covariates
As described previously [Levy et al., 2003], demographic and environmental factors, including age, sex, race, tooth-brushing frequencies and fluoride intake from water, were collected and included in our regression models. In brief, demographic information was obtained at the time of recruitment. Through questionnaires, environment factors such as tooth-brushing frequency and fluoride intake from water (considering intake amounts and the composite fluoride concentration from all major water sources used by children) were reported by parents.

Statistical Analysis
To assess the significance of these environmental factors, we first evaluated them using linear regression (with dependent variable being the counts of tooth surfaces with caries experience) and then with logistic regression (with dependent variable being the binary variable with caries affection status: yes or no) for verification purposes.

To avoid genetic heterogeneity among different ethnic groups, all genetic analyses were limited to Caucasians only. After removing unrelated and ungenotyped individuals, the final effective sample size in the analysis was reduced to 333 Caucasian parent-offspring trios (two parents plus one child, table 2). The Family-Based Association Test (FBAT) was used to test associations between candidate genes and each of the dental caries scores. FBAT is a variation of the standard transmission disequilibrium test, in which the statistical comparison is between alleles that were transmitted to the offspring from the parents versus those not transmitted. The association test for each single SNP was performed first, followed by haplotype analyses of multiple SNPs within those genes with significant results for one or more individual SNPs. In order to detect allelic associations with both caries susceptibility and caries resistance, we evaluated each caries score twice, first assessing transmission patterns to the children affected with caries (to detect susceptibility alleles) and second assessing transmission to children with no caries (for resistance).

Permutation procedures were conducted in PLINK (PLINK version 1.07, http://pngu.mgh.harvard.edu/~purcell/plink/) to generate empirical significance levels after correction of type I error inflations from multiple testing. We only permuted phenotypes while leaving the genotypes unchanged for each person and within each family. Thus, the LD structure as well as the Mendelian consistency across markers remained the same. In order to generate the empirical significance level under the null hypothesis (i.e. no association between disease trait and marker), every phenotype was permuted for 1,000 times followed by the association analyses between all permuted phenotypes and each marker (both single SNP and haplotype). Empirical p values were calculated as (R + 1)/(N + 1), where R is the number of times the permuted test is greater than the observed test in our discovery analysis and N is the number of permutations (N = 1,000 in the current study). A threshold of p = 0.05 was used as the cutoff for statistical significance across all analyses.

Results

Environmental Correlates
We first used multiple linear and logistic regressions to evaluate the effects of assessed environmental factors in 575 children of all races (table 3). Further, the analysis limited to 333 Caucasian children demonstrated very similar results (data not shown). As expected, age is positively correlated with all three caries scores in both regression models, except for pit and fissure caries with logistic regression. Figure 1 depicts in detail how caries experience increase with age across all three surface types. For example, the median score for smooth surface caries is 2 for children aged <5; this number jumps to 3 in children aged 5–6 and to 4 in children aged >6. Of note,
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Table 3. p values in regression analyses for environmental and demographic variables associated with caries scores

Table 4. Single SNP association analyses with dental caries scores

Entries in the table are in the format (p value in linear/logistic regressions); significant p values are in bold. Males and Caucasians samples were set as reference groups when studying the sex and race effects respectively. ‘+’ represents positive association (risk) and ‘–’ represents negative association (protective) between covariates and caries phenotype.

Entries in the table are the associated SNP alleles and their corresponding nominal p values and empirical p values by permutation test (in format of nominal p/empirical p); empirical p values are reported only for significant observations (nominal p ≤ 0.05, highlighted in bold).

among all surface caries, pit and fissure surfaces accounted for 61% vs. 39% smooth surface caries. This is in agreement with the common observation that pit and fissure caries is more common in children.

Unlike previous studies which identified higher caries prevalence rates in girls, there was no significant sex difference for caries in our cohort. Similarly, there was no significant difference observed for different race groups. However, this might be due to insufficient sample size for non-Caucasian children (5%). Frequent tooth-brushing and higher fluoride intake from water were both found in our study to act as protective factors against caries (table 3).

Single SNP Association Analysis
Next, we performed genetic association analysis testing the possible associations between single SNPs and each caries score. As shown in table 4, SNPs in three genes (DSPP, AQP5 and KLK4) showed consistent associations with protection against caries for all three caries scores (nominal p ≤ 0.05). Notably, for KLK4 the minor allele
(G) was associated with increased caries risk for phenotype d2fs-smooth surface, which supports the opposite A allele being protective. After multiple testing corrections by permutation, 6 out of total 10 significant observations above remained significant (empirical \( p < 0.05 \)). In particular, association signals for SNP rs2615487 at DSPP are significant for all 3 caries scores before and after the permutation adjustment.

### Haplotype Analysis

We conducted association tests between caries scores and haplotypes of 2 or more adjacent SNPs within the same gene. Interestingly, the haplotype analyses not only verified the importance of markers from previous single SNP analyses, but also revealed additional positive association signals: (1) For 2 SNPs (rs923911 and rs1996315) in AQP5, the first SNP demonstrated no association signals alone. However, when combined with the second SNP, both haplotypes (CA and CG) showed a consistent protective effect against caries for all 3 caries scores before and after multiple comparison correction. Both haplotypes remained significant for smooth surface caries after multiple genotyping adjustments (Table 5). (2) For 2 SNPs (rs2235091 and rs198969) in KLK4, we observed very similar patterns as were found in single SNP analysis. We identified two ‘protective haplotypes’ (AC and GC) against all 3 caries scores and one ‘risk haplotype’ (GG) for smooth surface caries (Table 6). After multiple comparison corrections, only ‘protective haplotype’ GC and ‘risk haplotype’ GG remained significant.

### Discussion

This project is one of the first comprehensive genetic studies of dental caries aimed at better understanding the underlying environmental and genetic components by screening the significant environment correlates and investigating individual SNPs and SNP haplotypes within candidate genes. All highlighted genes reported in this paper have strong implications for their biological plausibility in pathways potentially affecting dental caries.

The DSPP (a.k.a. dentin sialophosphoprotein) gene encodes two principal proteins of the dentin extracellular matrix of the tooth: the preprotein is secreted by odontoblasts and cleaved into dentin sialoprotein and dentin phosphoprotein. Dentin phosphoprotein is thought to be involved in the biomineralization process of dentin [Kim et al., 2005]. Defects in this gene are the cause of many diseases such as dentinogenesis imperfecta type 1 and dentin dysplasia type 2, both of which are autosomal dominant disorders [Bhandari and Pannu, 2008; Lee et al., 2009]. Researchers also found that mutations in DSPP lead to the production of abnormal DSPP-derived proteins or reduce the amount of these proteins in developing teeth. As a result, teeth have abnormally soft dentin. Teeth with defective dentin are discolored, weak and prone to breakage and decay. Lee et al. [2006] showed that when caries reaches the dentin, the dentin-pulp complex is stimulated to form sclerotic dentin as a defensive mechanism, and the DSPP gene is possibly involved in this response. In addition, other evidence also

<table>
<thead>
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<th>Caries score</th>
<th>Effect</th>
<th>AQP5 haplotype</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>d2fs-total</td>
<td>protective</td>
<td>C A</td>
<td>0.01/0.09</td>
</tr>
<tr>
<td></td>
<td>protective</td>
<td>C G</td>
<td>0.02/0.17</td>
</tr>
<tr>
<td>d2fs-pit/fissure</td>
<td>protective</td>
<td>C A</td>
<td>0.04/0.25</td>
</tr>
<tr>
<td></td>
<td>protective</td>
<td>C G</td>
<td>0.04/0.48</td>
</tr>
<tr>
<td>d2fs-smooth surface</td>
<td>protective</td>
<td>C A</td>
<td>0.003/0.01</td>
</tr>
<tr>
<td></td>
<td>protective</td>
<td>C G</td>
<td>0.004/0.03</td>
</tr>
</tbody>
</table>

Table 5. Haplotype association analyses of AQP5 with dental caries scores

<table>
<thead>
<tr>
<th>Caries score</th>
<th>Effect</th>
<th>KLK4 haplotype</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
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<td>protective</td>
<td>A C</td>
<td>0.04/0.40</td>
</tr>
<tr>
<td>d2fs-pit/fissure</td>
<td>protective</td>
<td>A C</td>
<td>0.03/0.63</td>
</tr>
<tr>
<td></td>
<td>protective</td>
<td>G C</td>
<td>0.0007/0.01</td>
</tr>
<tr>
<td>d2fs-smooth surface</td>
<td>protective</td>
<td>G C</td>
<td>0.002/0.01</td>
</tr>
<tr>
<td></td>
<td>protective</td>
<td>G G</td>
<td>0.03/0.01</td>
</tr>
</tbody>
</table>

Table 6. Haplotype association analyses of KLK4 with dental caries scores

We report both nominal p values and the corresponding empirical p values by permutation test (in format of nominal p/empirical p); significant observations (p ≤ 0.05) are highlighted in bold.
revealed that, in families with DSPP mutation, the softer malformed dentin is always associated with elevated risk of oral cavities [Kim et al., 2005].

Aquaporins encode a series of homologous membrane proteins that function as highly selective water channels. Aquaporin-5 (AQP5) is uniquely present in lacrimal and salivary glands, where it accounts for generation of saliva, tears and pulmonary secretions [Smith et al., 1999]. Also, this gene together with aquaporin-4 has been identified as playing a role in extracellular matrix hydration during tooth development. The most striking findings about AQP5 related to tooth decay were from Culp et al. [2005], who observed that in AQP5-deficient (AQP5–/–) mice there was a significant caries susceptibility increase accompanied by reduced salivary flow. Evidence indicated that the low salivary flow was caused by decreased water content of saliva.

Another gene, KLK4, was also highlighted in our study. It has been well characterized in a previous study as playing a pivotal role in enamel mineralization [Lu et al., 2008]. The kallikrein 4 gene (KLK4) is one of the fifteen kallikrein subfamily members located in a cluster on chromosome 19. The expression pattern of a similar mouse protein supports its role in the degradation of enamel proteins. During the enamel development process, MMP20, the gene which had borderline significant p value (0.05 < p < 0.1, date not shown) in our study, is expressed in earlier stages of enamel development, such as the secretory and transition stages. Conversely, KLK4 is expressed later during the transition through maturation stages as the enamel hardens. The principal functions of MMP20 and KLK4 in dental enamel formation are to facilitate the orderly replacement of organic matrix with mineral, generating an enamel layer [Bartlett et al., 2004]. Harder, less porous enamels are in turn more resistant to tooth decay. Interestingly, MMP20 and KLK4 are implicated in hereditary enamel defects known broadly as amelogenesis imperfecta [Ozdemir et al., 2005]. Individuals with amelogenesis imperfecta typically exhibit structurally abnormal enamel, which is under-mineralized and often susceptible to fracture and caries formation.

Several limitations of the study warrant discussion. First, the analytic method in this paper is a candidate gene approach where it is inherently limited by our a priori understanding of the biology of caries. A more comprehensive genome-wide approach is warranted to identify additional regions of the genome likely to contain caries genes. This hypothesis-free approach will allow for the identification of genes that may not have been obvious choices for involvement in the caries process. In this regard, our research collaboration has received funding through the NIH Genes and Environment Initiative to perform a caries genome-wide association analysis of 550,000 SNPs within the Iowa, West Virginia, and Pennsylvania study cohorts. Second, the SNP selection is quite arbitrary and does not well represent the full genetic variation within the gene. While SNPs were selected to provide the most genetic information, that is high heterozygosity and/or being within a large haplotype block, they likely do not represent all genetic variation for a given candidate gene. Hence lack of positive findings should be interpreted as inconclusive results. Third, applying permutation approach to correct false positive results from multiple comparisons will in no double help filter out spurious results caused by type I error inflation. However, risk of generating possible false negative results is probably the downside of this approach (or all approaches for multiple comparison correction). This is more likely when the sample size is smaller, which is the case of our data. In our preliminary association analysis based on just nominal p values (before multiple testing correction procedures), there are a few SNPs from two more genes being highlighted, MMP20 and TUFT1 (data not shown). MMP20 is involved in the breakdown of extracellular matrix of both dentin and enamel and hence could affect the carious resistance of both tissues. The latter one, TUFT1, is also a possible carious gene due to its important role during the development and mineralization of enamel through the tooth development, but its precise function is still unclear [Deutsch et al., 2002]. Unfortunately, neither of these genes became significant after the penalty of multiple comparisons. Therefore, replication of our analysis using independent data with larger sample size is a definitely imperative task. We are currently performing a similar analysis in a cohort of over 2,300 participants from more than 700 families from the Center for Oral Health Research in Appalachia (COHRA) project. Fourth, due to the low caries frequency in our cohort of children, we may not have enough power to detect the genetic loci associated with increased caries risk. This perhaps explains why we observed much more caries-protective alleles. Finally, as is well known, dental caries experience is a chronic, cumulative disorder for which time is a very critical component that should be considered in any related research. Like most of the other studies, the cross-sectional design of our analysis work has a variety of limitations. Our previous finding that genetic determinants affecting caries might vary by dentition makes us believe that different sets of genes can influence...
the susceptibility to caries at different time periods, even within the same dentition. Thus, studying the trajectory patterns of caries, especially how they related to the underlying genetic background, is quite valuable and exciting. Fortunately, the intrinsic longitudinal design of the Iowa Fluoride Study has offered this potential for our future work.

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Disclosure Statement

None of the authors have any conflicts of interest.

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