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EDITED BY
Sreekanth Kumar Mallineni,
Faculty of Dentistry at Al Zulfi, Majmaah University, Saudi Arabia

REVIEWED BY
Daniela Silva Barroso De Oliveira,
Federal University of Alfenas, Brazil
Ahmad Faisal Ismail,
Kulliyyah of Dentistry, International Islamic University Malaysia, Malaysia

*CORRESPONDENCE
Alexandre Rezende Vieira
arv11@pitt.edu

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Oral microbiome dental caries associated genotypes analysis of 6- to 19-year-old individuals shows novel associations

Alexandre R. Vieira* and Adriana Modesto

School of Dental Medicine, University of Pittsburgh, Pittsburgh, United States

The need to determine risk factors for complex diseases continues to drive efforts to identify etiological factors of common conditions. Molecular tools have created new opportunities to identify risk factors that may act interactively. The goal of this work was to explore potential interactions between oral microbial species and common genetic variants. Ninety-two 6- to 19-year-old individuals recruited through the University of Pittsburgh Dental Registry and DNA Repository project that had oral microbiome and genotyping of 44 single nucleotide polymorphism (SNP) data available were studied. Over-representation of alleles between individuals with or without particular microorganisms was determined using chi-square or Fisher's exact tests. An alpha of 0.001, to account for multiple testing (0.05/44), was considered statistically significant. Associations were found between *Candida albicans* and enamelin rs3796704 ($p = 0.0006$), and *Staphylococcus epidermidis* and tuftelin rs3828054 ($p = 0.001$). Microbiota and their metabolites might predispose to oral disease when interacting with the host genetic variation and future studies should address their causal roles in oral disease.

KEYWORDS

single nucleotide (nt) polymorphism (SNP), oral microbiology, dental caries, genetics, linkage disequiblibrium

Introduction

Single nucleotide polymorphisms (SNPs) have been associated with microbial commensal species, and these associations may mean an interplay between overlapping pathophysiological processes. Several SNPs are associated with the gut microbiome (1–6), and among these initially identified associations, some were associated with outcomes such as food intake, hypertension, atopy, chronic obstructive pulmonary disease, body mass index, and lipids (7). Combined analyses of the oral microbiota and genetic variation of the host have been done in humans (8), indicating that genetic variation is an explanation for a stable or recurrent oral microbiome in each individual. These initial analyses unveiled associations between rs1196764 in the locus of the adaptor protein, phosphotyrosine interacting with pleckstrin homology domain and leucine zipper 2 (*APPL2*) and *Prevotella jejuni*, *Oribacterium uSGB 3339*, and *Solobacterium uSGB 315* recovered from the tongue dorsum. The analysis also showed that carrying the variant allele of *APPL2* rs1196764

and one of the three bacterial species made an individual less likely to have dental calculus.

An opportunity exists to identify disease associations by determining underlying associations between multiple risk factors. Previous work has suggested a number of associations between common genetic variation in humans and dental caries (9, 10), as well as that certain microorganisms are associated with the disease (11). Therefore, we tested for associations between oral microorganisms and genetic variation of a well-characterized cohort of 6- to 19-year-old individuals to unveil unknown associations between oral microbiota and host SNPs.

TABLE 1 Single nucleotide polymorphisms (SNPs) studied.

Gene/locus	SNP	Reference
Defensin beta 1 (*DEFB1*)	rs11362	(13)
	rs1800972	(13)
Amelogenin (*AMELX*)	rs17878486	(14)
	rs946252	(14)
Ameloblastin (*AMBN*)	rs4694075	(14)
Enamelin (*ENAM*)	rs12640848	(14)
	rs3796704	(14)
Tuftelin (*TUFT*)	rs2337360	(14)
	rs7526319	(14)
	rs3828054	(14)
	rs3790506	(14)
Tuftelin interacting protein 11 (*TFIP11*)	rs134136	(14)
	rs5997096	(14)
Cyclin B1 (*CCNB1*)	rs875459	(15)
Basic transcription factor 3 (*BTF3*)	rs6862039	(15)
Zinc finger swim domain-containing 6 (*ZSWIM6*)	rs4700418	(15)
Prostrate androgen-regulated transcript 1 (*PART1*)	rs27565	(15)
T cell receptor alpha variable 4 (*TRAV4*)	rs8011979	(16)
	rs1997532	(16)
Estrogen-related receptor B (*ESRRB*)	rs6574293	(17)
	rs1077430	(17)
	rs2860216	(17)
	rs10132091	(17)
	rs1676303	(17)
	rs745011	(17)
LOC286467	rs4830231	(18)
Olfactory receptor family 13 subfamily H member 1 (*OR13H1*)	rs4829728	(18)
Leucine zipper down regulated in cancer 1 (*LDOC1*)	rs5907830	(18)
Sperm protein associated with the nucleous on the X chromosome N2 (*SPANXN2*)	rs5908778	(18)
Small integral membrane protein 10 like 2A (*SMIM10L2A*)	rs5930702	(18)
Glypican 4 (*GPC4*)	rs5977872	(18)
Immunoglobulin superfamily member 1 (*IGSF1*)	rs6637822	(18)
PHD finger protein 6 (*PHF6*)	rs6638230	(18)
Serine/threonine kinase 26 (*STK26*)	rs2748729	(18)
Aquaporin 5 (*AQP5*)	rs1996315	(19)
	rs296763	(19)
	rs461872	(19)
	rs3759129	(19)
	rs3741559	(19)
	rs467323	(19)
	rs10875989	(19)
Aquaporin 1 (*AQP1*)	rs17159702	(19)
Matrix metalloprotein 20 (*MMP20*)	rs1784418	(20)

Material and methods

Data from 92 6- to 19-year-old individuals (12) (**Supplemental Material**) that had their whole saliva samples characterized using the Ibis Universal Biosensor, which combines polymerase chain reaction (PCR) and mass spectroscopy (MS), to detect species composition in a sample. Dental caries experience data available (DMFT/DMFS scores) referred to caries experience only in the permanent dentition. In brief, individuals were asked to expectorate (spit) 1 ml of unstimulated saliva in a plastic vial. Participants went at least 60 min without eating before sample collection. Saliva samples were immediately placed in a container with ice and then stored at −80°C. Subjects were recruited at the Department of Pediatric Dentistry of the University of Pittsburgh School of Dental Medicine between May and October 2012 as part of the Dental Registry and DNA Repository (DRDR) project. This study was approved by the University of Pittsburgh Institutional Review Board (approval number 0606091). All parents of the participating children provided written informed consent for their participation in the study after they provided written assent for their participation. For DNA extraction, the saliva was placed into a sterile microcentrifuge tube containing ATL lysis buffer (Qiagen, Germantown, MD, cat# 19076) and proteinase K (Qiagen, cat# 19131). Samples were incubated at 56 °C until lysis. One-hundred microliters of a mixture containing 50 µl each of 0.1 mm and 0.7 mm Zirconia beads (Biospec cat# 11079101z, 11079107zx, respectively) were added to the samples which were then homogenized for 10 min at 25 Hz using a Qiagen TissueLyser. Nucleic acid from the lysed sample was then extracted using the Qiagen DNeasy tissue kit (Qiagen cat# 69506). For microbiota analysis, 10 µl of each sample was loaded per well onto the BAC detection PCR plate (Abbott Molecular, cat# PN 05N13-01). The BAC detection plate is a 96-well plate containing 16 primers that survey all bacterial organisms by using the omnipresent loci (e.g., 16S rRNA sequences), while some are targeted to specific pathogens of interest (e.g., the *Staphylococcus*-specific *tuf*B gene). The plate also includes primers for the detection of *Candida* species and some antibiotic resistance markers (e.g., mecA, vanA, vanB, and KPC). An internal calibrant of a synthetic nucleic acid template is also included in each assay, controlling for false negatives (e.g., from PCR inhibitors) and enabling a semi-quantitative analysis of the amount of template DNA present.

TABLE 2 Microbes identified in the sample.

Microbes	Number of 6- to 19-year-old individuals positive
Abiotrophia defectiva	1
Abiotrophia elegans	2
Acinetobacter johnsonii	1
Actinobacillus sp.	1
Actinomyces odontolyticus	1
Bacteroides thetaiotaomicron	10
Candida albicans	18
Candida dubliniensis	8
Candida rugosa	1
Candida tropicalis	1
Eikenella corrodens	1
Enterococcus casseliflavus	1
Enterococcus faecium	1
Eubacterium eligens	1
Fusobacterium nucleatum	2
Gemella haemolysans	1
Gemella sanguinis	5
Haemophilus influenzae	4
Haemophilus parainfluenzae	5
Lactobacillus acidophilus	1
Lactobacillus reuteri	1
Lactobacillus sp.	2
Lactobacillus vaccinostercus	1
Legionella feeleii	1
mecA	1
Moraxella lacunata	1
Neisseria canis	14
Neisseria canis/flavescens	2
Neisseria flava	7
Neisseria flavescens	2
Neisseria meningitidis	5
Neisseria mucosa	6
Neisseria sicca	5
Neisseria sp.	2
Novosphingobium aromaticivorans	1
Pasteurella multocida	1
Rothia mucilaginosa	5
Saccharomyces cerevisiae	4
Saccharomyces cerevisiae/paradoxus	26
Salmonella enterica	3
Salmonella houten	1
Shigella flexneri	1
Staphylococcus aureus	15
Staphylococcus auricularis/cohnii/kloosii	1
Staphylococcus capitis/caprae	2
Staphylococcus epidermidis	18
Staphylococcus hominis	4
Staphylococcus lugdunensis	1
Staphylococcus warneri	2
Stomatococcus mucilaginosus	32
Streptococcus agalactiae	1
Streptococcus constellatus	1
Streptococcus cristatus	1
Streptococcus gordonii	2
Streptococcus infantis	1
Streptococcus mutans	1
Streptococcus oralis	3
Streptococcus parasanguinis/pneumoniae	1
Streptococcus peroris	2
Streptococcus pneumoniae	48
Streptococcus pyogenes	1
Streptococcus salivarius	5
Streptococcus sanguinis	3
Streptococcus sp.	3
Streptococcus suis	2
Streptococcus vestibularis	4
Veillonella parvula	3

PCR amplification was carried out, and the products were desalted in a 96-well plate format and sequentially electrosprayed into a mass spectrometer. The spectral signals were processed to determine the masses of each of the PCR products present with sufficient accuracy that the base composition of each amplicon could be unambiguously deduced. Using combined base compositions from multiple PCRs, the identities of the pathogens and a semi-quantitative determination of their relative concentrations in the starting sample were established by using a proprietary algorithm to interface with the Ibis database of known organisms. To display the distribution of species per patient, we generated heat map graphics using the "image()" and "grid()" functions in the "graphics" package part of R base distribution (version 3.2.2) (R Foundation for Statistical Computing, Vienna, Austria) (12).

Further, genomic DNA was obtained from these same saliva samples and genotyping data for 43 single nucleotide polymorphisms (SNPs) were generated. These SNPs (**Table 1**) were selected based on previous data suggesting them to be marking genes that were associated with dental caries (13–20). TaqMan chemistry and endpoint analysis were used to determine genotypes (21). When a particular species was frequent enough in the group of 92 6- to 19-year-old

individuals (present in at least 18 individuals), chi-square or Fisher's exact tests were used to test for overrepresentation of alleles in individuals carrying particular species, with an alpha of 0.001 (0.05/43). This cutoff of 20% aimed to avoid multiple comparisons of very small frequencies.

Results

The 92 6- to 19-year-old individuals studied had a mean age of 12.74 years (ages ranged from 6 to 19 years), and 44 individuals were females. Most of them ($N = 63$) were white, and among the remaining individuals, 21 were Black and 8 were Asian. Twenty individuals were caries-free, and among the remaining individuals that had caries experience, DMFT scores (decayed, missing due to caries, filled teeth) ranged from 1 to 17 and DMFS scores (decayed, missing due to caries, filled surfaces) ranged from 1 to 41 (**Supplemental Material**). Sixty-seven microbial species were identified in the samples from the 92 6- to 19-year-old individuals studied (**Table 2**).

Five microbes (*Candida albicans*, *Saccharomyces cerevisiae/paradoxus*, *Staphylococcus epidermidis*, *Stomatococcus mucilaginosus*, and *Streptococcus pneumoniae*) that were at least frequent in 20% of the sample were used to create groups for testing for overrepresentation of alleles of the SNPs listed in **Table 1**, which are located in loci that were previously associated with dental caries (13–20). All genotypes were in Hardy-Weinberg equilibrium. Having *Candida albicans* was associated with *ENAM* rs3796704 ($p = 0.0006$). Half of the individuals colonized with *Candida albicans* carried the variant allele of *ENAM* rs3796704, whereas less than 10% of individuals without *Candida albicans* carried the allele. Being positive for *Staphylococcus epidermidis* was associated with *TUFT* rs3828054 ($p = 0.001$). No individuals colonized by *Staphylococcus epidermidis* carried the *TUFT* rs3828054 variant allele. No other comparison showed differences that were statistically significantly different (all data are available as **Supplemental Materials**). Microorganisms historically associated with caries lesions such as *Streptococcus mutans* and *Lactobacilli* were found in just a few individuals and did not provide a chance for testing for associations.

Discussion

Dental caries can be defined as having a complex mode of inheritance, which includes the influence of more than one gene with small effects and environmental factors. Putative associations between dental caries with certain microbes or with genomic variation have been reported. These results did not translate to new preventive strategies, in part due to the fact that we do not understand how these different contributing factors may interact. Here we report novel associations between microbial species found in the mouth and common genomic variants in the population that have been previously associated with dental caries. These kinds of analyses aim to provide new insight into how the host interacts with microorganisms and how these may impact health and disease.

The success of genome-wide association studies (GWAS) in determining associations between SNPs and disease has motivated the proposal of testing microorganism whole genomes as well. Initial attempts have identified associations between ranges of bacteria, viruses, and protozoa and traits under strong selection such as drug resistance. The challenge of identifying variants under moderate selection still exists, and solutions are needed for instances of increased population stratification in microorganisms due to selection and complex recombination patterns (22). To minimize the chance for spurious associations, we tested SNPs that were previously associated with dental caries, a meaningful oral health microbial-mediated outcome.

Candida albicans is suggested to play a role in the development of dental caries (23), and understanding this would provide useful new insights. We suggested that variation in enamel conformation, depending on genetic variation, may increase the risk of developing dental caries lesions (24). The association between *ENAM* rs3796704 and the presence of *Candida albicans* suggests that variation in enamel conformation may increase dental caries susceptibility in the presence of *Candida albicans* colonization. These possible interactions should be modeled in future studies.

The role of *Staphylococcus epidermidis* in dental caries is unclear, and it is possible that it exists in the presence of certain genetic variations. The meaning of the many species that can be found in the mouth after molecular technology became available is currently object of study. Interestingly, *Streptococcus mutans*, which is classically associated with initiation of dental caries lesions, was only detected in one individual out of 92, suggesting that the individuals studied here did not have newly forming dental caries lesions. We showed earlier (12) that when compared, the results between PCR-MS and 16S rRNA sequencing differ significantly. The samples we tested by PCR-MS displayed a range in the number of genera detected from 1 to 4, including both bacteria and fungi. Both PCR-MS and 16s rRNA sequencing detected a prevalence of *Streptococci*, *Neisseria*, and *Rothia* in the samples. Sequencing of the 16S rRNA gene suggests that *Prevotella* and *Veillonella* were not detected by PCR-MS; while staphylococci and fungi were not detected by 16S rRNA sequencing. Using 16S rRNA sequencing therefore should provide the opportunity for identification of additional associations.

Larger sample sizes will allow for these analyses to be conducted taking into consideration age, sex, ethnicity, and

dental caries severity. These data were available but were not used since initial comparisons were already with small numbers. Sex, age, and ethnicity variations were not accounted for in the comparisons presented here, and undetected population substructures can always unveil spurious associations. Another limitation is that the quantification of microorganisms is relevant data for interpreting associations with diseases and we utilized only representation of species in this report. Future approaches should include measures of quantification of microorganisms. Finally, some SNPs had lower amplification ratios, which also impacted some of the comparisons. In summary, associations between genomic variants that impact dental caries risk may be explained by individual microbial colonization, and genomic and microbiome host data should be considered when testing for associations between dental caries and other risk factors.

Data availability statement

The authors acknowledge that the data presented in this study must be deposited and made publicly available in an acceptable repository, prior to publication. Frontiers cannot accept a manuscript that does not adhere to our open data policies.

Ethics statement

The studies involving human participants were reviewed and approved by the University of Pittsburgh Institutional Review Board. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

ARV wrote the initial draft. AM critically revised and finalized the manuscript. Both authors contributed to design and resources. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fdmed.2022.875953/full#supplementary-material.

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