Common Polymorphisms in *IFI16* and *AIM2* Genes Are Associated With Periodontal Disease

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Background: The single nucleotide polymorphism (SNP) context of a previously identified periodontitis-associated locus is investigated, and its association with microbial, biologic, and periodontal disease clinical parameters is examined.

Methods: A 200-kb spanning region of 1q12 previously highlighted in a genome-wide association scan among 4,766 European American individuals (SNP rs1633266) was annotated. Two haplotype blocks were selected. Association of these polymorphisms with data on microbial plaque composition, gingival crevicular fluid (GCF)–interleukin (IL)-1β levels, and clinical parameters of periodontal disease were examined. Descriptive analysis of IFI16 and AIM2 protein expression in gingival tissues from healthy individuals (n = 2) and individuals with chronic periodontitis (n = 2) was done via immunohistochemistry.

Results: The highlighted locus is a 100-kb region containing the *interferon γ-inducible protein 16* (*IFI16*) and *absent in melanoma 2* (*AIM2*) genes. Two haplotype blocks, rs6940 and rs1057028, were significantly associated with increased extent bleeding on probing and levels of microorganisms *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Campylobacter rectus* ($P \leq 0.05$). Haplotype block rs1057028 was also significantly associated with pathogens *Fusobacterium nucleatum* and *Aggregatibacter actinomycetemcomitans*, increased GCF–IL-1β levels, and extent of probing depth ≥4 mm ($P \leq 0.05$). Prevalence of severe periodontitis (biofilm-gingival interface P3 classification) was positively associated with haplotype block rs1057028. Similar trends were observed for haplotype block rs1057028. IFI16 and AIM2 protein expression was observed in multiple cell types of gingival tissues, including inflammatory cells.

Conclusion: This study found *IFI16* and *AIM2* SNPs associated with higher levels of periodontal microorganisms and an increased percentage of periodontal disease clinical parameters, suggesting the need for functional studies and additional fine-mapping of variants in the 1q12-locus. *J Periodontol 2017;88:663-672.*

KEY WORDS

Genetics; immunity, innate; periodontitis; polymorphism, genetic.

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Periodontal disease is the polygenic condition of tooth-supporting structures.[1-4] Early studies in monozygotic and dizygotic twins showed that 33% to 48% of variance in periodontal disease expression was attributable to genetics.[1,5] Notably, alterations in genes encoding proteins involved in the immune response are shown to influence host microbiota and increase periodontal clinical parameters of disease. Individuals with variants in interleukin (*IL*)-*1α*, *IL-1β*, and *IL-6* are shown to have a unique periodontal microbiome with high levels of classic "red" and "orange" complex species that are known to be significantly associated with periodontal inflammation.[6] *IL-6* polymorphisms have also been moderately associated with diagnosis of periodontitis.[7] In addition, individuals with single gene mutations of β2 integrins leading to leukocyte adhesion deficiency-1 show increased bacterial loads, decreased complexity of biofilms, and severe periodontal bone loss.[8] Together, evidence supports the concept that genetic alterations controlling the immune response of the host can lead to alterations of microbial communities and predispose individuals to periodontal disease.

Genome-wide association studies (GWASs) and candidate gene studies have been used in an attempt to identify single nucleotide polymorphisms (SNPs) that either contribute to the pathogenesis and/or risk of developing periodontal disease. To date, four studies[9-12] have conducted GWAS analysis to identify SNPs associated with the American Academy of Periodontology (AAP) definition of chronic periodontal disease.[13] No single marker met the genome-wide significance criteria, although the following four loci met gene-centric statistical significance criteria: 1) *ninein* (*NIN*); 2) *abhydrolase domain containing 12B* (*ABD12B*); 3) *WAS protein homolog associated with actin, golgi membranes, and microtubules* (*WHAMM*); and 4) *adaptor-related protein complex-3 β-2 subunit* (*AP3B2*).[11] Therefore, a new approach was used to identify SNPs that were relevant to the pathogenesis of periodontal disease. This approach used a combination of the levels of eight classic periodontal pathogens and gingival crevicular fluid (GCF)–IL-1β to derive periodontal complex traits (PCTs) via principal component analysis.[14] The objective of this approach was to identify loci related to the biologic background and pathogenesis of periodontal disease. Approximately 2.5 million markers were evaluated among 975 European American adults. Several traits were derived by this analysis, with each trait having different eigenvalues (loadings) of the eight microorganisms and GCF–IL-1β. PCT1 (named the Socransky Trait) was defined by a microbial community structure with high positive loadings of all periodontal pathogens and correlated with clinical measurements of periodontal disease.[15] Six loci were associated with PCT1: 1) *C-Type Lectin Domain Family 19 Member A* (*CLEC19A*); 2) *T-Cell Receptor α Locus* (*TRA*); 3) *Glycoprotein, α-Galactosyltransferase 2, Pseudogene* (*GGTA2P*); 4) *Transmembrane 9 Superfamily Member 2* (*TM9SF2*); 5) *RNA Binding Motif Single Stranded Interacting Protein 3* (*RBMS3*); and 6) *interferon (IFN)γ-inducible protein 16* (*IFI16*)/*absent in melanoma* (*AIM*)*2*.[14] Clinical, microbial, and biologic characterization of individuals with SNP variants in these six individual loci is currently unknown. This present study further investigates *IFI16/AIM2* loci using bioinformatics and clinical, microbial, and biologic data.

Both *IFI16* and *AIM2* are members of the IFN-inducible PYHIN protein family that contain C-terminal DNA-binding hematopoietic expression, IFN-inducible nature, and nuclear localization (HIN) domain(s) and an N-terminal Pyrin domain that belongs to the death domain superfamily of signaling molecules.[16,17] Both *IFI16* and *AIM2* are intracellular recognition sensors that trigger inflammatory responses against DNA from host and microorganisms.[17] Increased expression of *AIM2* has been reported in a number of inflammatory diseases, including psoriasis, atopic dermatitis, venous ulcers, inflammatory disease, and periodontitis, suggesting involvement with inflammation.[18-22] Expression of *IFI16* in inflammatory diseases has been less explored, but increased expression is reported in inflammatory bowel disease.[22] To the best knowledge of the authors, no study has explored expression of *IFI16* in periodontal tissues. Because of the critical role of these proteins in innate immunity, the purpose of this study is to evaluate the relationship between SNPs in the *IFI16* and *AIM2* loci with periodontal microorganisms, levels of GCF–IL-1β, and clinical parameters of periodontal disease. Meanwhile, descriptive analysis of IFI16 and AIM2 protein expression in gingival samples derived from healthy individuals and individuals with periodontal disease showed expression in multiple cells, including inflammatory cells. Conclusively, a previous study by the authors[14] and the present study support that variants in *IFI16/AIM2* are associated with increased loads of periodontal pathogens and increased parameters of clinical disease.

MATERIALS AND METHODS

GWAS Population

A total of 4,766 Northern European descendants (2,264 males and 2,502 females, aged 53 to 74 years; mean age: 62.8 years) were enrolled in the Dental Atherosclerosis Risk in Communities (DARIC) cohort as described elsewhere.[23,24] Blood was collected as described for genotyping for ≈2.5 million markers.[9] GCF was collected at four gingival sampling areas from the mesio-buccal region of each first molar from each individual and stored for further

analysis of IL-1β levels.[23] Plaque samples were collected from the subgingival mesio-buccal site of the maxillary right first molar and stored for further DNA whole chromosomal checkerboard for the eight periodontal pathogens. Periodontal measurements (n = 4,766) in all teeth at six sites per tooth were collected and included number of missing teeth, gingival index, plaque index (PI), probing depths (PD), clinical attachment level (CAL), and bleeding on probing (BOP). All sites were examined by trained and calibrated examiners with >90% agreement.

Bioinformatic Approaches

Initial analyses of genome-wide imputed SNP data were done with a software package.[25] The results of that analysis revealed one genome-wide significant SNP in the *IFI16* region (lead SNP rs1633266). Markers were identified and visualized in linkage disequilibrium with this polymorphism.[14,26] Criteria used to prioritize and select SNPs of interest for this analysis were: 1) a statistical significance criterion ($P < 5 \times 10^{-5}$ considered as "suggestive" evidence of association); 2) biologic plausibility of genes in the region; 3) functional significance; and 4) linkage disequilibrium, the nonrandom association in the occurrence of alleles at two loci, represented by the square of the correlation coefficient between two indicator variables (r^2). SNPs with suggestive association ($P < 5 \times 10^{-5}$) with PCT1 were selected and carried forward for screening of missense SNPs with predicted functional protein damage.[27-29] Using these criteria, two SNPs of interest were identified, rs6940 and rs1057028 located in the IFI16 region. A search and evaluation was made of SNPs in perfect linkage disequilibrium (Northern and Western European ancestry panel, $r^2 = 1$, $D' = 1$ cutoff) with these two markers as previously described.[30] Protein information was gathered, and alignment of the different IFI16 isoforms [clustal 0 (1.1.1) multiple sequence alignment] was performed.[31] SNPs within these loci were carried forward to tests of association with clinical and biologic parameters, including plaque microorganisms, GCF–IL-1β levels, periodontal clinical measurements, and biofilm–gingival interface (BGI)–periodontal disease classification.[32] BGI classification was selected because it defines biologic phenotypes based on eight periodontal pathogens, serum immunoglobulin G (17 bacteria), 16 GCF mediators, PD, and BOP, representing the current disease activity and inflammatory condition of the individual. Previous GWAS studies from the study group of the authors have already shown that traditional American Academy of Periodontlogy (AAP)/American Dental Association (ADA) periodontal disease classification,[13] which uses CAL, a measurement of loss of tissue or history of disease, does not allow identification of groups with similar biologic characteristics.[9,14]

Plaque Microbial Analysis

A subset of 909 participants of the DARIC cohort was evaluated for plaque microbial composition using DNA–DNA checkerboard as previously described.[32] One plaque sample was used from each individual and assayed by DNA checkerboard for the eight periodontal pathogens. Microorganism levels were expressed as counts using known microbial standards for *Porphyromonas gingivalis* (*Pg*), *Prevotella intermedia* (*Pi*), *Treponema denticola* (*Td*), *Tannerella forsythia* (*Tf*), *Campylobacter rectus* (*Cr*), *Fusobacterium nucleatum* (*Fn*), *Aggregatibacter actinomycetemcomitans* (*Aa*), and *Prevotella nigrescens* (*Pn*). Total counts reflect a sum of these targeted pathogens for each individual.

GCF–IL-1β Levels

Four GCF strips were eluted and analyzed separately for each individual (n = 4,407). IL-1β levels were evaluated by enzyme-linked immune-absorbent assay according to instructions provided by the manufacturer, as previously described.[33] GCF analyte concentration data were pooled to provide a mean value for each individual in nanograms per milliliter.

Sample Collection for Immunohistochemistry

To describe tissue distribution of IFI16 and AIM2 in human gingival tissues, gingival biopsies were taken from two individuals with healthy periodontium and two with chronic periodontal disease according to AAP/ADA classification.[13] All participants enrolled in this study provided written informed consent, which was approved by the Institutional Review Board of the University of North Carolina at Chapel Hill, Chapel Hill, NC. Major exclusion criteria included: 1) symptoms of any systemic disease; 2) antibiotic use within 1 month prior to the examination; or 3) medical treatment for any known disease associated with periodontal disease within the last 3 months. Gingival biopsies were harvested either during routine periodontal flap surgeries from participants clinically diagnosed with chronic periodontitis (CP) or crown lengthening surgeries in healthy volunteers. A tissue biopsy sample (≈3 × 4 mm) was removed from underneath the papillae, buccally or lingually, to include the col area of depth of the osseous crest. On removal, gingival tissues were fixed in 10% neutral-buffered formalin overnight, dehydrated (70% alcohol), and embedded in paraffin for the immunohistochemical procedure.

Immunohistochemistry

Gingival tissue sections (5 μm thick) were obtained in the sagittal direction, including the epithelial and connective tissues. Slides were stained with rabbit polyclonal antiIFI16** and rabbit polyclonal anti-AIM2.†† Antirabbit horseradish peroxidase

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†† Abcam, Cambridge, MA.

(HRP)-3,3′-diaminobenzidine staining‡‡ was used according to instructions provided by the manufacturer, and slides were counterstained with hematoxylin. Photo images were captured using a microscope.§§

Statistical Analyses

General linear models‖‖ were used to examine associations among SNPs of interest and adjusted mean counts of microorganisms, GCF–IL-1β levels, and clinical measurements, adjusting for microbial plaque levels ($P \leq 0.05$). χ^2 tests were used to examine SNP associations with periodontal disease category (BGI classification) ($P \leq 0.05$).[32]

RESULTS

SNP Identification and Analysis

A sample of 4,766 Northern European descendants were genotyped and evaluated as PCTs, with PCT1 associated with periodontal clinical parameters of disease.[14] To select correlation of variants with clinical parameters, SNP prioritization was based upon statistical significance, linkage disequilibrium (r^2), biologic relevance, gene proximity, coding sequences, and functional prediction. *IFI16* rs1633266, an intron variant, was the lead SNP most significantly associated with the Socransky Trait ($P = 3.1 \times 10^{-8}$, see supplementary Fig. 1 in online *Journal of Periodontology*). Nine additional SNPs located in the *IFI16* region and one in the *AIM2* region (rs2793845) were in high disequilibrium ($r^2 \geq 0.8$) with the lead SNP in *IFI16,* rs1633266; one SNP in the *IFI16* region with an $r^2 \geq 0.6$, and 16 SNPs in the *IFI16* region with $r^2 > 0.4$ (see supplementary Fig. 1 in online *Journal of Periodontology*). *IFI16* and *AIM2* are both localized in the 1q25.2 locus and are transcribed in opposite directions (see supplementary Fig. 1 in online *Journal of Periodontology*). Among the SNPs that correlated with PCT1 ($P \leq 5 \times 10^{-5}$, suggestive evidence of association), 21 were localized in the *IFI16* locus and one closest to *AIM2* (within 2 kb downstream of the 3′ end of a transcript) (see supplementary Table 1 in online *Journal of Periodontology*). Analysis indicated the existence of two tight haplotype blocks in perfect linkage disequilibrium ($r^2 = 1$ and $D' = 1$), including *IFI16* rs6940 (missense) with neighboring gene *AIM2* rs2793845 and several additional intronic SNPs, whereas the second block identified by missense rs1057028 included rs1057027 and additional introns, all located in the *IFI16* region (Table 1).[30] Bioinformatic analysis of functional damages potentially caused by missense SNPs demonstrated that rs6940 (T >S) affected all four isoforms with a prediction of possibly damaging (score: 0.584 to 0.782), rs1057027 as benign, and rs1057028 (Y > N) as probably damaging to the protein function of isoform 3 (score: 0.995) (see supplementary Table 2 in online *Journal of Periodontology*). This analysis suggests that rs6940 (minor allele [T] frequency = 0.22) and rs1057028 (minor allele [T] frequency = 0.3) are potentially/probably damaging for protein function and, therefore, were selected for correlations with periodontal clinical parameters. Sequence alignment of isoforms shows that SNPs do not localize in known functional domains (PYRIN domain, HIN domains, and p53 interaction domain) (see supplementary Figs. 2A and 2B in online *Journal of Periodontology*). However, rs6940 is located between HIN200-1/p53 C-terminal binding and HIN200-2/p53 core domain binding and could be interfering with the three-dimensional structure and protein function. It is possible that not all four isoforms of IFI16 are affected to the same degree based on different protein sizes (see supplementary Fig. 2C in online *Journal of Periodontology*). Indeed, rs1057028 is predicted to probably damage isoform 3 only (see supplementary Table 2 in online *Journal of Periodontology*). Imputation quality was 0.9994 and 0.9999 for rs6940 and rs1057028, respectively. In sum, the present analysis identified two tight haplotype blocks with several SNPs in the *IFI16/AIM2* region that are potentially important in the pathogenesis of periodontal disease. SNPs rs6940 and rs1057028 are predicted to be potentially/probably damaging to the protein function.

IFI16 SNPs Are Associated With Parameters of Periodontal Disease

Correlation analysis of the clinical parameters showed that both haplotype blocks rs6940 and rs1057028 had a significant increase in percent sites with extent bleeding on probing (EBOP), and rs1057028 also had a significantly higher percentage of extent of PD ≥4 mm (EPDGE4) (Table 2). Increased trends were observed for mean PD and percent of extent of gingival score ≥1 (EGIGE1) (Table 2). This suggests that SNPs in the region of *IFI16* and *AIM2* affect the biology of the tissues, leading to an increase in the extent of periodontal disease. Further analysis of the microbiologic composition of plaque samples shows that several periodontal pathogen counts (plaque-adjusted) were also significantly higher in both haplotype blocks, including *Pg*, *Tf*, and *Cr*. Loads of *Pg* were more than 274 times higher for rs6940 and 90 times higher for rs1057028 comparing 2.2 individuals (homozygous for the minor allele) to 1.1 individuals (homozygous for the major allele). Additional organisms were significantly increased in rs1057028, including *Fn* and *Aa,* with similar trends observed for rs1057028 homozygous minor alleles (Table 3). This finding further supports that SNPs in the *IFI16* and *AIM2*

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Table 1.

SNPs in Haplotype Blocks Associated With the Missense SNPs rs6940 and rs1057028 From Individuals With Northern and Western European Ancestry

SNP	Proxy	Distance to Lead SNP (bp)	Predicted Function
rs6940	rs7532207	244	Intronic
	rs74359395	2,335	3′ downstream *IFI16*
	rs3737522	3,223	Intronic
	rs3018316	6,710	Intronic
	rs2793845	7,587	3′downstream *AIM2*
	rs2852695	8,735	Intronic
	rs2814770	11,896	Intronic
	rs2814771	12,430	Intronic
	rs12098223	12,974	Intronic
	rs3754460	17,822	Intronic
	rs74122232	18,245	Intronic
	rs1633266	18,691	Intronic
	rs1772407	18,744	Intronic
	rs3768519	19,444	Intronic
	rs1616024	20,370	Intronic
	rs3768523	20,670	Intronic
rs1057028	rs1057027	12	Coding
	rs861318	167	Intronic
	rs1633256	168	Intronic
	rs1772415	389	Intronic
	rs856057	654	Intronic
	rs856056	680	Intronic
	rs856055	796	Intronic
	rs856054	868	Intronic
	rs856053	885	Intronic
	rs1417804	1,594	Intronic
	rs1614182	2,137	Intronic
	rs1633262	2,499	Intronic
	rs1772408	3,260	Intronic
	rs1633265	3,338	Intronic
	rs2570916	10,257	Intronic
	rs855865	25,989	5′ upstream

Linkage disequilibrium with lead SNP: Utah residents (CEPH) with Northern and Western European ancestry (CEU) $D' = 1$, $r^2 = 1$.

region potentially affect the biologic host response of the individual, leading to increased numbers of periodontal pathogens present in plaque samples. Cytokine analysis showed that individuals with haplotype block rs6940 had a four-fold increased concentration in levels of GCF–IL-1β, with a trend of significance for rs1057028 of two-fold increase (Table 4). Because IL-1β is a well-known proinflammatory cytokine implicated in periodontal disease progression,[34] the finding of increased levels of this cytokine in the GCF in the presence of SNPs in the *IFI16* and *AIM2* regions suggest that potential defects in *IFI16/AIM2* can alter the inflammatory response of an individual and further influence disease status. Individuals with both haplotype blocks showed an increased percentage of severe periodontal disease (BGI P3) compared with healthy controls (Table 5), reaching statistical significance for haplotype block rs1057028 ($P = 0.02$). BGI classification accounts for PD and BOP (and not CAL), representing the current disease activity and inflammation of the individual. Because the presence of SNPs in the *IFI16* and *AIM2* regions lead to an increase in number of individuals with periodontal disease/inflammation, it suggests that the functional defect increased predisposition of the individual developing the multifactorial condition of periodontal disease. Taken together, the data suggest that the presence of these SNPs alters the normal host response, leading to an increased predisposition to develop periodontal disease, observed by higher numbers of periodontal microorganisms, increased measurements of periodontal disease, and higher number of individuals with clinical disease.

Descriptive Histologic Distribution of IFI16 and AIM2 in Gingival Tissues

The purpose of this approach was to describe IFI16 and AIM2 protein expression in cells of the periodontium. Immunohistochemistry analysis was performed in gingival tissue samples of individuals with healthy tissues (n = 2) (Figs. 1A through 1I) and chronic periodontal disease (n = 2) (Figs. 1J through 1R). Demographics of this population are shown in supplementary Table 3 in online *Journal of Periodontology*. Representative low-resolution (×10) images (Figs. 1A, 1D, 1G, 1J, 1M, and 1P) of healthy and periodontitis tissues show a similar pattern of expression among samples. Both proteins had a homogeneous distribution in the epithelial layer, with minimal to no expression in the keratin layer, among healthy and periodontitis samples (Figs. 1B, 1E, 1K, and 1N). IFI16 staining was dense in the basal layer (Figs. 1A and 1B). Migrating neutrophils expressing IFI16 and AIM2 were observed in the epithelial layer in a sample derived from individuals with periodontal disease (Figs. 1K and 1N). In the connective tissue (Figs. 1C, 1F, 1L, and 1O), endothelial cells and cells of the inflammatory infiltrate showed expression of both IFI16 and AIM2. Minor expression of these proteins was observed in fibroblasts in the connective tissue. All samples (n = 4) demonstrated a similar pattern of staining for both proteins. This descriptive analysis demonstrated that cells of the periodontal apparatus express IFI16 and AIM2 and, therefore, further supports a potential role of these proteins in the pathogenesis of periodontitis.

Table 2.

Clinical Measurements Among Individuals With SNPs in Haplotype Blocks, Mean (SD)

SNP	n	EPDGE4 (%)	Mean PD (mm)	EBOP (%)	EGIGE1 (%)	Mean CAL (mm)
rs6940*						
1.1	3,590	6.84 (0.16)	1.86 (0.01)	23.1 (0.33)	22.0 (0.46)	1.64 (0.01)
1.2	1,057	6.84 (0.30)	1.84 (0.02)	23.7 (0.61)	22.4 (0.84)	1.65 (0.02)
2.2	80	6.28 (1.13)	1.82 (0.06)	28.3 (2.28)†	24.9 (3.21)	1.51 (0.1)
rs1057028*						
1.1	3,199	6.84 (0.17)	1.86 (0.01)	23.1 (0.35)	22.1 (0.49)	1.64 (0.01)
1.2	1,367	6.64 (0.27)	1.84 (0.01)	23.1 (0.54)	22.1 (0.74)	1.63 (0.02)
2.2	161	8.34 (0.79)†	1.92 (0.04)	28.8 (1.60)‡	25.1 (2.24)	1.75 (0.07)

Genotypes: 1.1, homozygous for the major allele; 1.2, heterozygous; 2.2, homozygous for the minor allele. Data were adjusted for PI.
* Haplotype blocks for both SNPs include additional SNPs shown in Table 1.
χ^2 *P* values using 1.1 as the referent category: †$P \leq 0.05$; ‡$P \leq 0.01$.

Table 3.

Mean Levels of Periodontal Microorganism (SE) and Relative (fold) Changes for rs6940 and rs1057028 Haplotype Blocks (adjusted for plaque levels)

SNP	n	*Pg*	*Pi*	*Pn*	*Tf*	*Td*	*Cr*	*Fn*	*Aa*
rs6940*									
1.1	781	2.22 (0.06)	2.55 (0.07)	2.68 (0.07)	2.43 (0.06)	2.61 (0.07)	2.66 (0.07)	2.86 (0.08)	2.57 (0.06)
1.2	255	2.27 (0.10)	2.71 (0.13)	2.51 (0.13)	2.49 (0.11)	2.53 (0.12)	2.72 (0.11)	2.96 (0.13)	2.74 (0.10)
2.2	17	3.54 (0.43)‡	3.01 (0.54)	3.47 (0.52)	3.50 (0.45)†	3.11 (0.48)	3.69 (0.48)†	3.94 (0.55)	2.99 (0.44)
Fold change		274.34	58.41	120.34	191.54	64.87	180.11	194.47	52.20
rs1057028*									
1.1	711	2.24 (0.06)	2.62 (0.08)	2.71 (0.08)	2.42 (0.07)	2.63 (0.07)	2.67 (0.07)	2.86 (0.08)	2.59 (0.06)
1.2	300	2.21 (0.10)	2.48 (0.12)	2.42 (0.12)	2.46 (0.10)	2.47 (0.11)	2.65 (0.11)	2.90 (0.12)	2.58 (0.10)
2.2	42	2.89 (0.26)†	3.07 (0.32)	3.31 (0.31)	3.13 (0.27)†	2.92 (0.29)	3.34 (0.29)†	3.55 (0.33)†	3.25 (0.26)†
Fold change		91.55	56.83	82.21	103.40	33.64	95.42	99.37	93.48

Genotypes: 1.1, homozygous for the major allele; 1.2, heterozygous; 2.2, homozygous for the minor allele.
* Haplotype blocks for both SNPs include additional SNPs shown in Table 1.
χ^2 *P* values using 1.1 as the referent category: †$P \leq 0.05$; ‡$P \leq 0.01$; fold change comparing 2.2 versus 1.1.

DISCUSSION

The present study characterized clinical and biologic periodontal data among a sizeable group of participants and examined their association with SNPs in the *IFI16/AIM2* locus. Two haplotype blocks were found, one including a missense SNP rs6940 and a variant close to neighboring gene *AIM2* and a second block including missense SNP rs1057028, that were significantly associated with periodontal disease parameters, including increased extent PD and BOP, increased GCF–IL-1β levels, higher loads of periodontal pathogens, and higher prevalence of severe periodontal disease. Prediction analysis indicated that the function of IFI16 is altered by the presence of rs6940 and rs1057028. Several additional SNPs were in perfect linkage disequilibrium with the index SNPs. These polymorphisms are quite common in the general population with minor allele frequencies of 0.22 for rs6940 (minor allele: T) and 0.3 for rs1057028 (minor allele: T). It was identified that IFI16 and AIM2 are expressed in epithelial cells, fibroblasts, endothelial cells, and leukocytes of gingival tissues. Presence of these proteins in inflammatory cells of gingival tissues, including the finding of neutrophils in the epithelial layer (Figs. 1K and 1N) suggests a role of IFI16 and AIM2 in the response to periodontal pathogens. Taken together, these findings propose that these proteins and specific polymorphisms may have an important role in periodontal disease pathogenesis.

Previous GWAS analysis by the current group of authors has highlighted loci potentially associated with clinically derived-disease definitions, like chronic periodontal disease,[9,11] and high levels of specific periodontal pathogens.[35] However, no single marker met the strict genome-wide significance. Only four loci met gene-centric statistical significance.[11] This suggested

Table 4.

Mean log GCF–IL-1β Levels and Relative (fold) Changes Contrasting 2.2 Versus 1.1, According to rs6940 and rs1057028 Genotypes (adjusted for plaque levels)

SNP	n	Mean log IL-1β (SE)
rs6940*		
1.1	3,359	2.05 (0.01)
1.2	976	2.04 (0.01)
2.2	73	2.08 (0.04)
Fold change		3.05
rs1057028*		
1.1	3,006	2.05 (0.01)
1.2	1,255	2.04 (0.01)
2.2	147	2.10 (0.03)†
Fold change		5.13

Genotypes: 1.1, homozygous for the major allele; 1.2, heterozygous; 2.2, homozygous for the minor allele.
* Haplotype blocks for both SNPs include additional SNPs shown in Table 1.
† χ^2 $P \leq 0.05$ using 1.1 as the referent category; fold change comparing 2.2 versus 1.1.

Table 5.

Distribution of rs6940 and rs1057028 Haplotype Blocks According to BGI Periodontal Disease Classification (healthy [n = 571] versus severe periodontitis [n = 531])

	Individuals (%)	
SNP	Healthy	Severe periodontitis (P3)
rs6940*		
1.1	76.01	73.63
1.2	23.12	24.11
2.2	0.88	2.26
rs1057028*		
1.1	67.78	65.35
1.2	29.95	29.19
2.2	2.28	5.46†

Genotypes: 1.1, homozygous for the major allele; 1.2, heterozygous; 2.2, homozygous for the minor allele.
* Haplotype blocks for both SNPs include additional SNPs shown in Table 1.
† $P \leq 0.05$.

the existence of several distinct conditions with different genetic and biologic backgrounds with similar overlapping clinical presentations of periodontal tissue loss. Therefore, a new approach was used by defining the disease phenotype as complex traits as previously described.[14,36] With this approach, six PCTs were identified by levels of eight periodontal pathogens, local inflammatory response (GCF–IL-1β), and clinical data.[14] PCT1 was defined by a uniformly high periodontal pathogen load and significantly correlated with clinical periodontal disease parameters. Six loci were associated with PCT1, which included *IFI16/AIM2*. Although there was a degree of anticipation that the *IFI16/AIM2* haplotype blocks would be correlated with some periodontal microorganisms since the lead SNP was identified from an analysis that used a microbial community structure, individual microorganisms correlated with the haplotype blocks were not known. In addition, clinical measurements and disease significance in this population and tissue distribution of IFI16 and AIM2 were further characterized. Therefore, the present study further refines findings derived from the PCT1 analysis and defines the clinical and biologic characteristics of individuals with SNPs in the *IFI16/AIM2* region.

Both IFI16 and AIM2 are PYHIN inflammasome proteins and mediators of innate immune responses.[17] Inflammasomes are multiprotein oligomers that promote activation of inflammatory cytokines IL-1β and IL-18.[37] PYHIN inflammasome proteins bind microbial DNA and form caspase-1-activating inflammasomes (AIM2) or drive type I *IFN* gene transcription (IFI16).[17] IFI16 is also a mediator of the AIM2 inflammasome-dependent pathway by directly binding to AIM2.[38] Therefore, IFI16 has shown anti-inflammatory effects and AIM2 proinflammatory effects. This suggests that defects in expression or protein function of IFI16 could dampen the anti-inflammatory response and thereby increase the proinflammatory response. Studies show that AIM2 is increased in wound healing and in several inflammatory conditions, including psoriasis, atopic dermatitis, and venous ulcers.[18,19] Additional support for the role of AIM2 and IFI16 in inflammation is a recent demonstration of a strong increase of both proteins in the mucosa of individuals with active inflammatory bowel disease.[22] In accordance with the present findings, three studies have previously demonstrated the presence of AIM2 in gingival tissues.[20,21,39] AIM2 expression was increased in gingival tissues from individuals with CP compared with healthy controls and generalized aggressive periodontitis (AgP). In addition, *Pg* infection can activate the AIM2 inflammasome in vitro.[39] These findings further support an importance of AIM2 in the pathogenesis of periodontal disease. To the best knowledge of the authors, no other study has evaluated expression of *IFI16* in periodontal tissues. Further analysis and quantification of this protein in periodontal cells and tissues in various disease states is warranted.

The concept that the host genotype can influence microbiota and lead to disease has been reported previously. Counts of periodontal pathogens from the red and orange complex are reported to be significantly

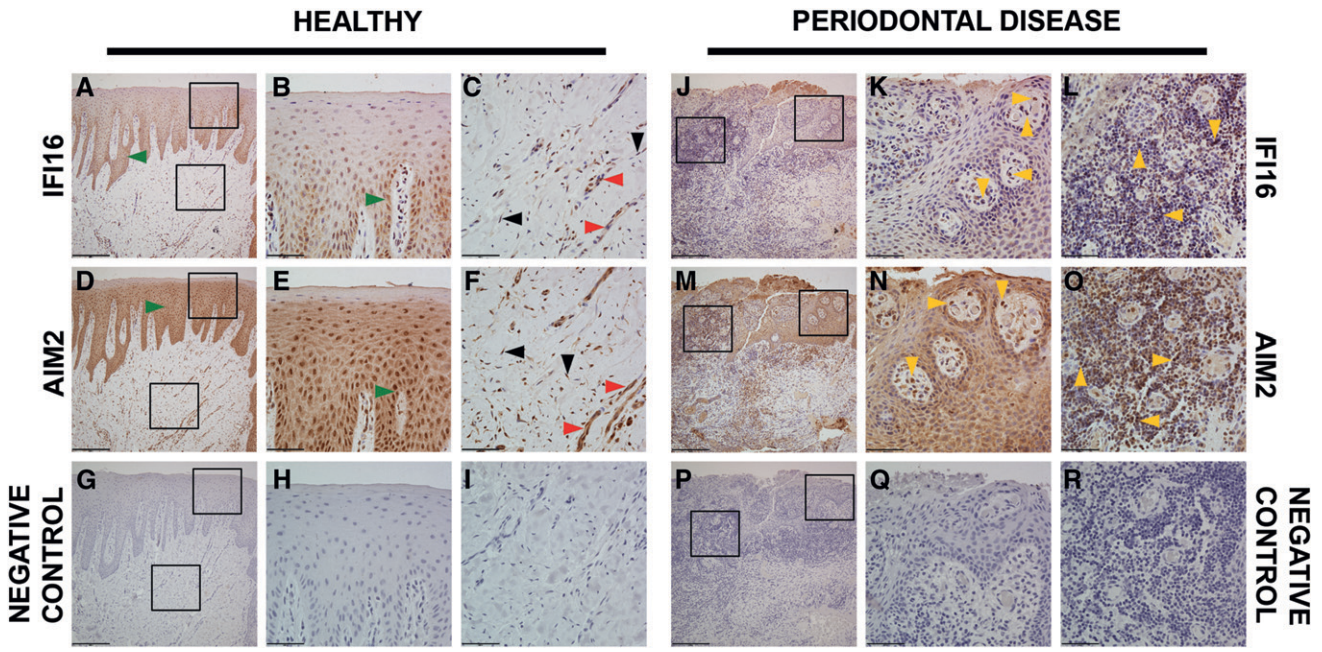

Figure 1.
Immunohistochemical detection of IFI16 and AIM2 in human gingival tissues. Representative images of tissue sections from a healthy individual ***(A through I)*** *and an individual with periodontal disease* ***(J through R)*** *according to the ADA/AAP classification, stained with the indicated antibodies (horizontal rows). Bottom row shows negative controls. A, D, G, J, M, and P represent original magnification ×10 (scale bar = 200 μm); B, E, H, K, N, and Q and C, F, I, L, O, and R represent original magnification ×40 (scale bar = 50 μm) of the square inserts located in the figures with original magnification ×10 in the epithelial and connective tissue layer, respectively. Green arrowhead = epithelial cells; black arrowhead = fibroblasts; yellow arrowhead = leukocytes; red arrowhead = endothelial cells.*

higher at PD >6 mm in *IL-1* genotype-positive individuals compared with genotype-negative individuals.[6] *IL-6* polymorphisms and haplotypes have also been associated with periodontitis, possibly due to the transcription of *IL-6*, which alters tissue levels.[7,40] An alteration in the immune response observed in leukocyte adhesion deficiency also leads to significant changes in the subgingival flora and severe periodontitis.[8] These results are in support of the current data and the concept that changes in the innate immune functions can alter the host response and facilitate development of diseases. A recent study found that *IFI16* rs6940 (also identified in this study) and *AIM2* rs855873 (also upstream of *AIM2*) were associated with increased susceptibility to Behçet disease, a systemic immune-mediated disease characterized by vasculitis and recurrent mucosal ulcerations.[41] The study shows rs6940 decreases expression of anti-inflammatory IFI16 and increases susceptibility to an immune-mediated disease.[41] The fact that Behçet is associated with epithelial ulceration and the present results show associations of these SNPs with BOP suggests that these variants may impair innate immune responses that maintain epithelial integrity at mucosal surfaces. In fact, individuals with Behçet disease have increased periodontal disease severity compared with healthy controls, suggesting both diseases share pathogenic aspects.[42,43] These findings support the hypothesis of a role of anti-inflammatory IFI16 in periodontal disease pathogenesis and that variants of this gene predispose individuals for an altered immune response to infection that ultimately leads to disease.

Functional analysis was done to help identify SNPs that have a high potential of altering protein function. The current analysis indicates that both missense SNPs rs6940 and rs1057028 are not localized in the known PYRIN, HIN-200, or p53-binding domains. However, predictions suggest that protein function is potentially/probably altered. Because rs1057028 is localized between both HIN domains it is possible that the variant induces a three-dimensional conformation change to the protein structure. SNP rs6940 is predicted to alter only isoform 3, which is probably related to the different sizes of the isoforms. A previous study suggests that instead of protein function, SNP IFI16 rs6940 decreases expression of *IFI16*.[41] It is possible that additional SNPs in high linkage disequilibrium with rs6940 are leading to this effect. However, no functional assays were performed in the study.[41] Because the SNP associated with AIM2 is not present in a missense region, predictions for potential alterations could not be performed. Future studies defining the impact of SNPs present in the two

haplotype blocks in protein expression and host response should provide additional evidence for the pathogenesis of periodontitis.

Although a biologically relevant region associated with elevated periodontitis parameters was identified, the present results have limitations. Validity of the reported SNP associations will need to be further examined and replicated in a new study. However, it is important to note that identification of these genes was not based on traditional AAP/ADA classification for CP and AgP, which is used in other GWASs.[10,44-46] Instead, these genes were first identified in the context of a PCT that used a combination of microorganisms and GCF–IL-1β levels.[14] In this model, periodontal disease is a group of distinct biologic conditions with overlapping clinical presentations. Therefore, future GWAS analysis may consider periodontal complex traits for identifying biologically relevant genes.

CONCLUSIONS

Together, the present results support a role of for IFI16/AIM2 in the pathogenesis of periodontal disease. This association was observed by correlation of SNP variants with increased measurements of microorganisms in the subgingival plaque, increased levels of GCF–IL-1β, increased periodontal clinical parameters of disease, and increased prevalence of severe disease.

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REFERENCES

1. Michalowicz BS, Diehl SR, Gunsolley JC, et al. Evidence of a substantial genetic basis for risk of adult periodontitis. *J Periodontol* 2000;71:1699-1707.
2. Michalowicz BS, Aeppli D, Virag JG, et al. Periodontal findings in adult twins. *J Periodontol* 1991;62:293-299.
3. Kornman KS. Mapping the pathogenesis of periodontitis: A new look. *J Periodontol* 2008;79(Suppl. 8): 1560-1568.
4. Weir BS. Bioinformatics and approaches to identifying polygenic susceptibility traits. *Ann Periodontol* 2002;7: 1-7.
5. Mucci LA, Björkman L, Douglass CW, Pedersen NL. Environmental and heritable factors in the etiology of oral diseases – A population-based study of Swedish twins. *J Dent Res* 2005;84:800-805.
6. Socransky SS, Haffajee AD, Smith C, Duff GW. Microbiological parameters associated with IL-1 gene polymorphisms in periodontitis patients. *J Clin Periodontol* 2000;27:810-818.
7. Nibali L, D'Aiuto F, Donos N, et al. Association between periodontitis and common variants in the promoter of the interleukin-6 gene. *Cytokine* 2009;45:50-54.
8. Moutsopoulos NM, Chalmers NI, Barb JJ, et al. Subgingival microbial communities in leukocyte adhesion deficiency and their relationship with local immunopathology. *PLoS Pathog* 2015;11:e1004698.
9. Divaris K, Monda KL, North KE, et al. Exploring the genetic basis of chronic periodontitis: A genome-wide association study. *Hum Mol Genet* 2013;22:2312-2324.
10. Teumer A, Holtfreter B, Völker U, et al. Genome-wide association study of chronic periodontitis in a general German population. *J Clin Periodontol* 2013;40:977-985.
11. Rhodin K, Divaris K, North KE, et al. Chronic periodontitis genome-wide association studies: Gene-centric and gene set enrichment analyses. *J Dent Res* 2014; 93:882-890.
12. Feng P, Wang X, Casado PL, et al. Genome wide association scan for chronic periodontitis implicates novel locus. *BMC Oral Health* 2014;14:84.
13. American Academy of Periodontology. American Academy of Periodontology Task Force report on the update to the 1999 classification of periodontal diseases and conditions. *J Periodontol* 2015;86:835-838.
14. Offenbacher S, Divaris K, Barros SP, et al. Genome-wide association study of biologically informed periodontal complex traits offers novel insights into the genetic basis of periodontal disease. *Hum Mol Genet* 2016;25:2113-2129.
15. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr. Microbial complexes in subgingival plaque. *J Clin Periodontol* 1998;25:134-144.
16. Hornung V, Ablasser A, Charrel-Dennis M, et al. AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. *Nature* 2009;458: 514-518.
17. Schattgen SA, Fitzgerald KA. The PYHIN protein family as mediators of host defenses. *Immunol Rev* 2011;243: 109-118.

18. Dombrowski Y, Peric M, Koglin S, et al. Cytosolic DNA triggers inflammasome activation in keratinocytes in psoriatic lesions. *Sci Transl Med* 2011;3:82ra38.
19. de Koning HD, Bergboer JG, van den Bogaard EH, et al. Strong induction of AIM2 expression in human epidermis in acute and chronic inflammatory skin conditions. *Exp Dermatol* 2012;21:961-964.
20. Sahingur SE, Xia XJ, Voth SC, Yeudall WA, Gunsolley JC. Increased nucleic acid receptor expression in chronic periodontitis. *J Periodontol* 2013;84:e48-e57.
21. Xue F, Shu R, Xie Y. The expression of NLRP3, NLRP1 and AIM2 in the gingival tissue of periodontitis patients: RT-PCR study and immunohistochemistry. *Arch Oral Biol* 2015;60:948-958.
22. Vanhove W, Peeters PM, Staelens D, et al. Strong upregulation of AIM2 and IFI16 inflammasomes in the mucosa of patients with active inflammatory bowel disease. *Inflamm Bowel Dis* 2015;21:2673-2682.
23. Beck JD, Elter JR, Heiss G, Couper D, Mauriello SM, Offenbacher S. Relationship of periodontal disease to carotid artery intima-media wall thickness: The atherosclerosis risk in communities (ARIC) study. *Arterioscler Thromb Vasc Biol* 2001;21:1816-1822.
24. Elter JR, Champagne CM, Offenbacher S, Beck JD. Relationship of periodontal disease and tooth loss to prevalence of coronary heart disease. *J Periodontol* 2004; 75:782-790.
25. Aulchenko YS, Struchalin MV, van Duijn CM. ProbABEL package for genome-wide association analysis of imputed data. *BMC Bioinformatics* 2010;11:134.
26. Pruim RJ, Welch RP, Sanna S, et al. LocusZoom: Regional visualization of genome-wide association scan results. *Bioinformatics* 2010;26:2336-2337.
27. Dayem Ullah AZ, Lemoine NR, Chelala C. A practical guide for the functional annotation of genetic variations using SNPnexus. *Brief Bioinform* 2013;14:437-447.
28. Dayem Ullah AZ, Lemoine NR, Chelala C. SNPnexus: A web server for functional annotation of novel and publicly known genetic variants (2012 update). *Nucleic Acids Res* 2012;40:W65-W70.
29. Chelala C, Khan A, Lemoine NR. SNPnexus: A web database for functional annotation of newly discovered and public domain single nucleotide polymorphisms. *Bioinformatics* 2009;25:655-661.
30. Johnson AD, Handsaker RE, Pulit SL, Nizzari MM, O'Donnell CJ, de Bakker PI. SNAP: A web-based tool for identification and annotation of proxy SNPs using HapMap. *Bioinformatics* 2008;24:2938-2939.
31. UniProt Consortium. UniProt: A hub for protein information. *Nucleic Acids Res* 2015;43:D204-D212.
32. Offenbacher S, Barros SP, Singer RE, Moss K, Williams RC, Beck JD. Periodontal disease at the biofilm-gingival interface. *J Periodontol* 2007;78:1911-1925.
33. Offenbacher S, Lin D, Strauss R, et al. Effects of periodontal therapy during pregnancy on periodontal status, biologic parameters, and pregnancy outcomes: A pilot study. *J Periodontol* 2006;77:2011-2024.
34. Engebretson SP, Grbic JT, Singer R, Lamster IB. GCF IL-1beta profiles in periodontal disease. *J Clin Periodontol* 2002;29:48-53.
35. Divaris K, Monda KL, North KE, et al. Genome-wide association study of periodontal pathogen colonization. *J Dent Res* 2012;91(Suppl. 7):21S-28S.
36. Suo C, Toulopoulou T, Bramon E, et al. Analysis of multiple phenotypes in genome-wide genetic mapping studies. *BMC Bioinformatics* 2013;14:151.
37. Martinon F, Burns K, Tschopp J. The inflammasome: A molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol Cell* 2002;10:417-426.
38. Veeranki S, Duan X, Panchanathan R, Liu H, Choubey D. IFI16 protein mediates the anti-inflammatory actions of the type-I interferons through suppression of activation of caspase-1 by inflammasomes. *PLoS One* 2011;6:e27040.
39. Park E, Na HS, Song YR, Shin SY, Kim YM, Chung J. Activation of NLRP3 and AIM2 inflammasomes by *Porphyromonas gingivalis* infection. *Infect Immun* 2014;82:112-123.
40. Fife MS, Ogilvie EM, Kelberman D, et al. Novel IL-6 haplotypes and disease association. *Genes Immun* 2005;6:367-370.
41. Ortiz-Fernández L, García-Lozano JR, Montes-Cano MA, et al. Variants of the IFI16 gene affecting the levels of expression of mRNA are associated with susceptibility to Behçet disease. *J Rheumatol* 2015;42:695-701.
42. Akman A, Kacaroglu H, Donmez L, Bacanli A, Alpsoy E. Relationship between periodontal findings and Behçet's disease: A controlled study. *J Clin Periodontol* 2007;34:485-491.
43. Arabaci T, Kara C, Ciçek Y. Relationship between periodontal parameters and Behçet's disease and evaluation of different treatments for oral recurrent aphthous stomatitis. *J Periodontal Res* 2009;44:718-725.
44. Shimizu S, Momozawa Y, Takahashi A, et al. A genome-wide association study of periodontitis in a Japanese population. *J Dent Res* 2015;94:555-561.
45. Freitag-Wolf S, Dommisch H, Graetz C, et al. Genome-wide exploration identifies sex-specific genetic effects of alleles do NPY to increase the risk of severe periodontitis in men. *J Clin Periodontol* 2014;41:1115-1121.
46. Shaffer JR, Polk DE, Wang X, et al. Genome-wide association study of periodontal health measured by probing depth in adults ages 18-49 years. *G3 (Bethesda)* 2014;4:307-314.

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