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The Effect of Single Nucleotide Polymorphisms (SNPs) in Toll-Like Receptors -2, -4, -9, and CD14 Genes in an African-American Population with Chronic Periodontitis

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THE EFFECT OF SINGLE NUCLEOTIDE POLYMORPHISMS (SNPs) IN TOLL-LIKE RECEPTORS -2, -4, -9, AND CD14 GENES IN AN AFRICAN-AMERICAN POPULATION WITH CHRONIC PERIODONTITIS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Dentistry at Virginia Commonwealth University.

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AIM: to determine if a relationship exists between TLR-2, TLR-4, TLR-9, or CD14 polymorphisms and risk for developing chronic periodontal disease in an African-American population. This is the first study conducted to determine role of SNPs in TLR genes and CD14 gene in a periodontally-diseased African-American population. Additionally, this is the first study to assess the role of TLR-9 polymorphism in periodontitis patients. METHODS: A total of 130 subjects were involved in the study. The chronic periodontitis (CP) group contained 73 subjects, and the healthy control (NP) group 57subjects. Genotyping was performed in TLR2 (G2408A), TLR4 (A896G),TLR9

(T1486C) and CD14 (C260T) genes by TaqMan® allelic discrimination using Assay-by-Design^{SM} SNP Genotyping Assays (Applied Biosystems). Accuracy of genotyping was confirmed by known DNA samples of each genotype and by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) analyses on selected samples. Fisher's exact test and chi-square analyses were performed to compare genotype and allele frequencies. Within disease groups, we investigated whether SNPs were related to disease severity by step-wise logistic regression adjusted for age, gender, and smoking status. RESULTS: There was a significant difference in the distribution of specific TLR9 (T1486C) genotypes between the periodontally diseased group versus the control group. Expression of TT genotype was more prevelant in periodontally-diseased individuals compared to periodontally-healthy subjects ($p<0.0001$) whereas individuals expressing C allele of the TLR9 SNP (CC&CT) were more frequently found in the control group after adjusting for age, gender, and smoking status ($p<0.0001$) There was no statistically significant difference in the distribution of genotypes between groups for any other TLRs or CD14 polymorphism. CONCLUSION: Based on findings of this study, homozygocity for the T allele of TLR 9 polymorphism was related to chronic periodontal disease susceptibility in African Americans. Additionally, presence of the C allele at TLR-9 appeared to confer resistance to periodontal destruction. Our results showed that specific SNPs in TLR-2, -4 and CD14 genes are not related to periodontitis in African Americans.

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Introduction

Periodontal disease is the result of a chronic bacterial infection involving gram-negative anaerobic pathogens. Bacteria colonizes on the tooth surface into biofilms (dental plaque) and initiates a host-driven inflammatory response leading to gingival inflammation. Dental plaque contains several hundred different bacterial species, and so far no single periodontopathogenic species has been identified as the sole causative factor for periodontitis. Initially, the biofilm consists of mainly gram positive aerobic bacteria. The colony then undergoes a maturing process to a predominantly gram-negative, facultative anaerobic aggregate.[1] As the biofilm ages, this increased bacterial insult to the host elicits an inflammatory response. Inflammatory response can lead to increased release of inflammatory mediators, including cytokines. Increased tissue concentration of inflammatory cytokines can cause damage to host tissues supporting the teeth on which the biofilm has colonized if left untreated.

Periodontal disease is a multifactorial response to bacterial infection which is influenced by individual host response, bacterial load, genetic susceptibility, and environment. Numerous reports have demonstrated a close association between periodontal tissue destruction and the development of a complex of microbial species within the plaque aggregate including *Porphyromonas gingivalis, Tannerella forsythia, Aggregatibacter* (formerly *Actinobacillus*) *actinomycetemcomitans, Treponema denticola, Prevotella intermedia,* and *Fusobacterium nucleatum.*[2,3,4] The process involved during this development was investigated by many authors. Haffajee and Socransky studied organisms responsible for periodontal destruction, and created a modality for classifying bacterial colonizers.[5] The so termed "Socransky complexes" were described as early or late colonizers according to the time frame in which they colonize in the plaque biofilm.[6,7]

Some individuals have a greater inflammatory response to periodontal-specific bacteria, and experience more destruction and increased bone loss compared to the average

individual response.[8] This increased destruction may be due to a genetic predisposition. Table 1 lists multiple syndromes attributed to increased susceptibility to infections. All syndromes listed in table 1 also demonstrate risk of accelerated periodontal destruction.

Genetic predisposition has been studied and reported to have an effect on host susceptibility to periodontal destruction.[9] Support for genetic susceptibility for the more common forms of adult periodontitis is increasing and suggests a multifactorial disease pattern. The results of twin studies clearly indicate that a significant part of the variance in clinical and radiographic measures of periodontitis may be explained by genetic factors.[10] Several immune response traits have been associated with clinical forms of periodontitis, and for some of these factors the underlying genetic determinants are known. Although it is unlikely that polymorphisms in all of these genetic determinants impart differential susceptibility to periodontal disease, it is reasonable to expect that multiple genes will be important and that knowledge of these may permit determination of individual susceptibility.[57]

Pathogen-associated molecular patterns (PAMPs) represent conserved bacterial components expressed by microorganisms that are not found in higher eukaryotic cells.[11] They are recognized by toll-like receptors (TLRs) and other pattern recognition receptors (PRRs) in plants and animals. Some of the periodontal bacterial-associated PAMPs include LPS, lipoproteins, fimbriae, and bacterial or viral DNA.[12,13,14,15,16,17] PAMPs can activate host immune responses that may lead to increased cytokine production from monocytes and macrophages, including IL-1, TNFα, and IL-6.[11] These cytokines are responsible for osteoclast activation, leading to bone resorption, and ultimately resulting in periodontal tissue destruction.[12] The balance between inflammatory and anti-inflammatory cytokine expression is vital in the maintenance of health.

Toll-like receptors (TLRs) are transmembrane pattern-recognition receptors (PRRs) that mediate intracellular signaling and antimicrobial responses upon recognition of conserved PAMPs, and play a major role in nonspecific (innate) immunity.[11] They contain an extracellular domain, and a cytoplasmic domain with homology to the mammalian IL-1 receptor.[11] TLRs recognize PAMPs that are expressed by a variety of microorganisms,

including pathogens known to be associated with periodontal disease,[14,15] forming a cellular recognition barrier for PAMPs related to multiple periodontopathogenic bacteria. TLRs also lack memory, which is characteristic to the innate immune system. Figure 1 illustrates known Toll-like receptors and their cellular locations.

TLR-4 is a principal signaling receptor for LPS which works synergistically with CD14.[12] TLR-4 is located at the cell surface as a transmembrane protein, and as such is an early interceptor of extracellular PAMPs. TLR4 (A896G) SNP is located in the ectoplasmic domain of the receptor, resulting in an aspartic acid to glycine substitution at position 299. The receptor CD14 is involved in lipopolysaccharide (LPS) signal transduction and therefore is essential for interfacing the innate immune system with microbial pathogens, as well as directing adaptive immune responses along specific T helper differentiation pathways through induction of cytokine expression.[11] The signal transduction of the LPS/LBP/CD14 ternary complex on effector cells is transferred via TLR-4 mediation. Upon stimulation, the TLR-4 complex leads to the activation of innate host defense mechanisms via the nuclear factor KB (NF-KB) pathway and the release of proinflammatory cytokines such as TNF-α, IL-1β, IL-6, and interferon-γ.[18] Increased expression of TLR-4 in gingival tissue from periodontal patients provides evidence that TLR-mediated host immune responses may play a role in the periodontal disease process.[13] Additionally, the A896G SNP was shown to be associated with ‘‘an endotoxin hyporesponsive phenotype’’ in concert with other genetic changes or acquired factors that influence the complex physiological response to LPS.[19] Recently, single nucleotide polymorphisms in different functional polymorphisms of TLR-4 gene have been described that are associated with impaired LPS signal transduction, leading to increased susceptibility to infections.[20] SNPs in TLR-4 genes were described by Folwaczny[21] in patients with chronic periodontitis. Lorenz et al[22] found a correlation between TLR-4 and presence of gram-negative bacteria. In another study, patients with SNPs in TLR-4 gene had greater risk of chronic periodontal destruction, but not aggressive periodontitis.[20]

CD14 C260T SNP is located in the promoter region. The polymorphism results in a cytosine to thymine substitution.[19] The SNP CD14-C260T is thought to enhance

transcriptional activity, and is associated with enhanced monocyte CD14 expression. Lipopolysaccharide is internalized by cells through the CD14 receptor. The CD14 receptor is expressed in considerable amounts by mature monocytes, macrophages and activated neutrophil granulocytes. In these cells, CD14 is anchored in the cell membrane. In addition, the soluble form (sCD14) can be found in plasma in response to LPS stimulation, where two major isoforms coexist. Lipopolysaccharide binds to the membrane-bound CD14 on monocytes and macrophages and activates these cells. Soluble CD14 plays an important role in the LPS-mediated activation of cells lacking membrane-bound CD14, so-called "CD14-negative cells" (endothelial, epithelial and smooth muscle cells). In addition, these CD14-negative cells are activated indirectly by cytokines from LPS-stimulated monocytes. But sCD14 can also activate monocytes independently of LPS. Thus, both mCD14 and sCD14 are competing for LPS and are able to bind it. Changes in CD14 expression and plasma sCD14 levels seem to be associated with an increasing number of disorders like septicemia[23] and periodontitis.[24] Recently, research in this field has focused on the gene of the CD14 receptor. A polymorphism in the CD14 gene has been reported to be associated with increased risk of acute myocardial infarct.[25] It was also suggested that the C260T change in the promoter region affects the level of CD14 gene expression.[26]

TLR-2 recognizes many bacterial, fungal, viral, and certain endogenous substances. Among these, peptidoglycan and lipoproteins, which are components in the cell walls of Gram-positive and Gram-negative bacteria, are the ligands for TLR-2. TLR-2 is a membrane protein, which is expressed on the surface of certain cells and recognizes foreign substances and passes on appropriate signals to the cells of the immune system. The TLR-2 SNP is located in the exon region, and represents a substitution for Guanine with Adenine at position 2408. It was shown that TLR-2 and TLR-4 are constitutively expressed in periodontal tissue and that TLRs can recognize a variety of bacterial components. Studies have found that there is no correlation of TLR-2 polymorphisms and chronic periodontitis in Japanese,[27] Chinese,[28] German,[21] or Turkish populations.[29]

TLR-9 is another toll-like protein responsible for cellular recognition of bacterial and viral DNA.[16] This receptor is expressed in immune cell rich tissues, such as spleen,

lymph node, bone marrow and peripheral blood leukocytes. The major human cell types that express TLR9, and therefore the cells that play a central role in TLR9 signaling, are B cells and plasmacytoid dendritic cells.[30] TLR-9 has been shown to be expressed in intracellular compartments such as endosomes.[12] The TLR9 (T1486C) SNP at position 1486 is located in the promoter region and represents a substitution of cytosine with thymine. Bacteria is first non-specifically captured into endosomes, where TLR-9 is recruited from the endoplasmic reticulum upon the PAMP uptake. Bacterial DNA is then exposed after degradation of bacteria in phagosomes/lysosomes or endosomes/lysosomes in the endoplasmic reticulum. TLR-9 activation then results in release of IL-6 from gingival fibroblasts.[31] TLR-9 signaling activates dendritic cells to produce proinflammatory cytokines and type I interferons (IFNs) through a set of transcription factors including NF-kB and IFN regulatory factors (IRFs). Once activated, the action of these transcription factors needs to be attenuated and/or terminated to prevent immunopathology and maintain tissue homeostasis.[32] Without downregulation of the transcription factors, increased production and concentration of cytokines in the periodontium can lead to loss of periodontal tissue. In the case of viral infection, viruses invade cells by receptor-mediated endocytosis, and the viral contents are exposed to the cytoplasm by fusion of the viral membrane and the endosomal membrane. Occasionally, the viral particles are degraded in the endosomal compartment, which results in exposure of TLR ligands such as dsRNA, ssRNA and CpG DNA. SNPs in TLR9 have also been described and found to be associated with various disease processes, including asthma,[33] graft versus host disease,[34] and other immune-related inflammatory disorders.[35,36] A 2008 study by Beklen found increased TLR-9 expression in periodontally diseased sites.[37] Additionally, another study by Yamazaki found increased expression of TLR-9 in periodontitis when compared with that of gingivitis.[38]

Fibroblasts, osteoblasts, and cementoblasts are all major components of the human periodontium. A study by Tabeta found that gingival fibroblasts respond to periodontopathic bacterial LPS via TLR-2 associated with mCD14 and TLR-4–MD-2 complex, resulting in the production of proinflammatory cytokines, which can lead to

periodontal tissue destruction.[39] While the primary role of cementoblasts and osteoblasts is to synthesize the components of cementum and bone matrix, respectively, it has been reported that osteoblasts exhibit functional expression of TLR-2, TLR-4, and TLR-9; and initiate the release of inflammatory cytokines and osteoclastogenesis by activating these receptors. CpG oligodeoxynucleotides interact with osteoblastic TLR-9 and increase the expression of molecules regulating osteoclastogenesis.[40] Expression of these toll-like receptors can lead to increased secretion of inflammatory mediators, resulting in increased host periodontal destruction. In addition to loss of bone supporting the teeth, untreated periodontal disease has been associated with various systemic diseases, such as cardiovascular disease,[41] respiratory disease,[42] diabetes,[43] and increased risk of preterm low birth weight in expectant mothers.[44,45] To date, studies investigating the relationship between TLR and CD14 gene polymorphisms and periodontal disease did not reach an agreement. We know that genetic background can be influenced by racial differences**.**

The purpose of this study is to determine if a relationship exists between TLR-2, TLR-4, TLR-9, or CD14 polymorphisms and risk for developing chronic periodontal disease in an African-American population. This is the first study conducted to determine role of SNPs in TLR genes and CD14 gene in a periodontally-diseased African-American population. Furthermore, this is the first study to assess the role of TLR-9 polymorphism in periodontitis patients. Our study provides further information pertaining to genetic susceptibility to periodontitis, allowing for earlier therapeutic intervention should an individual be determined at risk. Earlier intervention can prevent further periodontal destruction and lead to an overall greater level of health for the individual.

Methods and Materials

Subjects

A total of 130 African-American subjects between the ages of 29 to 74 years with available DNA samples were enrolled in this study. These subjects were divided into 2 groups: 71 Chronic periodontitis patients, and 59 periodontally healthy controls. The protocol was approved by an institutional review board and all subjects signed a consent form to participate in the study.

Chronic periodontitis is defined in this study as displaying at least 4 pockets ≥5mm which exhibit bleeding on probing, and at least 1 pocket ≥7mm. The control group consisted of individuals who did not have any periodontal probing depths greater than 3mm. The exclusion criteria were: manifestations of systemic disease including cancer, atherosclerosis, renal insufficiency, severe asthma, diabetes mellitus, HIV, immunosuppression, any patients being prescribed antibiotics for the past 6 months for any reason, any patients having systemic conditions that required antibiotic prophylaxis, pregnant patients, or any systemic diseases that would alter the course of dental treatment. Smoking was classified as current, former, or never smokers. Former smokers had quit at least 6 months ago but not more than 5 years ago, and never smokers had quit more than 5 years ago. Clinical measurements performed included Plaque Index, probing pocket depth, bleeding on probing, and clinical attachment level.

DNA Extraction and Genetic analysis

Blood was collected from each individual by a trained phlebotomist. The blood was centrifuged, and buffy coats were collected for examination. Buffy coats were stored at -70°C for future use. DNA was extracted from 200μL of buffy coats using DNA extraction kits from QIAGEN following manufacturer's instructions.

The SNPs that were investigated are as follows: TLR-2 G2408A (SNP CLUSTER ID: rs5743708), TLR-4 A896G (SNP CLUSTER ID: rs4986790), TLR-9 T1486C (SNP CLUSTER ID: rs187084), and CD14 C260T (SNP CLUSTER ID: rs2569190).

Genetic analysis was performed with TaqMan SNP assays (Applied Biosystems) using Real Time thermocycler AB 7500(Applied Biosystems). All primers and probes are predesigned using Primer Express program (Applied Biosystems) and labeled with VIC and FAM fluorescein dyes. PCR was performed in a volume of 20µL containing 5µL of DNA (2-4ng/µL), 13µL of PCR mix containing primers and probes and 2X TaqMan Universal PCR MasterMix, No AmpErase UNG(Applied Biosystems), and 2µL of H_20. The PCR conditions were as follows: Hold step for 10 min at 95°C to allow AmpliTaq Gold Enzyme (Applied Biosystems) activation followed by 40 cycles of initial denaturation step for 15 seconds at 92°C and annealing and extension step of 1 min at 60°C. The samples were run on 96-well plates. After completion of PCR amplification, an end-point plate reading was performed. Based on the signals generated from each well, specific alleles were determined using ABI PRISM Sequence Detection System (SDS) software.

To validate the results obtained from real time analysis, PCR-RFLP analyses were performed on selected samples using electrophoresis on 2% and 4% agarose gels. PCR amplifications were accomplished following published protocols.[21,46,47] Table 2 summarizes the primers, restriction enzymes and conditions for RFLP analysis**.**

Statistical analysis.

A thorough statistical power analysis was performed in the program Power and Precision Version 2 and was based on the following assumptions:

1) 80% power, an alpha of 0.05
2) Chi squared goodness of fit test
3) Allele frequencies from published studies
4) Available samples:
    a. 71 African American Chronic Periodontitis
    b. 59 African American Non-Periodontitis

The power analysis suggests that meaningful differences could be found in this pilot study of existing samples. The sample size is also sufficient for a basis of power analysis for future studies**.** A Pearson Chi-squared test was used to compare the distribution of the different genotypes among periodontally diseased patients versus healthy controls. The frequency of allele carriage and allele frequency in the test versus control groups was analyzed using Fisher's exact test. All statistics were considered statistically significant if they fell outside the 95% CI ($p<0.05$). Where significant p-values were generated, the odds ratio was subsequently calculated. Illustrative power calculations then were performed to estimate the relevance of the p values generated.

Results

Mean age of the subjects at time of initial examination was 39.71 for chronic periodontitis, and 33.75 for periodontally healthy individuals (Table 3). There were no statistically significant differences between the diseased versus the control group for age.

Smoking data was evaluated for the study population based on group classification. The distribution of smokers was significantly different between patient groups ($p<0.0001$), therefore all statistical analyses were adjusted for smoking data. (Table 4)

The frequencies of allele distribution within groups is noted in Table 5. There were no statistically significant variations between groups for genotypes of TLR-2 (G2408A), TLR-4 (A896G), or CD14 (C260T). There was, however, a statistically significant difference of TLR-9 (C1486T) genotypes between groups ($p<0.0001$).

The distribution of TLR-9 (C1486 T) genotypes was significantly different between the periodontally healthy subjects and the disease group. The frequency of individuals expressing the C allele of TLR-9 polymorphism (CC and CT combined total) was significantly higher in the control group (82.46%) compared to the disease group (45.21%) ($p<0.0001$) (Table 6).

The majority of the above mentioned individuals came from families afflicted with early onset periodontitis. Therefore, a question arises about whether the distribution of polymorphisms in families was related to being in these particular families. To investigate whether the relationship was related to family membership, TLR-9 distribution was analyzed in families with a healthy periodontal diagnosis (Table 7).

Chi squared tests were performed on the TLR-9 distribution data in table 7, and showed no statistical significance when familial data was compared.

Discussion

The purpose of this study is to determine if the SNPs in TLR-2, -4, -9, and CD14 genes differ among chronic periodontitis patients and controls in an African American population. Our results showed that there was no association between TLR-2, TLR-4, or CD14 genetic variants and chronic periodontitis susceptibility. It did, however, show that genetic variations at the TLR-9 promoter region (C1486T) containing the C allele (CC & CT genotypes) appeared to confer resistance to periodontal destruction in an African American population, while subjects homozygous for the T allele were more susceptible to chronic periodontitis. To our knowledge, this result has not been found prior to this study. Additionally, this is the first study to look at the role of TLR-9 C1486T SNP in periodontal disease. Another study conducted concurrently with this study evaluated the frequency of SNPs in an aggressive periodontitis population of African Americans and compared with the same control group, finding similar results.

The mechanical causes of periodontal disease due to bacterial insult have been well established. Several specific bacteria have been implicated as periodontopathogens such as *A. actinomycetemcomitans, T. denticola, T. forsythensis, P. gingivalis, P. intermedia, and F. nucleatum*.[2,3,4] These bacteria have specific pathologic processes (PAMPs) which can elicit a host inflammatory response.[12,13,14,15,16,17] Host innate immunity is designed to combat pathogenic processes with specific receptors, including TLR-2, TLR-4 , TLR-9, and CD14. Bacterial stimulation of TLR-9 (C1486T) stimulates release of cytokines and type I interferons (IFNs) through a set of transcription factors including NF-kB and IFN regulatory factors (IRFs). Once activated, the action of these transcription factors needs to be attenuated and/or terminated to prevent immunopathology and maintain tissue homeostasis.[32] With increased stimulation of TLR-9 and the NF- kB pathway, further release of transcriptional factors can lead to increased destruction of host tissues in the absence of proper immunoregulation.[32]

Genetic roles have been implicated for increased risk of individual periodontal destruction.[9] For example, twin studies conducted at VCU indicate that a significant portion of the differences in clinical and radiographic measures of chronic periodontitis may be explained by genetic dissimilarity.[10] Although it is unlikely that polymorphisms in all of these genetic determinants impart differential susceptibility to periodontal disease, it is reasonable to expect that multiple genes will be important and that knowledge of these may permit determination of individual susceptibility.[57]

Previous studies have established associations between toll-like receptor expression and disease processes, including periodontal disease. Genetic polymorphisms of TLRs can be seen in association with disease processes related to asthma,[33] graft-versus-host disease,[34] and arthritis.[36] Impaired LPS signal transduction related to single nucleotide polymorphisms in TLR4 have been shown to place the host at increased risk of bacterial infections.[39] Patients with increased levels of SNPs in TLR-4 (C399T) were also found to be at greater risk for chronic periodontal destruction.[20] Yoshioka found that plaque has an ability to stimulate TLR-4 activity, but not TLR-2 activity in gingivitis patients.[48] A further study conducted by the same group verified that TLR-4 mediated cytokine release by blood mononuclear cells was associated with stimulation by periodontopathogenic bacteria in plaque.[49] Other studies discovered the GG genotype or G-containing genotypes of TLR4 as risk markers for periodontitis in Dutch and Finnish patients.[50,51] The above mentioned results were in direct contrast to our findings, which showed no statistically significant relationship between SNP genotype at TLR-2, TLR-4, CD14 and periodontal disease susceptibility. This discrepancy could be explained because of a different genetic population with differing genetic makeup of these SNPs, or differing study designs with varying statistical analysis.

Other investigations have reported findings that are contrary to the above mentioned studies, stating that genotype is not associated with disease susceptibility. These study results coincide with our results for TLR-2, TLR-4, and CD14. For example, Folwaczny found functional effective mutations of Toll like receptor-2 and -4 genes that attenuate the response to PAMPs were equally prevalent among periodontitis patients with

any disease severity when compared with healthy control subjects, and reported these mutations had no influence on the individual's susceptibility to periodontitis.[21] Schulz studied a German population, and was unable to find a correlation between TLR-4 or CD14 genotypes and periodontal susceptibility.[52] CD14 also did not have any association with periodontitis in a Japanese population,[53] or the severity of periodontal disease in a Czech population.[54] CD14 (C260T) genotypes were found to have no significant influence on markers of inflammation or risk of coronary artery disease.[26] Additionally, studies did not find any association between CD14 and aggressive periodontitis, but did find an association with TLR-4 and periodontitis in patients living in the UK.[55] Our study results coincided with the above mentioned publications, finding no statistically significant relationship between individual allele genotypes for SNPs in TLR-2, TLR-4, or CD14.

It has recently been found that TLR-9 expression through stimulation by bacterial DNA increases neutrophil adhesion.[56] Neutrophil chemotaxis is the initial response in periodontal disease to combat periodontopathogenic bacterial aggregates. The innate immune system recognizes nonself, and initiates a host-driven response that results in neutrophil chemotaxis to the area of insult. Neutrophils then phagocytose the pathogenic processes (bacterial DNA in this example) for removal. Bacterial DNA activates NF-KB in human endothelial cells, the driving force in the initiation and progression of periodontal inflammation. Thus, bacterial DNA through TLR-9 promotes neutrophil adherence to endothelial cells, induces cytokine release and suppresses neutrophil apoptosis, indicating that bacterial DNA is capable of triggering and/or maintaining inflammation even in the absence of other bacterial constituents.[56] Scaling and root planing can reduce the bacterial aggregate, but the bacterial constituents will still be present in the surrounding connective tissue as well as the endosomal compartment. TLR-9 (C1486T) regulation of inflammatory response may be genetically driven and have an implication in host susceptibility to periodontal destruction. It can thus be hypothesized that differential expression of genes that elicit innate host response to periodontopathogens, such as those belonging to the toll-like receptors -2, -4, -9, and CD14, may result in an altered host susceptibility to periodontitis.

Conclusion

Within the limitations of this study, it is suggested that there is not a significant relationship between periodontal disease susceptibility in African-Americans whom possess genotypic variations of the SNPs of TLR2, TLR4, and CD14 after adjusting for age, smoking, and gender variations. It was, however, suggested through the results that African-American individuals whom possess the C allele on TLR9 are conferred a resistance to periodontal destruction, as opposed to those possessing the T allele. Additional studies with increased sample size are required in order to validate this finding.

Tables

Table 1. Disease conditions attributed to genetically transmitted alterations in immune response to microbial infection[57]				
Disease	**MIM number**	**Gene Map locus**	**Inheritance**	**Comments**
Leukocyte Adhesion deficiency type 1	116920	21q22.3	Autosomal recessive	Disorder of neutrophil function resulting from deficiency of beta-2 integrin subunit of the leukocyte cell adhesion molecule.
Familial disseminated atypical mycobacterial infection	209950	2q33-37	Autosomal dominant resistance allele; autosomal recessive susceptibility allele	*Bcg* gene is expressed in 2 allelic forms, a dominant resistance allele and parasitic infection, and affects macrophage function.
Chediak-Higashi svndrome	214500	1q42.1-42.2	Autosomal recessive	Clinical findings include neutropenia and abnormal susceptibility to infection.
Chronic Granulomatous disease	306400	Xp21.1	X-linked recessive	In this disorder, neutrophils can phagocytose bacteria but cannot kill them in the phagocytic vacuoles. The cause of the killing defect is an inability to increase the cell's respiration and consequent failure to deliver activated oxygen into the phagocytic vacuole

Specific granule deficiency (also called lactoferrin-deficient neutrophils)	245480	3q21-q23	Autosomal recessive	Lactoferrin has strong bacteriostatic properties and can deprive bacteria of the iron essential for growth. It may also protect cells from free radical damage. Patients with specific granule deficiency have normal neutrophil counts, but have a tissue neutrophil-specific absence of lactoferrin secondary to an abnormality of RNA production, possibly due to a defective granule-packaging gene.
Myeloperoxidase deficiency	254600	17q12-2	Autosomal recessive	Absence of myeloperoxidase, a dimeric protein that catalyzes production of intermediates with microbicidal activity against a wide range of microbes. Exaggerated superoxide production; several allelic variants have been identified.
Localized juvenile periodontitis	170650	4qll-113	Autosomal dominant	A major gene locus has been mapped to chromosome 4q. The genetic defect is unknown

Table 2. **Primers, restriction enzymes, and conditions for RFLP**

SNP	Forward Primer	Reverse Primer	Enzyme	Gels	Restriction Fragments
TLR4 A896G	**5'**GAT TAG CAT ACT TAG ACT ACT ACC TCC ATG **3'**	**5'**GAT CAA CTT CTG AAA AAG CAT TCC CAC **3'**	10U/µl Nco I at 37C overnight in 1.5 µl 10 X restriction buffer and 1.3 µl water	3% NuSieve gel or 4% MetaPhor agarose gel stained with ethidium bromide	Wild type (alleleA) 249bp Asp299Gly(alleleG)223+26bp
TLR2 A2408G	**5'** CAT TCC CCA GCG CTT CTG CAA GCT CC **3'**	**5'** GGA ACC TAG GAC TTT ATC GCA GCT C **3'**	Msp I at 37C	2% agarose gel	Wild type (allele G) 104bp + 25bp Arg753Gln(allele A) 129bp
TLR9 T1486C	**5'** TCC CAG CAG CAA CAA TTC ATT A **3'**	**5'** CTG CTT GCA GTT GAC TGT GT **3'**	20U of AflII (New England Biolabs) at 37C overnight	2% agarose 70 min at 75V	Wild type 327 and 172 bp Mutant 499bp
CD14 C260T	**5'** GGT GCC AAC AGA TGA GGT TCA C**3'**	**5** CTT CGG CTG CCT CTG ACA GTT-**3**	HaeIII	2% & 4% agarose	Wild type 159 Mutant 260

Table 3. Demographics data of the groups (age):

Disease Classification	Number	Age at initial examination	Age at final examination
Chronic periodontitis	73	39.71 ± 0.87	42.55 ± 0.67
Periodontally healthy	57	33.75 ± 1.23	37.81 ± 0.99

Ages of individuals at time of initial examination compared with age at final examination.

Table 4. Smoking data by disease classification

Classification	Chronic periodontitis	Periodontally healthy	Totals
Former smoker	12 16.90	8 14.55	20
Never smoker	28 39.44	40 72.73	68
Undetermined	4 5.63	1 1.82	5
Current smoker	27 38.03	6 10.91	33
Totals	71	55	126

Smoking data represented by questionnaire and interview of patients at time of periodontal examination by examiners in prior Virginia Commonwealth University study.

Table 5. Genotype frequency of TLR2, TLR4, TLR9, and CD14 between groups

Polymorphisms	Genotype	CP n (%)	NP n (%)	Allele	CP 2n (%)	NP 2n (%)
TLR2 (G2408A)	GG GA AA	69 (98.57) 0 (0) 1 (1.43)	2 (3.57) 3 (5.36) 51 (91.07)	G A	138 (98.6) 2 (1.4)	7 (6.3) 105 (93.7)
TLR4 (A896G)	AA AG GG	62 (84.93) 8 (10.96) 3 (4.11)	44 (83.02) 7 (13.21) 2 (3.77)	A G	132 (90.4) 14 (9.6)	95 (89.6) 11 (10.4)
TLR9 (C1486T)	CC CT TT	12 (16.44) 21 (28.77) 40 (54.79)	4 (7.02) 43 (75.44)* 10 (17.54)*	C T	45 (30.8) 101 (69.2)	51 (44.74) 63 (55.26)
CD14 (C260T)	TT CT CC	6 (15.79) 25 (65.79) 7 (18.42)	4(8.00) 36 (72.00) 10 (20.00)	C T	37 (48.7) 39 (51.3)	44 (44) 56 (56)

*p<0.0001

Genotype and allele frequencies for all SNPs investigated. n(%) values show frequency of genotype, first in raw number, followed by individual group percentage. 2n(%) values show frequency of each investigated allele, in the same format as n(%).

Table 6. **TLR-9 Genotypes (CT or CC) Versus TT**

Genotype	Chronic periodontitis	Periodontally healthy	Totals
CC + CT	33 45.21	47 82.46*	80
TT	40 54.79	10 17.54*	50
Totals	73	57	130

*p<0.0001.

Genotype of CC + CT frequency compared in chronic periodontitis group versus healthy controls.

Table 7. TLR-9 genotype distribution within families

Genotype	No familial relation	Familial relation	Totals
CC	2 10.00	2 5.41	4
CT	14 70.00	29 78.38	43
TT	4 20.00	6 16.22	10
Totals	20	37	57

Genotypes compared when occurring in same family versus different genetic lineage.

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