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Neutrophil Formylpeptide Receptor Single Nucleotide Polymorphism in Localized and Generalized Aggressive Periodontitis

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Abbreviations: Aggressive Periodontitis (AP), Aggressive Periodontitis Localized (LAP), Aggressive Periodontitis Generalized (GAP), *Aggregatibacter actinomycetemcomintans (A.a)* , Polymorphonuclear cells (PMNs) , Formylpeptide receptors (FPRs) , Single nucleotide polymorphisms (SNPs), Polymerase chain reaction (PCR)

Abstract

Background: Aggressive periodontitis (AP) is a destructive disease that affects around 2-10% of the population. There are two main sub-classifications of AP: Localized (LAP) and Generalized (GAP). However, very little is known about the etiologic differences between these two entities. Single nucleotide polymorphisms (SNPs) have been associated with AP in key genes. The objective of this study is to compare *FPR1* SNPs between LAP and GAP in African Americans.

Methods: Blood samples were obtained from African American subjects in New Orleans (LAP n= 27, GAP n=17, and healthy controls n=20), and genomic DNA was isolated. Polymerase chain reaction (PCR) and sequencing (for analysis of SNPs) was performed on a highly polymorphic fragment of the *FPR1* gene.

Results: No difference was found between subjects with LAP or GAP for 301 G>C, 348

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T>C, 546 C>A, 568 A>T, 576 T>C>G allele association. However, **546 C>A, 576 T>C>G** *FPR1* SNPs were associated with AP when compared to healthy controls. Specifically, **546 C and 576 G** alleles were more frequently associated with GAP, and the **546 C** allele was more frequently associated with LAP. None of the other three SNPs was detected, indicating no association with AP.

Conclusions: *FPR1* SNP **546 C>A, 576 T>C>G** *FPR1* SNPs were associated with AP in African Americans. Individuals who are homozygous (?) for 546C and 576 T may have increased susceptibility to developing this condition.

Introduction

Aggressive periodontitis (AP) is a disease that is characterized by rapid attachment loss and bone destruction in otherwise clinically healthy patients. In the United States, AP is most prevalent in African Americans, affecting around 2-10% of the population (Melvin, 1991) (Albander 1997). It primarily affects teenagers and young adults below 35 years of age (Albandar, 2002). Tooth loss due to this disease can have debilitating consequences for the future formation and function of the dentition.

Aggressive periodontitis can be sub-classified into localized (LAP) and generalized (GAP). LAP appears to affect the first molar/incisor regions with interproximal attachment loss on at least 2 permanent teeth, one of which is a first molar, with involvement of no more than 2 teeth other than the first molars and incisors. GAP patients have at least 3 permanent teeth affected by interproximal attachment loss other than the first molars and incisors (Lang, 1999). Brown et al. found that 35% of patients who initially were diagnosed with LAP progressed to GAP 6 years later.

Even though there is an established classification of these two entities based on the different clinical presentations, to date it is still unclear if these two conditions have different etiologies or contributing factors to explain their different presentations.

A classic finding of these diseases is the presence of a highly pathogenic biofilm, such as *Aggregatibacter actinomycetemcomintans (A.a)* (Califano, 2003). *A. actinomycetemcomintans* (*A.a*) serotype b strains have been more highly associated with AP than *A.a* serotype a or c (Ebersole, 1991). Wilson et al. reported that a humoral response to *A.a* includes the production of LPS-reactive IgG2 antibodies in patients with LAP. Based on these studies, some have correlated the increased level IgG2 as a key component to differentiate between LAP and GAP, and explained why LAP is limited to affect first molar and incisors. However, others have mentioned that these IgG2 antibodies have poor opsonizing capability against this specific antigen and may limit the humeral response from the host against *A.a* (Wilson, 1992). Very little is reported regarding the increased or decreased presence of IgG2 in GAP to support or contradict this theory. Overall AP is a complex multifactorial disease involving a complex interplay between the microbial challenge and host response.

Polymorphonuclear cells (PMNs) are a vital component of this host response. They are the first line of defense against bacterial infections. These cells rely upon high affinity formylpeptide receptors (FPRs) found on their surface to help locate and neutralize bacteria. Defects in these receptors can render the host more susceptible to

infection. As an example, mice devoid of FPRs exhibit significantly reduced resistance to *Listeria monocytogenes* (Gao, 1999).

The most frequent PMN anomaly reported in AP has been altered fMLP-mediated chemotaxis in African-American patients with the localized form of disease (Perez, 1991). The etiology of this altered chemotaxis is still unknown, although both innate genetic and acquired anomalies have been proposed. (Agarwal 1994) One possibility suggests the inability of the PMN to detect FMLP caused by a receptor defect (Van Dyke, 1891). In patients with AP, PMNs had a diminished number of high affinity FPRs (Perez, 1991). An alternate explanation would suggest an intrinsic cellular defect, causing a decrease in the number of FPR on the neutrophils surface. (Van Dyke, 1981)

AP tends to run in families, and its etiology is associated with a genetic component that could modify host response. Single nucleotide Polymorphisms (SNPs) are common genetic variations that occur when a single nucleotide in a specific position of the genome is changed for another. Several studies have reported SNPs that have been associated with AP in key genes (Vieira, 2014). Formyl peptide receptor is encoded by the *FPR1* gene, which is located on Chromosome 19.

The FPR1 gene is a highly polymorphic gene. Sahagun-Ruiz et al. (Sahagun-Ruiz, 2001) systematically analyzed polymorphisms in the *FPR1* open reading frame by direct sequencing of cloned alleles from random North American blood donors. They detected five common non-synonymous SNPs (c.32C>T, c.301G>C, c.568A>T,

c.576T>C>G and c.1037C>A) as well as two synonymous (silent) SNPs (c.348T>C and c.546C>A) that do not encode an amino acid substitution. Previous reports have suggested that AP is associated with specific patterns of single nucleotide polymorphisms (SNPs) of the *FPR1* gene, including SNPs c.568A>T and c.576T>C>G in African-Americans (Gwinn, 1999) (Zhang, 2003). Exploratory research has been conducted to investigate the relationship between *FPR* polymorphisms, defective *FPR* expression, PMN chemotactic defects and susceptibility to AP. These studies of an African-American population suggest that AP is associated with the silent SNP c.348T>C in the gene encoding the FPR (*FPR1*) as well as defective PMN chemotaxis (Maney, 2009). No study has directly analyzed the frequency of SNP's between LAP and GAP to determine if this may represent a difference in etiology, clinical presentation and/ or progression of the disease.

The aim of this study is to compare *FPR1* SNPs between LAP and GAP in an African American population, as well as to healthy controls.

Materials and Methods

Subject recruitment

African American subjects (n=24), aged 13-35years old, diagnosed with AP at LSUHSC School of Dentistry were recruited between January 2015 and February 2017 following an approved LSUNO-IRB protocol # 8796. Patients were diagnosed according to the criteria of the 1999 International Workshop for a Classification of

Periodontal Diseases and Conditions (Armitage, 1999). Additionally, unpublished data from a previous study of African American subjects diagnosed with AP (n=20) and matched healthy controls (n=20) were included in this study. These patients were previously recruited between January 2010 - January 2011. Written informed consent for blood sampling and DNA analysis was obtained from each subject. Patients were excluded if they had a history of prophylaxis within the last 3 months, any chronic medical condition, history of recent antibiotic therapy, or history of smoking.

Clinical examination

Demographic information including birth date, sex, race/ethnicity, tobacco exposure, and contact information was collected. All subjects received a full mouth periodontal and radiographic examination. Periodontal examination consisted of probing depth and attachment level measurements on 6 sites per tooth using a periodontal probe[§]. Bleeding on probing and plaque levels were also recorded. These measurements will be collected and be available for data exploration, but specific aims requiring these measurements are not planned.

Patients were classified as LAP when they presented with 4mm or more of attachment loss in at least two permanent first molars and incisors, including at least one first molar, but not more than two permanent teeth other than the first molars and incisors. GAP was diagnosed when at least three teeth other than first molars and incisors had an attachment loss of 4 mm or more. The control subjects (n = 20) were periodontally healthy. (Armitage 1999) (Tonetti 1999).

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Blood drawn and DNA isolation

Peripheral venous blood samples were drawn from each subject (approximately 5cc's). Genomic DNA was purified from the whole blood samples using a blood DNA purification kit according to the manufacturer's instructions[**]

PCR and Sequencing of the *FPR1* Coding Region

To detect *FPR1* SNP, PCR amplification of a 439–base pair (bp) fragment of *FPR1* was performed as previously described (Maney, 2009). The forward and reverse primers used were 5'TTCACCTCCACTTTGCCATT 3' and 3'TGACAGCAACGATGGACATG5', respectively. For every sample, PCR was carried out for 35 cycles in a mixture of 45 µl PCR Supermix (Invitrogen), 1 µl of each of the two primers, 1.5 µL of the DNA sample and 1.5 µl of water. Prior to initiating PCR, the reaction mixture was heated at 95ºC for one minute to dissociate antibody from the Taq polymerase. The parameters for one cycle of the PCR reaction include denaturation at 95ºC for one minute, annealing at 60ºC for one minute, and extension at 72ºC for one minute. PCR products were then purified using a commercially available kit[††].

[**] QIAamp DNA Blood Mini Kit, Qiagen, Valencia, CA.
[††] QIAquick PCR Purification Kit, Qiagen.

The samples were submitted for DNA sequence analysis to The Ohio State University Plant Microbe Genomics Facility. *FPR1* SNPs were manually identified using a computer program. Differences in nucleotide diversity for each SNP were analyzed by the Fischer exact test and Chi square test to determine associations between disease status and genotype. Then, frequencies were calculated and subjects were stratified into groups based on these genotypes frequencies.

RESULTS

Five previously associated SNP's (c.301G>C, c.568A>T, c.576T>C>G and c.348T>C and c.546C>A) were found in our population of AP cases (n=44) and controls (n= 20). No difference was found between subjects with LAP or GAP with respect to 301 G>C, 348 T>C, 546 C>A, 568 A>T, 576 T>C>G allele frequencies.

In contrast, significant differences between the AP cases (n=44) and controls (n=20) groups were observed in two *FPR1* SNPs **546 C>A and 576 T>C>G.** No significant differences were found with respect to the frequencies of *FPR1* SNPs 301 G>C, 348 T>C, 568 A>T in the fragments analyzed.

FPR1 SNPs Allele frequencies

Table 1.

Allele Frequencies of FPR1 5' SNPs in LAP and GAP

SNP		LAP		GAP		P-Value
		n	(%)	n	(%)	
301 G>C	G	37	(31.5)	22	(64.7)	0.817*
	C	17	(68.5)	12	(35.3)	
348 T>C*	C	40	(74.1)	25	(73.5)	1.000*
	T	14	(25.9)	9	(26.5)	
546 C>A*	C	40	(74.1)	28	(82.4)	0.440*
	A	14	(25.9)	6	(17.6)	
568 A>T	A	46	(85.2)	32	(94.1)	0.305*
	T	8	(14.8)	2	(5.9)	
576 T>C>G	T	22	(40.7)	11	(32.3)	0.306**
	C	7	(13%)	2	(5.9)	
	G	25	(46.3)	21	(61.8)	

*Determined by Chi Square

**Determined by the Fisher exact test.

Table 2.

Allele Frequencies of FPR1 5' SNPs in Cases and Controls

SNP		AP Cases		Controls		P Value*
		n	(%)	n	(%)	
301 G> C	G	59	(67)	33	(80)	0.198*
	C	29	(33)	8	(20)	
348 T>C	C	65	(73. 9)	26	(65)	0.980*
	T	23	(26.1)	14	(35)	
546 C>A	C	68	(77.3)	21	(52.5)	0.009*
	A	20	(22.7)	19	(47.5)	
568 A>T	A	78	(88.6)	37	(92.5)	0.723*
	T	10	(11.4)	3	(7.5)	
576 T>C>G	T	33	(37.5)	25	(62.5)	0.030*
	C	9	(10.2)	3	(7.5)	
	G	46	(52.3)	12	(30)	

*Determined by Chi Square

FPR1 SNPs Allele Association

Association of 546 C With AP

Three 546 C>A genotypes (C/C, C/A, and A/A) were detected in the AP case group and in the control group. The 546 C/C genotype was expressed by 28 of 44 case subjects and by 8 of 20 control subjects. There was a significant relationship between 546 C/C and experimental/ control status (P = ?; odds ratio, ?; Table ?).

Table 3.

Allele Association of FPR1 SNP 348T>C With AgP Cases in African Americans

Genotype	AP	n	(%)	Controls	n	(%)
546 C/C		28	(63.6)		8	(40%)
546 C/A		12	(27.3)		5	(25%)
546 A/A		4	(9.1%)		7	(35%)

The relationship between genotype and AP case/control status is significant (P = ?; Fisher exact test). Individuals with the 546 C/C genotype are at greater risk for AP than those with the 546 C/A and 546 A/A genotypes combined (odds ratio, ?% confidence

interval: ?).

Association of 576 C With AP

Six 576 T>C>G genotypes (T/T, C/C, G/G, C/G, T/G, and T/C) were detected in the AP experimental group. In the control group, five 576 T>C>G genotypes (T/T, G/G, C/G, T/G, and T/C) were detected. The 576 T/T (?) genotype was expressed by ? of 20 case subjects and by ? of 20 control subjects. There was a significant relationship between 576 ?/? and experimental/ control status (P = ?; odds ratio, ?; Table ?).

Table 4.

Allele Association of FPR1 SNP 576 T>C>G With AgP Cases in African Americans

Genotype	AP	n	(%)	Controls	n	(%)
576 T/T		8	(18.2)		8	(40)
576 C/C		2	(4.5)		0	(0)
576 G/G		14	(31.9)		2	(10)
576 T/G		15	(34)		7	(35)
576 T/C		2	(4.5)		2	(10)
576 C/G		3	(6.9)		1	(5)

The relationship between genotype and AP case/control status is significant (P = ?; Fisher exact test). Individuals with the 576 G/G genotype are at greater risk for AP than those with the 576 T/T, 576 C/C, 576 T/G, 576 T/C, 576 C/G genotypes combined (odds ratio, ?% confidence interval: ?).

***FPR1* SNPs Haplotype Association**

Table 5.

Haplotypes of **FPR1** SNPs c.546C>A and c.576T>C>G in African Americans

Haplotype	Frequency in Subjects with AP	Frequency in Subjects with LAP	Frequency in Subjects GAP	Frequency in Controls	P Value	Odds Ratio
546C.576T	21/44	14/27	7/17	10/20		
546C.576C	7/44	5/27	2/17	3/20		
546C.576G	32/44	19/27	13/17	10/20		

546A.576T	16/44	11/27	5/17	12/20		
546A.576C	2/44	1/27	1/17	0/20		
546A.576G	9/44	7/27	2/17	4/20		

Discussion

Many reports have discussed the susceptibility factors for aggressive periodontitis, including familial aggregation, polymorphonuclear neutrophils, antibodies to bacteria, smoking local contributing factor, herpes virus infections and decreased neutrophil abnormalities (Meng, 2007).

Many conditions in which neutrophils abnormalities are found have also been associated with oral complications. AP is an example of a rapidly destructive periodontal disease that is related to inadequate neutrophil function. While most of the patients with AP have neutrophil defects, the exact cause(s) of these defects may vary. Abnormalities such as decreased chemotaxis is a well-known functional defect suffered by patients with AP (Perez, 1991). To date, the exact mechanism of this dysfunction is not well understood, although several recently proposed mechanisms attempt to account for it. Gwinn and Maney suggest a defect of FPR (Gwinn, 1999) (Maney, 2009). Van Dyke proposed post-receptor pathways or an altered receptor for the complement fragment C5a25 (Van dyke 1983). Argarwal 1989 suggested cellular functions such as a decreased intracellular calcium mobilization (Argarwal 1989). Harvath proposed

elevation of TNF-α and IL-1 cytokines in the serum and presence of serum inhibitors of chemotaxis (Harvath, 1982).

Specifically, there are two main sub-classifications for AP, LAP and GAP. However, it remains unclear whether different etiologies or contributing factors explain the different clinical presentations of these two subgroups.

Our study is mainly focused in detecting any significant frequency alteration in *FPR1* SNPs between LAP and GAP. Studying the human genome to determine DNA variants as possible explanations to human diseases is well established; however, using genetic factors for multifactorial conditions presents a major challenge due the fact that both genetic and environment interact. The presence of a mutation or polymorphism in a single gene may not be associated with a specific disease, but when this one is combined with some variation in other genes, such changes may contribute to a disease genotype. (Shafer 1998)

Specific genotypes associated with SNPs in genes encoding proteins that play key roles in the pathogenesis of AP, have been reported to be associated with the increased odds of detecting *A actinomycetemcomitan*s, *Tannerella forsythia*, and *Porphyromonas gingivalis* (Nibali, 2007).

Consistent with our hypothesis, subjects with the LAP and GAP showed no difference with respect to 301 G>C, 348 T>C, 546 C>A\, 568 A>T, 576 T>C>G allele

associations. However, the present study is also consistent with previous studies' observations that *FPR1* SNP 576 T>C>G is associated with AP in African Americans. Subjects with the 576 (?) genotype seem to have a significantly increased risk for developing AP compared to those with the 576 ?/? or 576?/? genotype (odds ratio, ?.9?). These SNPs are localized to the second extracellular loop of *FPR1* and are non-synonymous, meaning they will cause a change in the amino acid sequence and, therefore, the protein formed.

The above is consistent with findings of a previous study (Zhang, 2003) in which SNPs 576 T>C> G were reported to cause a change in Asparagine to Lysine, altering the amide characteristic from hydrophilic to basic. This amino acid variation would affect ligand binding specificity and/or binding affinity that occurs in the extracellular domain (Quehenberger, 1993). Zhang et al. concluded that this SNP is accordingly significantly associated with AP phenotype in African Americans (Zhang, 2003)

The present study also identified synonymous SNP 546 c genotypes in the diseased cohort compared to the control group. Subjects with the 546 (?) genotype seem to have a significantly increased risk for developing AP compared to those with the 546 ?/? or 546?/? genotype (odds ratio, ?.?).

Although 546 C is a synonymous SNP that does not alter the amino acid, it is reasonable to hypothesize that it could be associated with other SNPs in the *FPR1* promoter or enhancer regions. Some association of this same kind has been made in

other studies where they have a concluded multiple SNPs within specific haplotypes containing variants of synonymous coding region SNPs and non-synonymous promoter SNPs could affect the biologic phenotype (Drysdale, 2000) (Mummidi, 2000). Zhang et al. did not report any associations of SNP 546 C>A with AP as our study shows. This discrepancy may be related to the DNA diversity in the specific study populations. Another possible explanation could be differences in the selection criteria of patients with aggressive periodontitis. Zhang et al. studied a more heterogeneous population that included African American, Brazilian and Turkish subjects, compared to our population which is a more homogenous population of African Americans localized in the New Orleans area.

Healthy controls were also analyzed to determine if these possible AP specific alterations are also present in otherwise healthy matched controls. Healthy controls showed no statistically significant alteration, suggesting that these proposed alterations may be specific to AP.

Within the limitations of this study, it is possible to state that the difference in the clinical presentations of LAP and GAP cannot be explained by an increase in the frequency of the SNPs we studied, since our results failed to find a difference between *FPR1* polymorphisms in patients with these two entities. However, as shown in previous studies, we found significant associations with SNP 546 and 576. It is possible that these variations in the gene play a role in or may be a risk indicator for the disease.

Statistical analysis showed an association between these alterations and the presence of the condition (P= 0.005)

In the long term, a better understanding of the etiology and pathogenesis of aggressive periodontitis could help develop laboratory tests for individuals at risk for AP. Targeted therapeutic intervention to eliminate pathogens prior to clinical expression of AP could mitigate periodontal destruction, which may improve prognosis, reduce tooth loss produced by AP, minimize functional and esthetic problems related to tooth loss and reduce the overall cost of dental care.

Conclusion

No difference was found between subjects with LAP or GAP with respect to 301 G>C, 348 T>C, 546 C>A, 568 A>T, 576 T>C>G allele association. However, SNPs 576 G and 546 C allele were more frequently associated with GAP when compared to controls. SNP546 C allele was more frequently associated with LAP. Considering the small population of this study, our findings should be confirmed by large scale, methodologically sophisticated studies.

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