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NO ASSOCIATION BETWEEN CALPROTECTIN (S100A8) SUBUNIT GENE POLYMORPHISM AND PATIENTS WITH CHRONIC AND AGGRESSIVE PERIODONTITIS IN DRAVIDIAN POPULATION

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ABSTRACT

Calprotectin is an inflammatory regulator whose elevated levels are implicated in periodontitis. S100A8 subunit of calprotectin has pro-inflammatory activity. The present study aims to determine the association of single nucleotide polymorphism (SNP) in the calprotectin subunit gene (*S100A8*) in participants from Dravidian ethnicity. The study included 236 individuals in total, of which 60 were patients with chronic periodontitis (CP), 50 were cases with aggressive periodontitis (AgP) and 126 were healthy controls. Genomic DNA was extracted from the white blood cells and the SNP rs3795391 (A→G) of calprotectin gene *S100A8* was screened by allele-specific polymerase chain reaction (AS-PCR). Our study showed lack of association of rs3795391 with chronic periodontitis and aggressive periodontitis (p=0.5764; >0.05). Thus rs3795391 SNP may not contribute to susceptibility of periodontitis in the patients of Dravidian ethnicity. Further study on a larger population is essential to understand the allelic significance of this SNP.

KEY WORDS: **Periodontitis, calprotectin, S100A8, rs3795391, Dravidian ethnicity.**

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INTRODUCTION

Periodontitis is a chronic inflammatory condition affecting the periodontal ligament, alveolar bone and cementum.[1] Although the presence of gram negative anaerobes is required for disease initiation and progression, recent concepts revolve around the basis of host-immune response playing a significant role in the periodontal destruction.[2] Thus the susceptibility to this condition depends on the varied factors like host-susceptibility other than the local factors alone. The two major destructive forms of periodontitis include chronic periodontitis (CP) and aggressive periodontitis (AgP). These periodontitis types have same immunopathological mechanisms and differ only in their degree of severity.[3] With genetic variations accounting for about 50% on susceptibility of individuals to develop periodontitis[4], it is well established that genes can influence the host-inflammatory response to periodontitis via overproduction of the pro-inflammatory molecules.A recent review by Gandhi and Kothiwale (2012) stipulate that gene polymorphism research in periodontitis on molecules involved in immunoregulation or metabolism has been on the spotlight.[5] Accordingly, the role of gene polymorphism in a metabolism-related receptor is targeted in the current study.Calprotectin, a prominent player in innate immunity is a calcium and zinc binding cytoplasmic protein.[6] This protein constitutes about 5% of polymorphonuclear neutrophils (PMNs) protein and 40-50% of cytosolic protein.[7] It is expressed in gingival keratinocytes, neutrophils, monocytes/macrophages and epithelial cells. Recruitment of monocytes and neutrophils to the inflammatory sites is mediated by adhesive interaction of calprotectin with the endothelium. Neutrophil activation and adhesion of monocytes to endothelium lead to release of more calprotectin which provides bacteriostatic and cytokine-like effects.[8] Its release from monocytes is also induced by P-LPS, TNF-alpha and IL-1 beta. In vitro studies on calprotectin gingival epithelial cells has shown that this protein enhance the barrier protection and innate immune functions against bacterial infection suggestive of a protective role in inflammation.[9] The protein also mediates trans-endothelial migration of myeloid cells and demonstrates chemotactic effect on neutrophils.[10-12] It has pro-apoptotic and pro-inflammatory activity in human periodontal ligament cells.[13] The level of calprotectin is thus shown to be associated with the severity and treatment outcome of periodontitis.[14] Calprotectin is a hetero-oligomer of EF-hand S100 calcium binding proteins namely S100A8 and S100A9, salient in innate immunity.[15] These subunits of calprotectin prevail as homodimers or complexes (S100A8/S100A9) depending on phase and type of inflammation and exhibit different functional properties. In the presence of calcium, calprotectin exists as heterotetramers and undergoes conformational changes allowing binding of more proteins.[16] In the presence of zinc, calprotectin inhibits MMPs (matrix metalloproteinases) which are zinc-dependent enzymes vital in angiogenesis, wound healing, inflammation, cancer, and tissue destruction. MMPs has the ability to cleave matrix component as they are known to hydrolyze components of the extracellular matrix[50]. In an chronic inflammatory periodontitis, abundance of S100A8 protein is predominantly involved in determining the host defence by inhibiting cell proliferation and apoptosis[17], whereas decrease in recombinant human S100A8 promoted migration of periodontal ligament cells.[18]The S100A8 subunit (earlier called as Myeloid/migration inhibitory factor-related protein-8, MRP8) of calprotectin is encoded by the *S100A8* gene located on human chromosome 1q21.3. S100A8 is produced mainly by polymorphonuclear neutrophils (PMN's) during inflammatory processes. It is expressed by activated macrophages, microvascular endothelial cells, osteoclast cells, gingival keratinocytes, neutrophil, monocytes. Activation of these cells by S100A8 protein further increases cell count in inflammatory sites.[19] It is dependent on IL-10 for expression in macrophages.[20] S100A8 subunit has pro-inflammatory activity including leukocyte recruitment, elevating the production of the cytokines and chemokines, effector function of neutrophils and regulation of adhesion and migration of leukocytes.[21] The endogenous damage-associated molecular pattern (DAMP) molecule - S100A8 stimulate the innate immune cells by binding to Toll-like receptor 4 (TLR4) and receptor for advanced glycation endproducts (RAGE). This activates the MAP-kinase and NF-kappa-B signalling pathways resulting in the augmentation of the pro-inflammatory cascade.[22,23] S100A8 also has anti-oxidant, anti-inflammatory and protective effect in endotoxemic mice.[24] The genetic variations influencing the severity and progression of periodontitis vary among racial and ethnic groups.[25,26] Literature search on the association of the gene polymorphism in the upstream region of *S100A8* gene with periodontitis revealed that there are no reports in the South Indian population till date. Thus the aim of the present study is to find whether the calprotectin subunit *S100A8* gene polymorphism rs3795391 (A→G) is associated with chronic and aggressive periodontitis.

MATERIALS AND METHODS

Study Population and subject selection

A cross-sectional analysis involving individuals of Dravidian ethnicity from the state of Tamil Nadu in the southern part of India is implemented in the present study. A total of 236 individuals who reported to the Department of Periodontics - Saveetha Dental College, Chennai were included in this study. The subjects were stratified into chronic periodontitis group (n=60), and aggressive periodontitis group (n=50) based on the American Academy of Periodontology (AAP) criteria 1999.[27] Medical history and clinical examination of the participants were recorded. The control group in the study (n=126) comprised individuals who did not present a history of previous periodontal disease as determined by the absence of gingival inflammation, clinical attachment loss (CAL), and no sites with a probing depth (PD) >3mm. All participants included in this study belong to age group of 30-55 years. A detailed history of previous dental treatment, family history of periodontal disease, smoking habits as well as general health concerns were obtained from all the subjects. Except for the presence of periodontitis, individuals who were smokers, with a previous history

of systemic health problems or systemic medication which affects periodontium, with previous periodontal treatment in the recent 6 months, pregnant women and lactating mothers, and medically compromised individuals were excluded from the study. Both the patients and the control subjects belong to the Dravidian ethnicity. Other populations were excluded from the study. Informed consent was obtained from all subjects. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Institutional Human Ethical Committee.

Sample collection and DNA extraction

A total of 5 ml of venous blood was collected by vein puncture and dispersed into a sterile tube containing a pinch of EDTA. It was mixed thoroughly to avoid clot formation. DNA isolation was done using salting out method according to the modified Miller *et al.,* 1998 protocol.[28] The buffy coat of nucleated cells was separated from the peripheral blood. The RBCs were lysed by adding 10 ml of RBC lysis buffer (0.17 M Tris-base, 0.155 M NH_4Cl), incubated in water bath for 10 minutes, followed by centrifugation at 3000 rpm for 20 minutes and the supernatant was discarded to remove remaining RBCs. The clear white pellet containing WBCs were digested using 3 ml of WBC lysis buffer (1 M Tris-HCl, 0.5 M EDTA, 1 M NaCl) and 200 µl of 10% SDS and incubated for 16 ± 2 at 37°C in water bath. To the cell lysate 1 ml of 6M NaCl was added, gently inverted several times and centrifuged at 3000 rpm for 20 minutes. The supernatant was transferred to a sterile tube and double the volume of ice cold absolute ethanol was added and inverted gently. The precipitated DNA was transferred to a sterile microcentrifuge tube, washed with 70% ethanol and dissolved in 1X TE buffer and stored at -20˚C until use.

SNP Genotyping

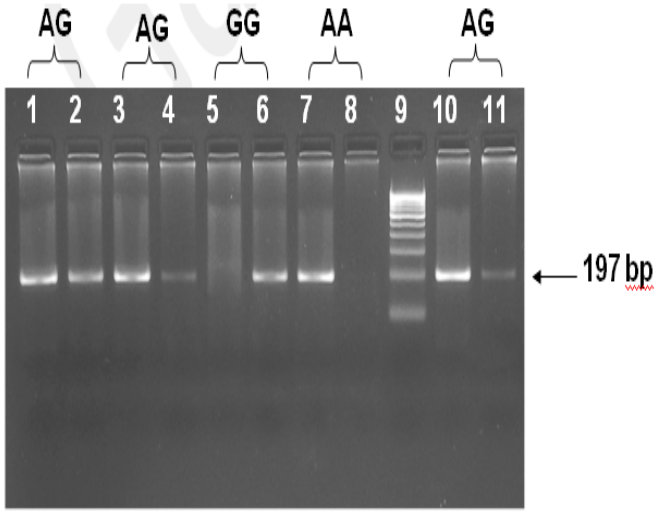
The S100A8 rs3795391 SNP was genotyped using the following allele specific primers:Forward- F1-5'–AGGGCAGTACGCAGAGGAT-3', F2-5'-AGGGCAGTACGCAGAGGAC-3' and Reverse - 5'-GGTGGTAAGGAAAGTCTACGG-3'. PCR was performed with the extracted genomic DNA by initial denaturation at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 30s, annealing at 61.8°C for 45s, extension at 72°C for 45s and a final extension step of 8 min at 72°C using Eppendorf Master Gradient Thermocycler, Germany. The PCR amplicons with fragment length of 197 bp were confirmed using 2% agarose gel and visualized by UV fluorescence. For confirmation of genotyping results, 10% of samples were randomly selected and were genotyped again using the same protocol which showed 100% concordance.

Statistical analysis

Statistical analysis of data was performed using the SPSS software version 19.0 for Windows (SPSS, Inc., Chicago, IL, USA). The genotypic distribution of this study was evaluated for Hardy-Weinberg Equilibrium (HWE). Data on frequency distribution collected for chronic periodontitis and aggressive periodontitis patients were pooled to single periodontitis group and analysed statistically. The Chi-square test was done to determine the strength of association between SNP rs3795391 and periodontitis by comparing chronic periodontitis and control group; aggressive periodontitis and control group; chronic periodontitis and aggressive periodontitis groups; and periodontitis and control group. The odds ratio (OR) was calculated with 95% confidence intervals (CI). A P-value < 0.05 was considered statistically significant.

Figure I

Agarose gel electrophoresis of AS-PCR assay showing the rs3795391 polymorphism



Lane: 1-2, 3-4 and 10-11 - heterozygous (AG); 5-6 - homozygous mutant (GG); 7-8 - homozygous normal (AA); 9 - 100 bp DNA marker

The association statistics of rs3795391 polymorphism with periodontitis obtained by chi-square analysis is represented in the Table III. The values were calculated with degrees of freedom (Df) = 1. The 2-sided p-values calculated were greater than 0.05. The P-values of genotypes test by 3 x 2 chi-square test (chronic periodontitis, aggressive periodontitis and periodontitis: P = 0.7848; P = 0.6691; P = 0.6125 respectively) calculated with Df= 2 indicated no association.

Table I
Clinical characteristics of the study population

Clinical characteristics	Control			Chronic Periodontitis			Aggressive Periodontitis		
No. of Subjects	Male	Female	Total	Male	Female	Total	Male	Female	Total
	69	57	126	29	31	60	29	21	50
Mean age (± SD)	35.36 ± 6.09			38.39 ± 6.47			27.86 ± 6.18		
CAL(mm)	-			4.76 ± 1.28			5.61 ± 2.47		
PD (mm)	1.86 ± 1.03			5.37 ± 1.21			6.23 ± 1.97		
Plague index	0.89 ± 0.21			1.94 ± 0.45			1.02 ± 0.38		
Gingival index	0.84 ± 0.19			1.85 ± 0.27			0.97 ± 0.59		

CAL –Clinical Attachment Loss; PD – Probing Pocket Depth

Table II
Frequency distribution of rs3795391 genotypes and alleles in controls and patients

rs3795391 Genotypes and Alleles	Control	Chronic Periodontitis	Aggressive Periodontitis	Periodontitis
	n (%)	n (%)	n (%)	n (%)
AA	91 (72.2)	44 (73.3)	34 (68.0)	78 (70.9)
AG	34 (27.0)	16 (26.7)	16 (32.0)	32 (29.1)
GG	1 (0.8)	0 (0.0)	0 (0.0)	0 (0.0)
AA *vs*	91 (72.2)	44 (73.3)	34 (68.0)	78 (70.9)
AG+GG*	35 (27.8)	16 (26.7)	16 (32.0)	32 (29.1)
A	216 (85.7)	104 (86.7)	84 (84.0)	188 (85.5)
G	36 (14.3)	16 (13.3)	16 (16.0)	32 (14.5)

****Combined effect of AG+GG genotype due to absence of homozygous polymorphic GG genotype in patients group***

Table III
Association statistics of S100A8 SNP rs3795391 with the risk of Periodontitis in Dravidian population

rs3795391 Genotypes and Alleles	Control vs CP*			Control vs AgP*			Control vs Periodontitis*		
	P-value	χ^2	OR (95% CI)	P-value	χ^2	OR (95% CI)	P-value	χ^2	OR (95% CI)
AA	Reference			Reference			Reference		
AG	0.939	0.006	0.97 (0.49-1.95)	0.5254	0.403	1.26 (0.62-2.57)	0.7476	0.104	1.10 (0.62-1.94)
GG	0.4876	0.482	-	0.5416	0.373	-	0.3557	0.853	-
AA *vs*	Reference			Reference			Reference		
AG+GG	0.8738	0.025	0.95 (0.47-1.89)	0.5776	0.31	1.22 (0.60-2.49)	0.8234	0.05	1.07 (0.61-1.88)
A	Reference			Reference			Reference		
G	0.8044	0.061	0.92 (0.49-1.74)	0.6827	0.167	1.14 (0.60-2.17)	0.9361	0.006	1.02 (0.61-1.71)

χ^2 - Chi-square values OR (95% CI) - Odds Ratio (95% confidence interval) with degrees of freedom = 1

Two-sided chi-square test comparing the control group with periodontitis group and subgroups (CP-Chronic periodontitis and AgP-Aggressive periodontitis) Fischer's exact test carried out with the allelic distribution of the control group and compared with each patients group: chronic periodontitis (P=0.8738), aggressive periodontitis (P=0.7394) and periodontitis (P=1.0). Similarly for the genotypes, frequency distribution of the patient group (chronic periodontitis, aggressive periodontitis and periodontitis) was tested for association with rs3795391 by comparing with the control group by 3 x 2 Fischer's test (P = 1.0; P = 0.6691; P = 0.8786 respectively). However, no statistical significance was evident. The genotypes and alleles of the chronic periodontitis vs aggressive periodontitis groups were also evaluated (AA vs AG+GG: χ^2 = 0.376; P = 0.5397), (A vs G: χ^2 = 0.312; P = 0.5764). The chi-square P-values among all three groups were greater than 0.05 in our study population.

RESULTS

The clinical characteristics of the subjects who participated in this study are depicted in Table I. The *S100A8* fragment of 197 bp containing rs3795391 polymorphism was amplified by AS-PCR and resolved on 2% agarose gel electrophoresis (Fig. I). The genotypic and allelic distribution (Table II) in all the study groups were consistent with Hardy-Weinberg Equilibrium (Healthy controls P = 0.2529, chronic periodontitis P = 0.2334, aggressive periodontitis P = 0.178 and periodontitis patients P = 0.0742). The distribution of genotypes and alleles did not show any significant differences between the healthy control group and the periodontitis subgroups (CP or AgP) or combined. Number of subjects with AG genotype was higher in aggressive periodontitis group (32.0%) of all the others. No individuals belonging to the patients group had the homozygous GG genotype.

DISCUSSION

Calprotectin is a proven inflammatory biomarker in various diseases such as inflammatory bowel disease,[29] rheumatoid arthritis[30] and atherosclerosis.[31] Since periodontitis is also a chronic inflammatory condition, significant upregulation of calprotectin in gingival crevicular fluid (GCF) positively correlated with chronic periodontitis and generalized aggressive periodontitis as well as amplified the clinical indicators

such as probing depth and bleeding on probing is documented.[32-34] This may raise a question on the scientific rationale of association of calprotectin gene polymorphism with periodontitis and therefore it is prudent to evaluate the association. The single nucleotide polymorphisms of various genes and their implications in periodontitis were recently reviewed by Karthikeyan *et al.,* (2014).[35] Investigations in the year 2015 concerning association of SNPs in the periodontitis related genes have shown both positive and negative correlations with periodontitis.[36-39] Noteworthy association of single nucleotide polymorphism at +3954 (C>T) within the IL1B gene with chronic periodontitis (p=0.007) was reported in South Indian population.[40] The AA allele at -1087 locus of IL-10 gene showed significant association in non smoking patients with severe chronic periodontitis of Dravidian ethnicity, whereas CTLA-4 gene SNP rs4553808 was insignificant in patients with AgP and CP who were from the same ethnicity.[41,42] A study on the effect of nucleotide substitution (A→ G) in the intron 1 of calprotectin subunit gene *S100A8* in AgP patients was first demonstrated in the Chinese population.[43] Later, association studies of *S100A8* upstream region polymorphisms (rs3795391, rs3806232 and rs3885688) in patients with chronic periodontitis and aggressive periodontitis were carried out in the same population.[44] Recently, in Han Chinese population significant correlation of the S100A8 gene (rs3795391 A/G) polymorphism was observed absent in osteoarthritis patients compared to control groups even in the dominant and recessive modes.[45] To the best of our knowledge, there are no such studies in any other population. Screening for SNPs aids in analysing the susceptibility profile of individual. If shown association it would help in early diagnosis and therapeutic intervention and hence we studied the association of rs3795391 polymorphism with periodontitis cases of Dravidian ethnicity. Smoking has an important role in the susceptibility, pathogenesis, progression and treatment outcome of periodontal disease.[46] Also there have been studies which have demonstrated that genetic association with chronic periodontitis is more evident in smokers than in non-smokers.[47] Hence, smokers were excluded from this study. One of the limitation of our study is low sample size and thus the results needed to be viewed with extreme caution. In our study, we only screened for the rs3795391 polymorphisms in calprotectin subunit gene *S100A8*. The data was analysed using a logistic regression model and the allelic genotypes of different groups were observed and compared. In the association statistics of *S100A8* SNP rs3795391 with periodontitis, the OR and p-values of two sided Chi-square (Table 3) indicates that this particular polymorphism may not be associated with the risk for this disease (P > 0.05). The heterozygous genotype AG was slightly elevated in aggressive periodontitis group (32%) than the chronic periodontitis group (26.7%), but was not statistically significant (P = 0.5397). Our findings are contradictory to the reports of the Chinese population which showed a positive correlation between rs3795391 and periodontitis in males.[44] This SNP was proposed to have no effect on the structure of gene products, but suggested to cause quantitative differences in gene expression and might affect the susceptibility of AgP.[48] But, unlike an increased AA genotype frequency in AgP patients compared to control group in a study by Sun *et al* in 2011 suggesting S100A8 to influence susceptibility and severity of AgP, decreased AA percentage was noted in our study (Control group = 72.2%, AgP patients = 68%).[49] The resultant data did not show any significant difference in the rs3795391 distribution between the controls and the cases.

CONCLUSION

The rs3795391 polymorphism in calprotectin gene does not serve a function in periodontal disease susceptibility in the Dravidian population. Future studies with larger sample size in different population are necessary to validate our findings.

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CONFLICT OF INTERESTS

Authors have no conflict of interests.

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