Clinical Relevance of Cytokines Gene Polymorphisms and Protein Levels in Gingival Cervical Fluid from Chronic Periodontitis Patients

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ABSTRACT

Background: Cytokines are suggested to play a role in periodontitis. **Objective**: To determine and compare the levels of Interleukin-1 beta (IL-1β) and Tumor necrosis factor alpha (TNF-α) in gingival crevicular fluid (GCF) samples amongst healthy individuals and those with chronic periodontitis. Further to compare the GCF cytokine levels in three genotype classes defined by the respective gene polymorphisms. **Methods:** The study was conducted on 41 chronic periodontitis patients and 40 healthy volunteers. IL-1β and TNF-α were quantified in GCF by cytometric bead array. DNA was extracted from peripheral blood samples and genotyping of IL1B +3954C/T (rs1143634) IL1B -511G/A (rs16944), TNFA -1031T/C (rs1799964) and TNFA -863C/A (rs1800630) polymorphisms were performed using Sanger sequencing and Taqman SNP genotyping assays methods. **Results:** Both IL-1β and TNF-α levels were significantly higher in chronic periodontitis group compared to the controls. IL-1β and TNF-α levels did not significantly differ in genotype classes of the respective polymorphism (IL1B -511G/A, TNFA -1031T/C and TNFA -863C/A). However, individuals with CT genotype of IL1B +3954C/T showed higher levels of IL-1β in the gingival crevicular fluid (ANOVA $p<0.05$). **Conclusion:** The results of this study revealed the presence of higher levels of IL-1β and TNF-α in subjects with periodontitis and genetic control of IL-1β levels in our samples of Indians.

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Keywords: Gene, Gingival Crevicular Fluid, Interleukin-1 beta, Polymorphism, Allele, Tumor Necrosis Factor- alpha

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INTRODUCTION

Periodontitis is a chronic inflammatory disease of microbial aetiology. Cytokines play an important role in the pathogenesis of periodontitis (1). The detection of cytokines has been performed for use as biomarkers of periodontitis initiation and progression. Identification of these biomarkers has been performed in gingival crevicular fluid and saliva samples (2,3). Gingival crevicular fluid (GCF) is an inflammatory exudate that derived from extracellular fluid of gingiva. In healthy gingiva, very low volume of the GCF is produced (3-4 μL/hr) (4). Gingival crevicular fluid is a preferred sample for identifying metabolic and other mediators from the gingiva during periodontal disease, however due to its low volume available for sampling, detection of the mediator/ biomarker levels is a challenge (5). Cytometric bead array provides a specific and highly sensitive (pg/ml) method for detection of multiple biomarkers in a small volume of Gingival Crevicular Fluid (<50 μL).

Interleukin-1 and TNF-alpha gene polymorphisms have been identified to be associated with periodontitis susceptibility in different ethnic populations across the world (6-8). Our previous study reported a statistically significant association between IL1B +3954 single nucleotide polymorphism and periodontitis susceptibility (9) and no association of periodontitis susceptibility with TNFA (-863,-1031) genotypes in a sample population from South India (10). The objective of this study was to determine and compare the levels of Interleukin 1 beta (IL-1β) and Tumor necrosis factor alpha (TNF-α) in gingival crevicular fluid samples by cytometric bead array. Further to compare the GCF cytokine levels in three genotype classes defined by the respective gene polymorphisms.

MATERIALS AND METHODS

Subject Selection. A total of 81 individuals aged above 18 years were recruited from the Out Patient Department of Periodontology, Faculty of Dental Sciences, Sri Ramachandra University, Chennai. The study was approved by the Institutional Ethics Committee of Sri Ramachandra University, Chennai (IEC-NI/10/APR/15/05). Prior to the commencement of study, informed written-consent was collected from all the participants. Of the 81 individuals, 29 were women and 52 men. The healthy group consisted of 40 individuals and 41 belonged to the chronic periodontitis group. Individuals were diagnosed to have generalized chronic periodontitis based on attachment loss of >1 mm in at least 30% of the sites examined (11) and radiographic evidence of bone loss, presence of at least ten natural teeth. Individuals with probing pocket depth of ≤ 3 mm, no attachment loss, absence of gingival bleeding on probing, absence of any clinical signs of gingival inflammation and no previous history of periodontal disease were included as controls (healthy gingiva). Current and former smokers, individuals with known systemic diseases, pregnant women, individuals who were on any medication were excluded from the study.

Clinical Examination. A full mouth periodontal examination was performed. The following indices: Plaque Index (12), Gingival Index (13), Oral Hygiene Index (OHI-S) (14), and clinical parameters - Clinical Attachment Level (CAL), probing pocket depth at six sites per tooth excluding third molars. All the clinical parameters were assessed by a single examiner using a UNC 15 manual periodontal probe.

Quantification of IL-1β and TNF-α in GCF. Five microliter of Gingival crevicular fluid was collected by micropipettes [5 microliter) with five markings for one microliter each (Sigma Aldrich)]. The GCF sample was collected by the extra-crevicular placement of the pipette at the entrance of the sites selected. Sites with the deepest probing pocket depth in each quadrant were sampled and pooled in each periodontitis patient. The sites with pocket depth of less than 3 mm, absence of bleeding on probing, and absence of clinical signs of gingival inflammation were chosen for sampling in the healthy subjects. The samples were transferred into a 1.5 mL Eppendorf tube containing 200 microliters of buffer and stored immediately at -80 degrees till processing. The GCF samples were transferred into a 1.5 ml Eppendorf tube containing 200 µL of buffer and stored immediately at -80 degrees until further processing. Cytometric bead array (BD™ CBA) was performed using a customized kit from, BD Biosciences, as per manufacturer's protocol.

In brief, standards were prepared for IL-1β and TNF-α using serial dilution (1:2 to 1: 256). About 50 µL of the test samples were mixed with equal volume of capture beads specific for IL-1β and TNF-α and incubated in the dark at room temperature for one hour. 50 µL of the mixed PE detection reagent was added to the samples and incubated for two hours at room temperature. One ml of wash buffer was added to each sample and centrifuged for five minutes at 200×g. The supernatant was discarded and the beads were re-suspended with 300 microliters of wash buffer. The samples were acquired on the same day on the flow cytometer (FACS Aria, BD Biosciences). Analysis of the samples was done using FCAP Array™ (BD Biosciences, Version 3) software and the levels of IL-1β and TNF-α protein were expressed as pg/ml.

Genotyping of IL1B and TNFA Gene Polymorphisms. About 3 ml of peripheral blood was collected into EDTA-Vacutainer (Beckton-Dickinson, Franklin Lanes, NJ, USA). DNA extraction was done in 200 µL of the blood using commercially available extraction kits (Nucleo-pore DNA Sure Blood Mini Kit, Shivaji Marg, New Delhi, India) as per manufacturer advised protocols. The quality of extracted DNA was checked using the NanoDrop ND-1000 (Thermo Fisher Scientific, USA). IL1B +3954C/T (rs1143634) and IL1B -511G/A (rs16944) were determined by PCR and DNA sequencing. The detailed protocol has been described in our previous report (9). In brief, the polymorphic genotypes at IL1B (- 511) [rs16944], and IL1B (+ 3954) [rs1143634] were determined by PCR and DNA sequencing. PCR was performed on 50–100 µg of DNA in a 10 µL reaction containing 5 µL PCR Master Mix (Merck PCR Master Mix Kit; Merck, Rahway, NJ), 2.8 µL sterile water, and 30 pM of forward and reverse primers using an Eppendorf thermocycler (Eppendorf, Hamburg, Germany). Cycle sequencing, for the PCR products, to determine the polymorphisms was performed using an ABI PRISM Big Dye Terminator Ready Reaction Cycle Sequencing Kit (Life Technologies, Carlsbad, CA, USA). Sequencing was done in a final volume of 10 µL containing 0.5 µL of ready reaction mix, 1.5 µL of sequencing dilution buffer, 1 µL of primer (forward/reverse), 6 µL of sterile water, and 1 µL of PCR product. The cycle sequenced products were then subjected to purification before the sequencing. Analysis of sequencing results was done using Applied Biosystems Seqscape v2.7 software (Life Technologies, Carlsbad, CA, USA) to determine the base transition in the sites of interest.

TNFA -1031T/C (rs1799964) and TNFA -863C/A (rs1800630) were genotyped using TaqMan genotyping method. Details of the primers and probes used for the assay are

given in our previously published report (10). The methodology in brief is described below. The final volume of each reaction was 5 µL, containing 2.5 µL of TaqMan genotyping Master Mix (Applied Biosystems, Foster City, CA, USA) 0.25 µL of TaqMan primer and probe mix (Applied Biosystems, Foster City, CA, USA), 2.25 µL of DNA (10 ng/µL). Fluorescence was measured with a Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and analyzed with its System software, SDS version 2.3. At least two non-template controls without DNA were included in each of the 384 well-plates.

Statistical Analysis. The mean and standard deviation for all the continuous variables was determined. The IL-1β and TNF-α protein levels were compared with the genotype classes of their respective genes using one way Analysis of Variance (ANOVA). The results were assumed to be statistically significant at $P<0.05$.

RESULTS

The demographic data of the study population are summarized in Table 1.

Table 1. Baseline clinical characteristics of study subjects and their IL-1β and TNF-α levels.

Clinical Indices	Control (n=40)	Chronic Periodontitis (n=41)	P Value [b]
Age range (yrs.)	20-55	23-58	
Mean age	31.53 ± 8.3	41.39 ± 8.6	<0.001
Gender			0.282
Female	17 (42.5%)	12 (29.3%)	
Male	23 (57.5%)	29 (70.7%)	
OHI Score [a]	0.354±0.163	2.848±0.788	<0.001
Gingival index [a]	0.363±0.131	1.877±0.346	<0.001
Plaque index [a]	0.426±0.215	1.789±0.321	<0.001
Probing pocket depth (mm)[a]	1.203±0.209	4.052±0.481	<0.001
CAL (mm)[a]	0.000±0.000	3.606±1.210	
TNFA pg/mL[a]	1.18±0.892	2.17±1.56	0.001
IL-1B pg/mL[a]	35.03±25.87	231.61±132.91	<0.001

[a]Mean ± SD; SD: standard deviation, [b]P value <0.05- statistically significant.

Levels of IL-1β and TNF-α in GCF samples of controls and patients with periodontal disease are documented in Table 1. Both IL-1β and TNF-α levels were significantly higher in chronic periodontitis group compared to the controls. Correlation of IL-1β and TNF-α levels with the genotypic classes of their respective gene polymorphisms are presented in Table 2 and Table 3, respectively.

Table 2. IL1B levels of the study subjects according to IL1B genotypes.

Genotype	N	Mean (95% CI)	F Value	P Value[b]
IL-1 B -511				
CC	34	128.96 (82.49-175.41)		
CT	45	140.32 (96.86-183.78)	0.129	0.87
TT	2	99.65 (500.90-700.21)		
IL-1 B +3954				
CC	31	96.50 (58.0-134.99)	3.980	0.04[b]
CT	50	158.13 (115.32-200.95)		

[b] P value <0.05 – statistically significant

Table 3. TNFA levels of the study subjects according to TNFA genotypes.

Genotype	N	Mean (95% CI)	F Value	P Value[b]
TNFA -863				
CC	46	1.71 (1.27-2.15)		
CA	29	1.53 (1.15-1.91)	0.513	0.601
AA	6	2.13 (0.07-4.20)		
TNFA -1031				
TT	22	1.71 (0.91-2.52)		
CT	53	1.56 (1.26-1.87)	1.417	0.249
CC	6	2.54 (1.07-4.02)		

[b] P value <0.05- statistically significant

IL-1β and TNF-α levels did not differ significantly in genotype classes of the respective polymorphism (IL1B -511G/A, TNFA -1031T/C and TNFA -863C/A). However, individuals with CT genotype of IL1B +3954C/T showed higher levels of IL-1β in the gingival crevicular fluid (ANOVA $p<0.05$).

DISCUSSION

The results of the present study demonstrated that the gingival crevicular fluid levels of IL-1β and TNF-α are significantly elevated in chronic periodontitis group as compared to control individuals. Although the genotypes of TNFA polymorphisms did not

correlate with gingival crevicular fluid levels of TNF-α, the CT genotype of IL1B +3954C/T showed higher levels of IL-1β in the gingival crevicular fluid.

There exists a large number of reports supporting the association of single nucleotide polymorphisms in Interleukin 1 alpha, Interleukin 1 beta, Tumor necrosis factor alpha genes and susceptibility to chronic periodontitis in the population of different ethnicities (15-18) across the world. However, studies evaluating the SNPs functionality of the SNPs in these genes in the different ethnic populations are very few in number (19-21). Our research group has published previous reports on the association of SNPs at IL1B (+3954) (9) and lack of association of SNPs at TNFA (-1031,-863) (10) with chronic periodontitis susceptibility in a sample population from South India. The present pilot study evaluated the functionality of the genotypes of the above mentioned SNPs by correlating the polymorphic genotypes with gingival crevicular fluid levels of Interleukin 1 and Tumor necrosis factor alpha. Results of our previous study revealed that the IL-1β levels were correlated with IL1B +3954C/T genotypes in individuals with chronic periodontitis in a sample South Indian population. In aggressive periodontitis and different forms of arthritis, the plasma levels of different cytokines and their receptor agonists were found to be correlated with the respective genotypes (22). In contrast to this, no correlation between IL1B +3954C/T genotypes and IL-1β levels was reported in GCF samples of chronic and aggressive periodontitis conditions in a Turkish population (23).

A comparison of TNF-α level in gingival crevicular fluid among genotype classes of TNFA (-1031,-863) did not show significant differences between chronic periodontitis and control participants of the present study. In a study performed by Yucel *et al.* wherein TNFA (-308) genotypes were correlated with TNF alpha levels in individuals with chronic and aggressive forms of periodontitis in individuals of Turkish origin the authors reported that TNFA -308G/A genotypes failed to correlate with TNF-α levels (8). To the best of our knowledge, the present study is the first to correlate the genotypes with cytokine levels in gingival crevicular fluid using the cytometric bead array in a sample of South Indian population.

Gingival crevicular fluid represents a more ideal choice for identifying cytokines generated during periodontitis as compared to saliva, since the sample is obtained from the specific site of disease activity. The small volume of GCF limits studies with traditional ELISA methods and warrants use of newer methodologies. Hence, a sensitive and specific method of detection of protein levels in a small volume of fluid is necessary. A technique that has gained a rapid acceptance in the recent times especially when markers in large numbers have to be identified and quantified in low volumes of the biological sample is the cytometric bead array (24,25). Using Cytometric bead array, a strong correlation between degree of periodontitis and serum levels of IL-2 and IFN-γ was established in HIV positive patients with periodontitis (26). In the present study we have adopted the Cytometric bead array to determine the cytokine levels in GCF samples. The reports on usage of this method to detect the cytokine levels in GCF samples amongst periodontitis patients are scarce. A recent study evaluated Interleukin-17 levels in GCF of periodontitis subjects before and after treatment (27). A study in smokers with chronic periodontitis evaluated various cytokine levels in gingival tissue extract samples and reported the lack of correlation between these cytokine levels with number of cigarettes that consumed per day (28).

In summary, this study evaluated the feasibility and utility of cytometric bead array to detect levels of multiple cytokines in gingival crevicular fluid. Further, this study

revealed the presence of higher levels of IL-1β and TNF-α in periodontitis subjects and genetic control of IL-1β levels in Indian population. A limitation of this study would be the small sample size which if improved upon might provide a better understanding of the role of genetic regulation of cytokines in periodontitis pathogenesis.

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