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Cytokines and Inflammatory Mediators for Monitoring the Status of Periodontitis

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Abstract

Objectives: C-Reactive Protein (CRP) is a sensitive and dynamic systemic marker of inflammation. Pro- and anti-inflammatory cytokines have been well documented with initiation, control and susceptibility to periodontal diseases. This study applied high-sensitivity CRP (Hs-CRP), pro- and anti-inflammatory cytokines to predict risk of periodontal diseases.

Methods: The 2200 subjects (1200 healthy subjects and 1000 chronic periodontal patients) were recruited from community of Hong Kong, Department of Periodontology and Oral Medicine, Xiangya Hospital, Central South University, Hunan, China, respectively. Blood was drawn from subjects and DNA was extracted. The polymorphic sites of C-Reactive Protein (CRP), pro-inflammatory (Interleukin-1α (IL-1α), IL-1β, Tumor Necrosis Factor-α (TNF-α), IL-6 and IFN-γ) and anti-inflammatory cytokines (IL-4, IL-10) were amplified and measured *via* polymerase chain reaction for further analysis. Chi-square test and logistic regression analysis were applied to analyze gene type distribution differences, allele frequencies and carriages rates between healthy and disease groups. Soluble protein levels of cytokines were evaluated by the T-test.

Results: Genotype frequencies of CRP, pro- and anti-inflammatory cytokines was statistically higher in periodontal disease patients than healthy controls ($p<0.05$). Prevalence of periodontal diseases was statistically correlated with carriage of single nucleotide polymorphisms of the cytokine parameters.

Conclusion: Hs-CRP and cytokine gene polymorphisms may be applied as biomarkers to predict periodontitis susceptibility, clinical behavior and severity.

Keywords: C-Reactive protein; Pro-inflammatory cytokines; Anti-inflammatory cytokines; Periodontitis; Gene polymorphisms

Introduction

The presence of periodontitis is mainly caused by the interaction of various factors, including the susceptibility of the host, the presence of pathogenic organisms, and the absence of beneficial species [1,2]. The main cause of plaque-induced inflammatory periodontal diseases is the result of bacteria, but progression and clinical characteristics of these diseases are influenced by both acquired and genetic factors that can modify susceptibility to infection [3]. Genetic factors have been demonstrated to play a significant role in the risk of periodontal diseases [3,4].

C-Reactive Protein (CRP) is an acute phase protein and its levels increase rapidly during infection and inflammation [5]. Thus, CRP is recognized as a sensitive and dynamic systemic marker of inflammation. High Sensitive CRP (Hs-CRP) is reported to be a strong predictor of cardiovascular risk, in spite of conventional cardiovascular risk factors [6]. The A/G polymorphism at position -717 of the human CRP gene was found to be associated with coronary heart disease. The infection-induced elevation of systemic CRP might account for the relationship between inflammatory and cardiovascular diseases.

Cytokines are soluble proteins that are secreted by cells to act as a messenger for transmitting signals to other cells. They initiate, mediate and control immune and inflammatory responses, as well as regulate growth and differentiation of cells. Gingival epithelial cells produce a broad range

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of cytokines, among which, Interleukin-1α (IL-1α), Interleukin-1β (IL-1β), Tumor Necrosis Factor-α (TNF-α), Interleukin-6 (IL-6) and Interferon-γ (IFN-γ) are classified as pro inflammatory cytokines, and Interleukin-4 (IL-4) and Interleukin-10 (IL-10) are categorized as anti-inflammatory cytokines [6-8].

IL-1 consists of at least two separate gene products, IL-1α and IL-1β, which have common biological activities but limited homology at nucleotide and peptide levels [9,10]. Concentrations of IL-1α and IL-1β are significantly greater at diseased sites ($p<0.05$) [11]. In comparison to other pro-inflammatory cytokines, IL-1β was most prevalent in the presence of active inflammation [12]. The gene encoding IL-1 is assigned to chromosome 2q13 to 21 [10,13,14]. The carriage of certain alleles of IL-1α and IL-1β is associated with the incidence and the severity of periodontal diseases, in particular Chronic Periodontitis (CP), because these carriers produce more IL-1 in response to plaque than genotype negative individuals by different studies [3,15-18].

TNF-α is located in 6p21.3 of chromosome 6 within the major his to compatibility complex [19,20]. Eight Single Nucleotide Polymorphisms (SNP) in the promoter region of this gene have been studied at positions -1031T/C, -863C/A, -857C/T, -575G/A, -376G/A, -308G/A, -244G/A, and -238G/A [21,24]. Many researchers investigated the possible link between the -308 polymorphism in the TNF-α gene and periodontitis because a G to A polymorphism at the -308 position of the TNF-α promoter region was suggested to influence TNF-α production and monocytes of patients with periodontitis [25-28].

The IL-6 gene is assigned to chromosome 7p21. Various SNPs in the promoter region of this gene have been studied at positions -174G/C, -190C/T, -572C/G, -597G/A, -1363G/T, -1480C/G and -6106A/T [29-31]. Periodontitis patients carrying one or two copies of the rare allele in the IL-6 (-174) polymorphism displayed significantly higher serum IL-6 and C-reactive protein concentrations [32]. Carriers of the rare allele at this position was associated with less reduction in probing depths among chronic periodontitis patients after delivery of standard non-surgical periodontal therapy [32].

The mRNA expression and/or concentration of IFN-γ in gingival crevicular fluid, gingival tissues, and serum were able to affect gingivitis, probing depths and alveolar bone loss [33,34]. Polymorphism in gene IFN-γ was found to be functionally relevant and causes differences in the immunoregulatory activity of its cytokine molecules. The T allele of the IFN-γ 874 T/A is found in high producers of IFN-γ [35,36]. The gene for IL-4 is localized in chromosome 5q31.1. The presence of IL-4-producing cells and the percentage of IL-4-expressing cells were significantly higher in established and advanced periodontitis lesions than in gingivitis tissues. IL-4 levels in the serum of patients were higher in chronic periodontitis but these levels did not correlate with the degree of bone loss or pocket formation. Identified promoter SNP at position (-590) and a 70-bp Variable Numbers of Tandem Repeat (VNTR) polymorphism at intron 2. However, the reports about the connection of IL-4 polymorphism and periodontitis seem controversial [37-39].

The gene encoding IL-10 was mapped to chromosome 1q31-32 [40]. The (-1082) G/A locus was not associated with chronic periodontitis susceptibility in most Caucasian populations except in one Swedish study but was linked to chronic periodontitis severity [41]. The (-1082) single nucleotide polymorphism was associated with high *in vitro* interleukin-10 production [42,43]. There was a complete absence of the N-allele carriage at position -1082 among the Japanese in contrast to Caucasians where the -1082 N-allele is the most occurring variant [3,42,43].

Since Hs-CRP, pro- and anti-inflammatory cytokines play a significant role in inflammatory diseases; the aim of this study is to investigate the association between polymorphisms in gene of Hs-CRP, pro and anti-inflammatory cytokines and chronic periodontitis subjects in Chinese populations.

Materials and Methods

Selection of subjects and inclusion criteria

This study took place from 2010 to 2017, recruiting a total of 2200 subjects: 1200 healthy subjects and 1000 chronic periodontal patients. The healthy control group, comprising 1200 subjects, was randomly recruited from community of Hong Kong, and Changsha while patients of the study group were recruited from and Department of Periodontology and Oral Medicine, Xiangya Hospital, Central South University, Hunan, China.

All subjects were screened against the inclusion criteria of the study prior to enrollment in the study. For eligibility to be included as healthy subjects, participants could be either male or female, between the ages of 23 to 66, non-smoking, have no past history of smoking, Body Mass Index (BMI) of 25, no systemic diseases, sites % with gingival recession <5, no severe caries and no periodontal diseases. For the study group, prescreening was conducted to confirm the following criteria: between the age of 30 to 70, probing depth >5 mm, and clinical attachment loss >4, gingival recession, and tooth mobility (for confirmation of chronic periodontal diseases).

Among the 1200 control samples, 432 were female and 768 were male, with an average age of 45.6 years old. The study group comprising 1000 Chinese periodontitis patient (410 females and 590 males) was on average 49.2 years of age. Approval was obtained from the Ethics Committee, Xiangya Hospital, Central South University, and informed consent was obtained from subjects prior to study start-up.

Oral clinical examination

Prior to enrollment in the study, potential healthy and study subjects were invited to take a full mouth examination at Keenlink Dental Clinic, Hong Kong, Department of Periodontology and Oral Medicine, Xiangya Hospital, Central South University, Hunan, respectively (Table 1) for prescreening against the inclusion in the study. In both prescreening sites, oral examinations were carried out by one periodontitis with a minimum of 10 years of experience. An intra oral examination was performed to determine periodontal conditions, including supragingival/sub-gingival calculus, gingival recession, Bleeding on Probing (BOP), Probing Depth (PD), Clinical Attachment Loss (CAL), gingival recession and tooth mobility.

In the prescreening process for inclusion of subjects in the healthy group, potential subjects were recruited to the study if all inclusion criteria for healthy subjects were met. Study group subjects were recruited after the oral examination to determine if chronic periodontitis was present. The basis of diagnosing chronic periodontitis was made following the criteria defined by the American Academy of Periodontology in 2017 [3].

Study subject sample size calculation

The sample size calculation for this study was decided based on

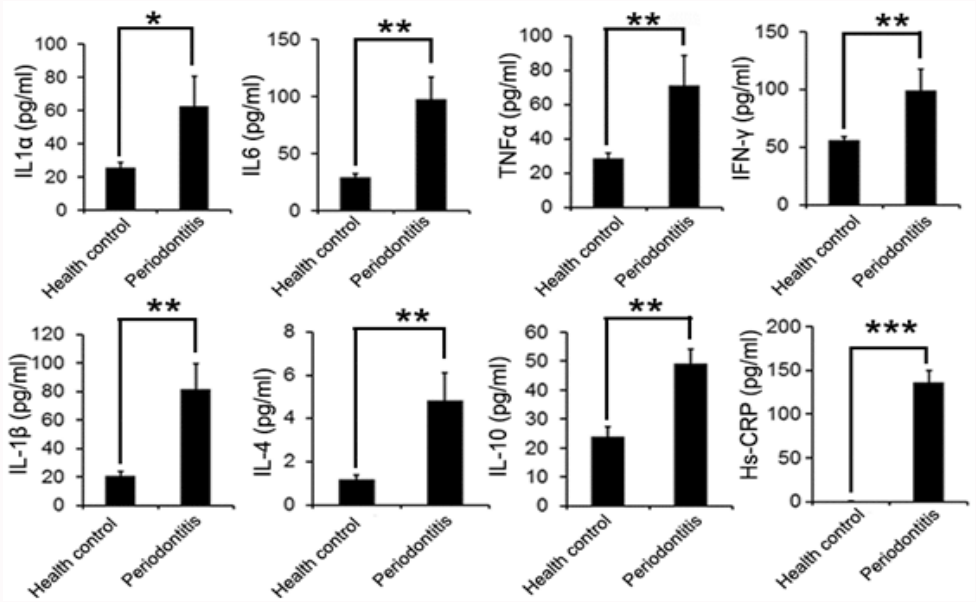

Figure 1: Protein expression (Mean ± SD) of various cytokines in patient and control groups as measured by ELISA. *p<0.05, **p<0.01, ***p<0.001.

Table 1: The clinical data (Mean ± SD) of 1200 control subjects and 1000 periodontitis patients.

Parameters	Control subjects (N=1200)	Periodontitis patients (N=1000)
Age (years)	45.6 ± 8.6	49.2 ± 11.2
Age range (years)	24-65	30 -68
Male/female	768/432	590/410
PD (mm)	2.2 ± 0.9	5.98 ± 2.5*
Sites % with BOP	35.3 ± 7.2	78.2 ± 19.8*
Sites % with gingival recession	0.9 ± 0.5	38.9 ± 25.9*
Sites % with calculus	30.2 ± 8.6	65.0 ± 12.5
Clinical attachment loss (mm)	1.1 ± 0.76	5.8 ± 1.8

Significant difference from the control subjects, *p<0.05

the reports of [37]. In the report, the sample size was calculated based on a 0.05 level of significance for two arms to achieve 90% power [37]. The required number of periodontal subjects for the study was determined according to a data survey in Hong Kong that measured the periodontal Pocket Depth (PD) and Bleeding on Probing (BOP).

Blood sample preparation

Two tubes of blood were collected in Essence Medical Laboratory, Hong Kong from all subjects by direct venipunctures from each subject: 20 ml in lithium heparin tubes and 10 ml in clot blood tubes (BD Vacutainer, NJ USA), respectively. The blood samples were centrifuged for 10 min at 1,500 rpm. Serum and plasma was removed for Enzyme Linked Immunosorbent Assay (ELISA) analysis. The remaining cellular components were transferred to a 50 ml centrifuge tube with an addition of red blood cell lysis buffer, up to a final volume of 45 ml. The mixture in the tube was inverted several times, and then centrifuged for 10 min at 1,500 rpm. The supernatants were discarded and the remaining components were washed with 0.9% PBS used for DNA extraction.

Isolation of purified DNA

Genomic DNA was extracted from collected blood samples using a commercially available Genomic DNA Blood Mini Kit (QIAGEN, MD, USA), following the manufacturer's protocols. The concentration of DNA was estimated by measurements of OD260 by a spectrophotometer (U1800, Hitachi, Japan). The extracted DNA was labeled and stored at -80ºC.

Polymerase chain reaction (PCR) for amplification of polymorphic sites

The extracted genomic DNA from samples was amplified with a PCR kit (Promega Corporation, USA) consisting of nuclease-free water and PCR Master Mix-according to provided protocols. All procedures were carried out in a sterile and stable environment to prevent external contamination.

PCR was undertaken in a thermal cycler (MJ, USA) with a mixture containing 20 µl of nuclease-free water, 25 µl of PCR Master Mix, 0.5 µl of each designed cytokine genes primer (Invitrogen, USA) [33,37,44-50]. All primers were designed using the Roche UPL Primer Design Program, and 4 µl of the extracted DNA sample were mixed to undergo thermal cycling. All products from the thermal cycling were labeled accordingly and stored at -80ºC until use. In this study, GAPDH were used (Table 2) with the forward primer 5'-AGAAGGCTGGGGCTCATTTG-3' and reverse primer 5'-AGGGGCCATCCACAGTCTTC-3'. GAPDH is a constitutive housekeeping gene for PCR of DNA, utilized for comparison of changes in specific gene expressions [37,45,50].

Table 2: PCR conditions for various genes.

Gene	Product size (bp)	Denaturation	Annealing	Extension	Cycles
IL-1α	229	94ºC,1 min	55ºC, 30s	72ºC, 60 s	35
IL-1β	305	94ºC, 5 min	56ºC, 45s	72ºC, 60 s	35
IL-6	296	95ºC, 60 s	60ºC, 60s	72ºC, 60 s	35
TNF-α	133	94ºC, 1 min	61ºC, 1min	72ºC, 60 s	35
IFN-γ	366	95ºC, 5 min	56ºC, 30s	72ºC, 5 min	30
IL-4	195	95ºC, 5 min	51ºC, 60s	72ºC, 60 s	35
IL-10	139	94ºC, 30 s	60ºC, 45s	72ºC, 60 s	35
CRP	376	95ºC, 60 s	61ºC, 60s	72ºC, 5 min	35
β-actin	211	94ºC, 30 s	55ºC, 30s	72ºC, 60 s	30

Restriction digest using *Fnu4H1*, *AvaI*, *Nla III*, *Alw*, *Mnl I*, *AvaII* and *Bsh1236* for IL-1α, IL-1β, IL-6, IFN-γ, IL-10, TNF-α, IL-4 and CRP respectively

The amplified fragments generated from PCR were digested:

1. The 229bp fragment on IL-1α was recognized by *Fnu4H1* (Fermentas Life Sciences, USA) [51,52];

2. The 305bp fragment on IL-1β was recognized by *AvaI* (Fermentas Life Sciences, USA) [49,52];

3. The 296bp fragment on IL-6 was recognized by *NlaIII* (Fermentas Life Sciences, USA) [31,46];

4. The 296bp fragment on IFN-γ was recognized by *Alw* (Fermentas Life Sciences, USA) [47,53];

5. The 139bp fragment on IL-10 was recognized by *Mnl I* (Fermentas Life Sciences, USA) [48,54];

6. The 296bp fragment on TNF-α and 195bp fragment on IL-4 were recognized by *AvaII* (Fermentas Life Sciences, USA) [3,46,53]; and

7. The 376bp fragment on CRP was recognized by *Bsh1236* (Fermentas Life Sciences, USA) [55].

For each digest, 10 µl of amplified PCR product was mixed with 2.5 µl to 5 µl of the corresponding restriction enzyme, 10 µl of nuclease free water and 0.5 µl to 1 µl of restriction enzyme buffer and incubated for over 4 h at 37ºC (Table 3). All digestion reagents were kept on ice before incubation to prevent denaturation. To ensure amplicons were consistent throughout the procedure, all samples were digested twice.

DNA gel electrophoresis and visualization

The 10 µl of digestion product and 1 µl of ready load 1 Kb DNA Ladder (Invitrogen, Spain) were loaded into 2% to 4% agarose gel (Invitrogen, Spain) containing 0.5 µg/ml of ethidium bromide. The gel underwent electrophoresis at 100 volts; 100 milliampere for 30 min afterwards, the gel was visualized using a Dolphin-DOC ultraviolet illuminator (Wealtec, South Africa).

Sera measured by ELISA

100 µl of the standard group solutions and serum of each subject was pipette into a 96 well plate included in the cytokines ELISA kit (Diaclone, France) [56]. The plate was incubated for 2 h to 3 h at 350 rpm and washed with washing buffer three times. Then the wells were dried and 200 µl of substrate Tetramethylbenzidine (TMB) was added into each well for 20 min in the dark at room temperature. The plates were read at 450 nm wavelength using Universal Microplate Reader (Sunrise, TECAN, and Austria). The levels of cytokines in the samples were obtained by comparison with the standard curve generated from standards supplied by the manufacturer [56]. ELISA was performed according to the manufacturer's protocols from cytokines ELISA Kit (Diaclone, France) and the serum samples. The normal detection ranges of biomarker were showed in Table 4.

Statistical analysis

SPSS 22.0 for windows (IBM, SPSS, USA) was used for calculations of the chi-square and independent t-tests. The chi-square test (χ^2) was applied to examine the differences in Hs-CRP, genotype distributions, allele frequencies and carriages rate between healthy and disease groups. The alleles were calculated as odds ratio (or) with 95% confidence intervals (95% CI). The soluble protein levels of cytokines were evaluated with an independent t-test. A *p*-value of <0.05 was considered statistically significant.

Results

The chronic periodontitis study group showed a significantly greater mean of PD (5.98 ± 2.5 mm *vs.* 2.2 ± 0.9 mm), CAL (5.8 ± 1.8 mm *vs.*1.1 ± 0.76), and a higher percentage of sites with BOP (78.2 ± 19.8% *vs.* 35.3 ± 7.2%) and gingival recession (38.9 ± 25.9% *vs.* 0.9 ± 0.5%) than the healthy control group ($p<0.05$) (Table 1). Confirmatory of the inclusion of systematically healthy individuals, the blood results for both the healthy and study group were predominantly within the normal ranges. Statistical differences, however, in neutrophils (33.6% *vs.* 70.0%), lymphocytes (49.2% *vs.* 26.0%) and monocytes (14.0% *vs.* 3.0%) were observed ($p<0.05$) in the periodontitis patients compared to the healthy controls (Table 4).

All protein expressions of patients and control samples for IL-1α (62.65 ± 11.93p g/ml *vs.* 25.28 ± 4.51p g/ml), IL-1β (81.73 ± 17.94p g/ml *vs.* 20.55 ± 3.37p g/ml), IL-6 (97.57 ± 18.92p g/ml *vs.* 28.77 ± 3.13p g/ml), TNF-α (70.99 ± 13.18p g/ml *vs.* 28.45 ± 4.77p g/ml), IFN-γ (99.44 ± 10.52p g/ml *vs.* 55.85 ± 8.26p g/ml), IL-4 (4.85 ± 1.26p g/ml *vs.* 1.18 ± 0.21p g/ml), IL-10 (49.23 ± 4.88p g/ml *vs.* 23.85 ± 3.55p g/ml), and Hs-CRP (136.50 ± 12.92p g/ml *vs.* 0.61 ± 0.25p g/ml) measured by ELISA confirmed a statistically significant difference ($p<0.001$). Agarose gel electrophoresis (Wealtec, South Africa) and visualization demonstrated that the pro-inflammatory cytokines IL-1α and TNF-α and the anti-inflammatory cytokines IL-4 and IL-10 were associated with chronic inflammation in patients ($p<0.0001$) (Figure 1).

The homozygous A alleles of CRP-717 were represented by a DNA band of 376bp, and homozygous G alleles presented 2 DNA bands of 376 and 319bp. The homozygous G alleles of CRP-717 were

Table 3: The primer sequences and restriction enzyme used for detection of cytokine DNA polymorphism genes.

Ctokines	Primers	Sequence	Position	Restriction Enzyme	Digestion Time (hours)	References
IL-1α	Forward	5'-ATGGTTTTAGAAATCATCAAGCCTAGGCA-3'	-889	Fnu4H1	>12	[51]
	Reverse	5'-AATGAAAGGAGGGGAGGATGACAGAAATGA -3'				
IL-1β	Forward	5'-TGGCATTGATCTGGTTCATC-3'	-511	AvaI	>12	Nestor et al.
	Reverse	5'-GTTTAGGAATCTTCCCACTT-3'				
IL-6	Forward	5'-TTGTCAAGACATGCCAAGTGCT-3'	-174	Nla III	4	[18]
	Reverse	5'-GCCTCAGAGACATCTCCAGTCC-3'				
TNF-α	Forward	5'-GAAGCCCCTCCCAGTTCTAGT TC-3'	-238	AvaII	4	Sleijfferset al.
	Reverse	5'-CACTCCCCATCCTCCCTGGTC-3'				
IFN-γ	Forward	5'-GCTGTCATAATAATATTCAGAC-3'	-874	Alw	4	Inoue et al.
	Reverse	5'-CGAGCTTTAAAAGATAGTTCC-3'				
IL-4	Forward	5'-TAAACTTGGGAGAACATGGT-3'	-590	Ava II	>12	[18]
	Reverse	5'-TGGGGAAAGATAGAGTAATA-3'				
IL-10	Forward	5'-CTCGCTGCAACCCAACTGGC-3'	-1082	MnlI	4	[54]
	Reverse	5'-TCTTACCTATCCCTACTTCC-3'				
CRP	Forward	5'GACTCCTGCCTGAAGCTTTACATA-3'	-717	Bsh1236	4	[55]
	Reverse	5'-ATACATGTGCCATGCTGGTGTG-3'				

Table 4: Complete blood count (Mean ± SD) of control subjects (N=1200) and periodontitis patients (N=1000).

Parameters	Control	Periodontitis	Normal Range	Unit
White blood cells	6.0 (± 0.98)	9.2 (± 2.1)	4.00-11.00	109/L
Red blood cells	4.8 (± 0.25)	5.0 (± 0.10)	3.8-6.0	1012/L
Hemoglobin	14.5 (± 0.80)	13.5 (± 0.43)	11.5-16.5	g/dL
Platelets	295 (± 30.2)	265 (± 23.31)	150-400	109/L
Neutrophils	70.0 (± 11.32)	*33.6 (± 8.3)	4.0-75.0	%
Lymphocytes	26.0 (± 7.18)	*49.2 (± 10.69)	20-45	%
Monocytes	3.0 (± 1.2)	*14.0 (± 2.89)	4.7-12.2	%
Eosinophils	0.83 (± 0.21)	1.5 (± 0.52)	0.7-7.0	%
Basophils	0.17 (± 0.09)	0.33 (± 0.24)	0.1-1.2	%

Significant difference from the control, *p<0.001

found to be significantly higher in periodontitis patients compared to healthy subjects, corresponding to 72% *vs.* 54%, respectively (p=0.008; OR=2.19; 95% CI=1.22-3.94). Heterozygote's for CRP at position -717 site of the CRP gene displayed a combination of both G and alleles. A comparison of A/A genotype in periodontitis patients (67%) with respect to healthy controls (55%) demonstrated a higher frequency (p=0.0004). The odds ratio for carriage of CRP -717 allele (A/A and G/G genotypes combined in comparison to A/A genotype) was computed to be 2.80 (95% CI=1.58 to 4.99) for the patient group (Table 5).

Digesting *Fnu4H1* for IL-1α formed the DNA products of 153- and 76-bp for homozygous C allele, and 124-, 76- and 29-bp for homozygous T alleles. The results of detection frequency of the homozygous C allele of IL-1α were similar in the chronic periodontitis study group (64%) as the healthy control group (42%) (χ^2, p=0.002). Varying results were demonstrated for the C/C genotype, where frequency was 54% in periodontitis patients in comparison to 18% in the control group. The odds ratio for carriage of IL-1α allele (T/T and C/T genotypes combined to compare with the C/C genotype) was 5.35 (95% CI: 2.81 to 10.18, χ^2: p<0.0001) in periodontitis subjects (Table 5).

The frequency of homozygous T alleles ofIL-1β (305bp) digested with Ava1 were discovered to be higher in the study group (66%) compared to healthy controls (49%) with significance (p=0.02; OR=2.02; 95% CI=1.14-3.57). The T/T genotype was detected to be higher also the periodontal study group compared to healthy subjects, despite an absence of significance (55% *vs.* 35%, p=0.05). The odds ratio for IL-1β allele (C/C and C/T genotypes combined compared with the T/T genotype) was 2.27 (95% CI: 1.28 to 4.01) in periodontitis patients (Table 5).

Digesting *Nla III* for IL-6 formed the DNA products of 296 76-bp for homozygous G allele [57]. Significant differences were detected for the frequency of the homozygous G allele of IL-6 in the chronic periodontitis study group (65%) than the healthy control group (43%) (χ^2, CI=1.39-4.36, p=0.002). Varying results were demonstrated for the G/G genotype, where a lower frequency was discovered for periodontitis patients (24%) than the control group (50%). The odds ratio for carriage of IL-6 allele (C/C and G/C genotypes combined to compare with the G/G genotype) was 0.32 (95% CI: 0.17-0.58, χ^2: p<0.001) in periodontitis subjects (Table 5).

Digesting *AvaII* for TNF-α formed the DNA products of 70- and 63-bp for homozygous G alleles, and 63-, 49- and 21-bp for

Table 5: Genotype and allele frequency of cytokines in periodontitis patients (CP) and control subjects.

Genotypes	CP Patients *n*=1000 (%)	Healthy subjects *n*=1200 (%)	CP *versus* Controls OR (95% CI)	CP *versus* Controls X^2 *P* values	Alleles	CP patients *n*=2000 (%)	Healthy subjects *n*=2400 (%)	CP *versus* Controls OR (95% CI)	CP *versus* Controls X^2 p values
CRP-717									
*A/A	670 (67)	660 (55)	2.8 1.58-4.99	0.0004	***A**	1440 (72)	1764 (54)	2.19 1.22-3.94	0.008
G/G	150 (15)	132 (11)			**G**	560 (28)	636 (46)		
A/G	180 (18)	408 (34)							
IL-1α									
*C/C	540 (54)	216 (18)	5.35 2.81-10.18	<0.0001	***C**	1280 (64)	1248 (42)	2.46 1.39-4.34	0.002
T/T	270 (27)	132 (11)			**T**	720 (36)	1152 (58)		
C/T	190 (19)	852 (71)							
IL-1β									
*T/T	300 (55)	456 (35)	2.27 1.28-4.01	0.005	***T**	1120 (66)	1416 (49)	2.02 1.14-3.57	0.02
C/C	120 (12)	252 (21)			**C**	880 (34)	984 (51)		
C/T	580 (33)	492 (44)							
IL-6									
*G/G	240 (24)	600 (50)	0.32 0.17-0.58	0.0002	***G**	1100 (65)	1512 (43)	2.46 1.39-4.36	0.002
C/C/	70 (7)	276 (23)			**C**	900 (35)	888 (57)		
G/C	690 (69)	324 (27)							
TNF- α									
*G/G	450 (45)	192 (16)	4.30 2.21-8.35	<0.0001	***G**	1100 (55)	768 (32)	2.59 1.46-4.62	0.0012
A/A	310 (31)	564 (47)			A	900 (45)	1632 (68)		
A/G	240 (24)	444 (37)							
IFN-γ									
*T/T	550 (55)	660 (35)	2.27 1.28-4.01	0.005	*T	1280 (65)	1584 (32)	3.95 2.19-7.10	<0.0001
A/A	220 (22)	276 (43)			A	720 (35)	816 (68)		
T/A	230 (23)	264 (22)							
IL-4									
*C/C	30 (3)	312 (26)	0.09 0.03-0.30	0.0001	*C	440 (22)	1248 (52)	0.26 0.14-0.48	<0.0001
T/T	530 (53)	240 (20)			T	1560 (78)	1152 (48)		
T/C	440 (44)	648 (54)							
IL-10									
*A/A	650 (65)	1080 (90)	0.21 0.09-0.45	0.0001	*A	1300 (65)	2280 (95)	0.1 0.04-0.26	<0.0001
G/G	290 (29)	84 (7)			G	700 (35)	120 (5)		
A/G	60(6)	36 (3)				1440 (72)	1764 (54)	2.19 1.22-3.94	0.008

CRP: G/G+A/G *vs.* A/A; IL-1α: T/T+C/T *vs.* C/C
IL-1β: C/C+ C/T *vs.* T/T; IL-6: C/C+G/C *vs.* G/G
TNF-α: A/A+A/G *vs.* G/G; IFN-γ: A/A+T/A *vs.* T/T
IL-4: T/T +T/C *vs.* C/C; IL-10: G/G+A/G *vs.* A/A
OR: Odds Ratio; CI: Confidence Interval
Significant difference from healthy controls, *p<0.05

homozygous A alleles. The homozygous G allele of TNF-α was shown to be higher in the study group (55%) than healthy controls (32%) (χ^2: *p*=0.0012, OR: 2.59, CI: 1.46 to 4.62). The detection frequency of the G/G genotype in periodontitis patients was higher than the controls (45% *vs.* 16%, p<0.0001). For the periodontal study group, the OR was 4.30 (95% CI: 2.21 to 8.35) for carriage of TNF-α allele (A/A and A/G genotypes combined for comparing G/G genotype) (Table 5).

The homozygous T-alleles of IFN-γ were represented by a DNA band size of 366bp, digested by *Alw*. A significantly higher detection frequency (*p*<0.0001) was noted for the patient group (65%) compared to healthy subjects (32%) (OR=3.95, 95% CI: 2.19 to 7.10). Comparison of the T/T genotype of IFN-γ indicated a higher frequency also for periodontal patients (55%) than healthy subjects (35%), albeit a lack of significance. The odds ratio for carriage of IFN-γ allele (A/A and T/A genotypes combined compared with the T/T genotype) was 2.27 (95% CI: 1.28 to 4.01) in the study group of periodontal patients (Table 5).

The homozygous T alleles of IL-4 were represented by a DNA band size of 195bp, and homozygous C-alleles were represented by DNA bands sizes 18 and 177bp [41]. The chronic periodontitis patient group had a higher detection frequency of the homozygous T alleles of IL-4 (78%) than the control group (48%), and lower frequency for

the homozygous C-alleles for periodontitis patients (22%) compared to healthy subjects (52%). A comparison of the study and healthy group demonstrated significant differences (p<0.0001, OR=0.26, 95% CI: 0.14 to 0.48). Comparing T/T genotype, the study group presented a higher frequency of 53% than 20% in healthy controls (p<0.0001). The OR for carriage of IL-4 allele (T/T and T/C genotypes combined compared with the C/C genotype) was 0.09 (95% CI: 0.03 to 0.30) in the study group of periodontal patients (Table 5).

The homozygous A alleles of IL-10 were represented by a DNA band size of 139bp, homozygous G alleles were represented by DNA band sizes 106 and 33bp. Compared to the study group (65%), the detection frequency of the homozygous A alleles of IL-10 was higher in healthy controls (95%). Statistical significance was observed between the study and control group (p<0.0001, OR=0.10, 95% CI: 0.04 to 0.26). A significantly lower frequency was detected for the A/A genotype for periodontitis patients than healthy controls 65% *vs.* 90%, (p<0.001). The OR for carriage of IL-10 allele (G/G and A/G genotypes combined compared with the A/A genotype) was 0.21 (95% CI: 0.09 to 0.45) in periodontitis patients (Table 5).

Discussion

The presence of inflammation in diseased periodontal tissues or gingival crevicular fluid can be observed with increased levels of CRP and pro-inflammatory cytokines IL-1α, IL-1β, TNF-α, IFN-γ and IL-6 [58,59]. Recent study results showed that subjects with CP have increased levels of inflammation as a results of increased serum levels of Hs-CRP, IL-1, IL-6, TNF-α, and IFN-γ in comparison to healthy individuals. Hs-CRP and IL-6 are generally the most sensitive markers of the acute phase response to infections and inflammation [55].

Anti-inflammatory cytokines IL-4 and IL-10 work by balancing raised levels of pro-inflammatory cytokines. The progression of inflammation in periodontal diseases can be explained by an absence of response of the anti-inflammatory cytokines IL-4 and IL-10 in chronic periodontitis [58-60]. Genetic polymorphisms cause a change in the protein or its expressions altering the immune response.

The results of this study parallel past and current research documenting the prevalence of elevated levels of CRP inflammatory diseases as periodontitis [5]. Statistical differences were observed for A/G allele frequencies for the CRP gene between periodontal patients and orally healthy subjects (p=0.008). A higher frequency of homozygous G/G genotype and heterozygous A/G genotype was detected in orally healthy individuals than orally-diseased persons (25% *vs.* 15%; and 33% *vs.* 18%). The CRP -717 A/A genotype was more highly associated with periodontal-diseased individuals (67%) than healthy subjects (42%) (OR=2.80; 95% CI: 1.58 to 4.99). Similarly, ELISA investigations for the protein expression of the Hs-CRP cytokines also demonstrated significant differences in the study and healthy subjects. A higher association of A/A genotype in periodontal patients may be an indicator of periodontal-diseased tissues in the Chinese population (Figure 1).

Significant differences in detection of C and T-alleles of IL-1α (-889) were also discovered in the study and control groups. As observed, genetic polymorphism likely differs between ethnic populations as the carriage rate of C allele was shown to be lower in our population of Chinese subjects than other Asian cohorts [61,63]. The C-allele frequency in chronic periodontal patients was comparably higher than healthy controls (64% *vs.* 42%), while the reverse was true for the frequency of T-allele, where the orally healthy control group (58%) was observed to be higher than the patient group (36%). In line with other studies, the IL-1α CC genotype (OR=5.35; 95% CI=2.81 to 10.18) was significantly associated with chronic periodontitis [46,64].

The association of IL-1β SNPs with periodontal diseases has been commonly reported in Caucasian populations [12,65]. While varying results have been published for the Chinese population [66,68]. The association of SNP IL-1β (-511) and periodontitis was examined in this study. A statistically significant correlation for the SNP IL-1β (-511) and periodontitis was established for the patients of this study, correlating with severity of periodontitis. The IL-1β SNP was shown to be elevated in the patient group, compared to healthy controls (p=0.005). The carriage rate of the T-allele was found to be higher in orally diseased patients of the study, compared to orally healthy individuals (66% *vs.* 49%). A higher detection rate for the TT genotype was also found (55% *vs.* 35%). These results highlight a similar role of IL-1β in inflammation in periodontal diseases of Chinese patients as Caucasians.

The pro-inflammatory cytokine TNF-α possesses a wide range of immunoregulatory functions, including production of secondary mediators [69]. Ample research has studied the polymorphic site position 308 and concluded a lack of association with periodontitis. In contrast, the results of this present study was able to demonstrate a higher frequency of homozygous (G/G) genotype and an association of TNF-α G/G genotype with chronic periodontal diseases (OR=4.30; 95% CI=2.21 to 8.35). Taken together, TNF-α may be considered a risk genotype for periodontitis susceptibility.

The results of the current study showed a similar association of IL-6 in the Chinese population with periodontitis to Caucasians and Brazilians [70,71]. The study and control groups demonstrated significantly differing carriage rates for both G and C alleles, rendering at 65% and 43% for C allele and 35% and 57% for G allele. The heterozygous genotype IL-6-174 (G/C) was observed to have a higher frequency in the study cases in contrast to healthy controls (69% and 27%). IL-6 -174 G/C genotype could be considered a risk genotype for periodontitis susceptibility.

Previous case control studies have demonstrated varied results in the relationship between the IL-4 gene polymorphism and susceptibility to chronic periodontitis [16,39,72,73]. The present study showed an association of IL-4 C/C genotype (OR=0.09; 95% CI: 0.03 to 0.30) with a lower frequency in the study subjects. The T/T genotype was more prevalent in the study patients (53%) than healthy controls (20%), suggesting this may be a risk genotype for periodontitis susceptibility. A comparatively lower C/C genotype in patients (3%) compared to healthy controls (26%) may be an indicative factor against the development of the disease [74-86].

In this study, the IL-10 (-1082) polymorphism demonstrated a lower frequency of A/A genotype with periodontitis patients than healthy subjects (65% *vs.* 90%). A higher frequency of IL-10 (-1082) G/G genotype (29%) was found in the study group compared to healthy subjects (7%), which may be considered a risk genotype for periodontitis susceptibility. The results of this study were in accordance with past studies: subjects who exhibited the G/G genotype were significantly larger in subjects with chronic periodontal diseases than in periodontal-healthy individuals [3].

Conclusion

The results of this study demonstrate that Hs-CRP in

combination with cytokine gene polymorphisms may be associated with periodontitis susceptibility, clinical behavior and severity. Application of these biomarkers may be used as putative risk indicators for chronic periodontitis in the Chinese population.

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