

Cytokine gene polymorphism (interleukin-1 β +3954, Interleukin-6 [–597/–174] and tumor necrosis factor- α –308) in chronic periodontitis with and without type 2 diabetes mellitus

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ABSTRACT

Background: Pro-inflammatory cytokine gene polymorphisms are potential candidates for susceptibility for both type 2 diabetes mellitus (DM) and chronic periodontitis (CHP). This study explored the association of interleukin-1 beta (IL-1 β) +3954, interleukin-6 (IL-6) –597/–174 and tumor necrosis factor-alpha (TNF- α) –308 single nucleotide polymorphisms in CHP with and without type 2 DM in Malayalam speaking subjects of Dravidian ethnicity.

Materials and Methods: This case control study consisted of 51 chronic periodontitis with type 2 diabetes mellitus (CHPDM) and 51 CHP patients as cases and 51 healthy subjects as controls. Polymorphisms were identified by polymerase chain reaction amplification followed by restriction enzyme digestion and gel electrophoresis.

Results: IL-1 β (+3954) TT genotype and T allele were significantly associated with CHPDM group when compared with CHP ($P = 0.001$), whereas CC genotype and allele C was higher in CHP subjects ($P = 0.001$). For IL-6 (–597) frequency of genotype GA/AA ($P = 0.04$) and allele A ($P = 0.01$) was lower in CHPDM group, and for TNF- α –308 the frequency of genotype GA ($P = 0.01$) and allele A ($P = 0.01$) was higher in CHP subjects when compared with controls.

Conclusions: In Malayalam speaking Dravidian population, IL-6 (–597) genotype GA/AA and allele A appears to be protective for CHP with type 2 DM. Allele C of IL-1 β +3954 and allele A of TNF- α –308 appears to be risk factors for CHP individuals.

Key words: Cytokines, periodontitis, polymorphism, single nucleotide, type 2 diabetes mellitus

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Periodontal disease is a chronic multifactorial disease characterized by destruction of supporting periodontal tissues. Microbial biofilm and host susceptibility plays an important role in the initiation and progression of periodontitis. Recent studies^[1-4] have demonstrated that chronic periodontitis (CHP) is a potential risk factor for systemic diseases/conditions such as cardiovascular disease, diabetes mellitus (DM), atherosclerosis etc. Epidemiologic

survey^[5] demonstrated that there is an increased prevalence of periodontitis among patients with uncontrolled DM. Periodontal disease is recognized as the sixth complication of diabetes.^[6]

Cytokines appears to play a central role in the pathogenesis of chronic inflammatory diseases such as CHP and type 2 DM. Single nucleotide polymorphisms (SNPs) in the genes coding for pro inflammatory cytokines (interleukin-1 [IL-1], IL-6 and tumor necrosis factor-alpha [TNF- α]) are potential candidates for susceptibility to these chronic inflammatory diseases.^[7-9]

The IL-1 gene complex (IL-1 α , interleukin-1 beta [IL-1 β] and IL-1 Receptor antagonist) is clustered on chromosome 2q13-21 and are polymorphic in several loci. IL-1 β +3954 TT genotypes increased the risk for periodontitis in Chile population,^[10] whereas in Malayalam speaking Dravidian population, allele C of IL-1 β +3954 appeared to be an important risk factor for CHP.^[11] In diabetics

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with periodontal disease, allele C at IL-1 β (-511) and IL-1 β (+3954) was overrepresented among African American population.^[12,13]

SNPs of IL-6 at positions -174, -572 and -597 in the promoter region of chromosome 7p15-21 were associated with either periodontal disease^[14] or diabetes.^[15]

TNF- α gene is present on chromosome 6q21.3. The -308 A allele of the TNF- α gene may be a predictor for type 2 DM.^[16] Several studies^[17,18] proved that TNF- α polymorphisms are the factors affecting periodontal disease, whereas conflicting reports^[19,20] are also available.

Based on available evidence, it is biologically plausible that patients with diabetes who are carriers of pro-inflammatory cytokine polymorphisms may exhibit more severe periodontitis and common genetic factors may be responsible for cross-susceptibility between periodontitis and diabetes. To the best of our knowledge, only few studies^[10,12,21] have investigated the association of cytokine (IL-1 α , IL-1 β and IL-6) gene polymorphisms with CHP in type 2 diabetic patients in different population groups. So far, there are no reports on association between cytokine gene polymorphism and chronic periodontitis with type 2 diabetes mellitus (CHPDM) in any of the Indian ethnic groups including Dravidians. Hence, we undertook this study to assess the association of IL-1 β +3954, IL-6 -597/-174 and TNF- α -308 SNPs in CHP and type 2 DM in Malayalam-speaking Dravidian ethnicity.

MATERIALS AND METHODS

This case-control study was conducted from September 2009 to August 2010 in Department of Periodontics, Government Dental College, Calicut, Kerala, in collaboration with Department of General Medicine, Government Medical College, Calicut, Kerala and Human Molecular Genetics Laboratory, Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram and Kerala, India. The study protocol, prepared in accordance with the Declaration of Helsinki of 1973 (as revised in 2002), was approved by the Institutional Ethics Committee. All included subjects had signed the informed consent. The study consist 153 subjects of Malayalam-speaking Dravidian ethnicity. The case groups included a total of 102 subjects out of which 51 patients had CHPDM and 51 patients had CHP without type 2 DM (CHP). The mean age for CHPDM and CHP group was 42.24 and 36.39 years, respectively and for controls it was 37.27 years.

All study subjects were selected from a homogeneous population with similar ethnic and socio-economic status, level of education and other demographic characteristics.

The diagnostic criteria for periodontitis was based on American Academy of Periodontology criteria 1999^[22] and

clinical case definitions proposed by the Centers for Disease Control and Prevention working group for use in population based surveillance of periodontitis.^[23] DM was defined as serum glucose ≥ 126 mg/dL after fasting for a minimum of 8 h or self-reported current use of hypoglycemic medication or insulin.^[24] Patients with type 2 DM were recruited from the subjects who reported to the out-patient wing of Department of General Medicine, Government Medical College, Calicut who were then screened for CHP. CHP subjects without type 2 DM were selected from the subjects who reported to the out-patient wing of Department of Periodontics, Government Dental College, Calicut after ruling out type 2 DM at the time of recruitment into the study. Exclusion criteria were the presence of rheumatoid arthritis, obesity, acute infections, systemic antibiotic treatment or non-steroidal anti-inflammatory medication within the previous 6 months and any condition that precludes periodontal probing. The control group was selected from healthy volunteers from faculty, staff and residents of Government Dental College, Calicut.

A complete clinical examination was carried out by a single trained examiner (Nitin Sharma). A standardized periodontal probe with William's graduated markings was used. Measurements of probing depth, clinical attachment level (CAL), plaque index,^[25] calculus index (calculus component of the Simplified Oral Hygiene Index)^[26] and modified gingival index^[27] were recorded.

A peripheral blood sample (3 ml) was collected from all subjects by venipuncture and transferred to plastic falcon tubes containing ethylenediaminetetraacetic acid. A modified, standard, organic extraction method was used for deoxyribonucleic acid extraction.^[28] Genotyping was performed at positions IL-1 β +3954, IL-6 -597 and TNF- α -308 according to methods based on polymerase chain reaction and restriction enzyme digestion described elsewhere.^[11,29,30] Sequence of primers and restriction enzymes used are shown in Table 1.

We chose IL-6 position (-597) of instead of (-174) since both these sites were found to be in complete linkage disequilibrium based on extensive analysis of the control population in one of our investigators lab (personal communication from Dr. Moinak Banerjee).

Statistical analyses of results were performed using a Statistical Package for Social Sciences 17. Differences between the groups were determined using the one-way analysis of variances test followed by Bonferroni correction for continuous variables and Chi-square test for categorical variables. Hardy-Weinberg equilibrium was tested for genotype frequencies by Chi-square test with one degree of freedom. The strength of the association was determined using an odds ratio (OR) at 95% confidence intervals (CI). Statistical significance was set at $P < 0.05$.

RESULTS

The periodontal parameters of the study subjects are shown in Table 2. There were no statistically significant difference between CHPDM and CHP groups for mean plaque index ($P=0.875$), probing pocket depth (PPD) ($P=1.000$) and CAL ($P=0.907$) scores. Calculus index scores were significantly lower in CHPDM as compared with CHP group ($P<0.001$). The modified gingival index scores were significantly higher for CHPDM as compared to CHP group ($P<0.001$).

No statistically significant differences were found in the distributions of the IL-1 β +3954 genotypes TT, CT and CC between subjects in both case groups when compared with controls [Tables 3 and 4]. However, allele frequency for IL-1 β +3954 showed significant difference between subjects with CHP without type 2 DM, with higher frequency of T allele in control group ($P=0.038$) [Tables 3 and 4]. Statistically significant differences were found in the distributions of the genotypes TT, CT and CC ($P=0.01$), as well as for alleles C and T ($P=0.001$) for IL-1 β +3954 among CHPDM as compared to CHP group [Tables 3 and 4]. Frequency of allele T was higher in CHPDM group and allele C was higher in CHP subjects ($P<0.001$, OR = 4.4, 95% CI = 1.70-11.38).

For IL-6 (-597), the control group showed statistically significant higher frequency for genotype GA and AA whereas the CHP group showed higher frequency of genotype GG ($P=0.048$). Analysis of allele distribution between these groups showed significant greater frequency for allele G in the case group ($P=0.018$) [Tables 3 and 5].

For TNF- α -308, there was a slight difference in genotypes and allele distribution between CHPDM and control group. Genotype GA ($P=0.05$) as well as allele A ($P=0.05$) was higher in case group [Tables 3 and 6]. Genotype GA was found to be higher ($P=0.01$) in CHP group when compared to controls. The genotype and allele distribution for TNF- α +308 was similar in CHPDM and CHP groups.

DISCUSSION

Mounting evidence suggest that a bidirectional link exists between CHP and DM. Polymorphisms in the genes coding

for pro-inflammatory cytokines are potential candidates for susceptibility to both diabetes^[8] and periodontitis.^[7] SNP is likely to be a valuable tool to assess risk allele for a disease as they represent variation in population. The total sample size of our study was higher than many of the earlier studies.^[31-33]

In this study, the CHPDM group had more number of severe CHP subjects ($P=0.015$) as compared with CHP group. Extent and severity of periodontitis in type 2 DM subjects was more than CHP group [Table 2]. This is in accordance with a previous study^[34] showing that diabetes is a risk factor for periodontitis. The CHPDM subjects had lower calculus index score ($P=0.001$) and higher scores for modified gingival index ($P=0.000$) than CHP group. There was no statistically significant difference in plaque scores between these groups, which suggest that factors other than pathogenic microorganisms may determine the actual clinical presentation of CHP in type 2 diabetic subjects [Table 2].

We examined IL-1 β , IL-6 and TNF- α gene polymorphism as these pro-inflammatory cytokines play a central role in the pathogenesis of both CHP and type 2 DM. IL-1 α is about 15-fold less potent than IL-1 β which stresses the importance of IL-1 β in pathogenesis of periodontal disease than IL-1 α .^[35] In our study, no significant association was found for IL-1 β +3954 gene polymorphism among CHPDM and control groups [Tables 3 and 4], but the genotype TT tend to be higher in CHPDM group. The frequency of T allele was higher in CHPDM group (22%) as compared to controls (15%), but the difference was not statistically significant [Tables 3 and 4]. Similarly, no association was reported between diabetes and IL-1 β +3954 gene polymorphisms in Chile population.^[10] To the best of our knowledge, there are no published data that have investigated the role of IL-1 β +3954 polymorphism in the pathogenesis of type 2 DM. IL-1 β +3954 SNP has been associated with the risk of type 1 DM with higher frequency of T allele in Hungarian children.^[36] Similarly, genotypes TT, CT and CC for IL-1 β +3954 was not associated with CHP. However, the difference in distribution of alleles C and T was statistically significant [Tables 3 and 4]. This finding is consistent with the result of a previous study^[11] conducted in the same population. However, contrasting results are

Table 1: The primer sequences used for the study

SNP	Primers	Annealing temperature (°C)	Restriction enzymes	Allele size
IL1 β +3954 (rs1143634)	Forward 5'-GTTGTCATCAGACTTTGACC-3' Reverse 5'-TTCAGTTCATATGGACCAGA-3'	50	Taq I α	C allele 136 and 114 bp T allele 250
IL6-597 (rs1800797)	Forward 5'-CTCCTCTAAGTGGGCTGAAG-3' Reverse 5'-CAAGCCTGGGATTATGAAGA-3'	56.7	FokI	G allele 212 bp A allele 125 and 87 bp
TNF- α -308 (rs1800629)	Forward 5'-AGGCAATAGGTTTTGAGGGGCAT-3' Reverse 5'-TTGGGGACACAAGCATCAAGG-3'	61	Noc I	G allele 147 bp A allele 127 and 20 bp

SNP=Single nucleotide polymorphism, IL=Interleukin, TNF- α =Tumor necrosis factor-alpha

also available in the literature questioning the exact role of +3954 IL-1 β gene polymorphism in periodontal disease pathogenesis.^[31,37]

The analysis of IL-1 β +3954 genotype and allele distribution in CHP with and without type 2 DM subjects revealed few exciting relationships. The IL-1 β (+3954) TT genotype was significantly associated with CHPDM group, whereas the IL-1 β (+3954) CC genotype was higher in CHP group ($P < 0.01$). A similar statistically significant pattern was observed in the distribution of allele frequencies. T allele was more in CHPDM group ($P < 0.001$, OR = 4.4, 95% CI = 1.70-11.38) [Tables 3 and 4]. This implies that the protective effect of T allele observed in the control group has been masked in CHPDM subjects.

A probable explanation could be that IL-1 β (+3954) polymorphisms are in linkage disequilibrium with other unidentified polymorphisms that are more frequent in subjects with diabetes. The protective effect of the TT genotype and allele on periodontitis could have been neutralized in combination with other polymorphisms present in subjects with diabetes.

In this study we found statistically significant association between IL-6 -597 gene polymorphism and CHPDM group [Table 3 and 4]. IL-6 (-597), the genotype GA/AA and allele A was significantly higher in controls as compared with CHPDM subjects [Table 5]. IL-6 -597 gene polymorphism was found to be protective. This is in contrast to a previous study conducted in Chinese population where no statistically significant difference was observed for SNP IL-6 (-597) in CHPDM subjects.^[21] However in Caucasian Danes, IL-6 -174 G/C and -597 G/A

promoter polymorphisms showed associations with type 2 diabetes ($P < 0.002$).^[38]

IL-6 (-597) gene polymorphism was not significantly associated with non-diabetic CHP. However, the G allele frequency of IL-6 (-597) in CHP group was tend to be higher than that in the controls, but there was no statistically significant difference [Tables 3 and 5]. In accordance with our result, no association was reported between CHP and control groups for IL-6 -597 G/A and -174 G/C polymorphisms in Czech patients.^[14] Their findings suggested that presence of specific promoter haplotype could increase or reduce the risk of developing CHP.

Several studies^[39,40] found an association between IL-6 gene polymorphism at position -174 and periodontal disease and their reports are conflicting that which genotype (GG or GG/CG) is involved in the risk of periodontal damage. A recent meta-analysis^[41] indicated that the IL-6 -174 G allele could not modify the risk of CHP whereas our results indicate that G allele of IL-6 (-597) could be associated with CHP. In our study population, both CHPDM and CHP groups had similar genotype, as well as allele distribution for IL-6 -597 SNP. This is in consistent with a previous study^[21] conducted in Chinese population. This may support the hypothesis that a common genetic factor might be involved in the pathogenesis of CHP and type 2 DM to some extent.

There was a slight difference in the distributions of genotypes and alleles for TNF- α -308 among CHPDM and control group [Table 3], with higher genotype GA and allele A in the case group. To the best of our knowledge, there is no study in the literature investigating the frequency of TNF- α -308 polymorphism in CHPDM.

Similarly genotype GA and allele A was significantly associated with CHP alone [Tables 3 and 6]. This is consistent with the previous study,^[31] in which they found an association between TNF- α -308 G > A polymorphism and advanced CHP, whereas other researchers^[19,42] could not confirm a link between TNF- α polymorphism and periodontitis.

Serum TNF- α is elevated in patients with type 2 DM and result in inhibition of insulin activity.^[43] TNF- α also inhibits insulin secretion through an action on beta cells. The role of TNF- α in periodontal pathogenesis has been

Table 2: Comparison of periodontal parameters between the groups (mean $\pm$ SD)

Periodontal parameters	Group (n=51)		
	CHPDM	CHP	Controls
Plaque index [□]	1.30 $\pm$ 0.45	1.39 $\pm$ 0.44	0.34 $\pm$ 0.24
Calculus index-simplified [□]	1.31 $\pm$ 0.64 [†]	1.72 $\pm$ 0.52 [†]	0.16 $\pm$ 0.36
Modified gingival index [□]	1.6 $\pm$ 0.36 [‡]	1.24 $\pm$ 0.35 [‡]	0.4 $\pm$ 0.12
PPD (mm) [□]	2.8 $\pm$ 0.35	2.83 $\pm$ 0.58	2.1 $\pm$ 2.9
CAL (mm) [□]	3.33 $\pm$ 0.51	3.16 $\pm$ 0.95	0.97 $\pm$ 0.98

[□]One-way ANOVA test ($P < 0.001$), Calculated after *post hoc* adjustment,

[†] $P < 0.001$ for calculus index-simplified, [‡] $P < 0.001$ for modified gingival index.

CHPDM=Chronic periodontitis with type 2 diabetes mellitus, CHP=Chronic periodontitis, SD=Standard deviation, PPD=Probing pocket depth, CAL=Clinical attachment level

Table 3: Intergroup analysis of IL-1 β +3954, IL-6-597 and TNF- α -308 gene polymorphisms between cases and controls

Group comparison	P value*					
	IL-1 β +3954		IL-6-597		TNF- α -308	
	Genotype	Allele	Genotype	Allele	Genotype	Allele
CHPDM versus control	0.36	0.203	0.048	0.018	0.05	0.054
CHP versus control	0.11	0.038	0.12	0.077	0.014	0.017
CHPDM versus CHP	0.01	0.001	0.5	0.535	0.5	0.579

* $P < 0.05$ (Chi-square test), IL=Interleukin, TNF- α =Tumor necrosis factor-alpha, CHPDM=Chronic periodontitis with type 2 diabetes mellitus, CHP=Chronic periodontitis

Table 4: Genotype and allele frequencies of IL-1 β +3954

Group	Genotype n (%)			Allele n (%)	
	CC	CT	TT	C	T
CHPDM	33 (64.71)	14 (27.45)	4 (7.84)	80 (78.43)	22 (21.57)
CHP	45 (88.24)	6 (11.76)	0 (0.00)	96 (94.12)	6 (5.88)
Control	37 (72.55)	13 (25.49)	1 (1.96)	87 (85.29)	15 (14.71)

IL=Interleukin, CHPDM=Chronic periodontitis with type 2 diabetes mellitus, CHP=Chronic periodontitis, CC=Cytosine cytosine, CT=Cytosine thymine, TT=Thymine thymine

Table 5: Genotype and allele frequencies of IL-6-597

Group	Genotype n (%)			Allele n (%)	
	GG	GA	AA	G	A
CHPDM	39 (76.47)	12 (23.53)	0 (0.00)	90 (88.24)	12 (11.76)
CHP	36 (70.59)	15 (29.41)	0 (0.00)	87 (85.29)	15 (14.71)
Control	29 (56.86)	19 (37.25)	3 (5.88)	77 (75.49)	25 (24.51)

IL=Interleukin, CHPDM=Chronic periodontitis with type 2 diabetes mellitus, CHP=Chronic periodontitis, GG=Guanine guanine, GA=Guanine adenine, AA=Adenine adenine

Table 6: Genotype and allele frequencies of TNF- α -308

Group	Genotype n (%)			Allele n (%)	
	GG	GA	AA	G	A
CHPDM	45 (88.24)	6 (11.76)	0 (0.00)	96 (94.12)	6 (5.88)
CHP	43 (84.31)	8 (15.69)	0 (0.00)	94 (92.16)	8 (7.84)
Control	50 (98.04)	1 (1.96)	0 (0.00)	101 (99.02)	1 (0.98)

TNF- α =Tumor necrosis factor-alpha, CHPDM=Chronic periodontitis with type 2 diabetes mellitus, CHP=Chronic periodontitis, GG=Guanine guanine, GA=Guanine adenine, AA=Adenine adenine

extensively reviewed and local overproduction of TNF- α by the innate host defenses is a major contributor to alveolar bone destruction. Evidence from animal study^[44] indicated that persistent TNF- α expression in the diabetes state is associated with prolonged inflammation in response to bacterial infection.

To some extent, similar distribution of genotype and allele frequencies of IL-6 (-597/-174) and TNF- α (-308) in both CHP with and without type 2 DM subjects, may support the notion that a common genetic factor might be involved in the cross susceptibility of these chronic inflammatory diseases. To confirm these cross susceptibility through genetic factors, assessment of level of pro inflammatory cytokines like IL-1 β , IL-6 and TNF- α could have strengthened the evidences. Further studies with large sample size evaluating the entire cytokine gene cluster are required to further confirm the cross susceptibility of these chronic inflammatory diseases.

CONCLUSION

In Malayalam speaking Dravidian population, for IL-6 (-597), the genotype GA/AA and allele A appeared to be protective for CHPDM. For IL-1 β +3954, allele T may be overrepresented in CHPDM and allele C showed an association with CHP independent of type 2 DM. Also the allele A of TNF- α -308 appeared to be important risk factor for CHP with and without type 2 DM individuals. IL-6 -597/-174 and TNF- α -308 showed an overlapping

association in CHP with and without type 2DM. The function of these unique distributions needs further research with large sample size. This study opens further avenues in investigating the role of these pro inflammatory cytokines in independent groups of type 2 DM and CHP and contributes to the understanding of genetic differences in type 2 DM and CHP patients.

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