Research Article

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Cyclooxygenase 2 gene polymorphisms and chronic periodontitis in a North Indian population: a pilot study

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Purpose: Cyclooxygenase (COX) enzyme catalyzes the production of prostaglandins, which are important mediators of tissue destruction in periodontitis. Single nucleotide polymorphisms of COX2 enzyme have been associated with increasing susceptibility to inflammatory diseases. The present study evaluates the association of two single nucleotide polymorphisms in *COX2* gene (-1195G>A and 8473C>T) with chronic periodontitis in North Indians.
Methods: Both SNPs and their haplotypes were used to explore the associations between COX2 polymorphisms and chronic periodontitis in 56 patients and 60 controls. Genotyping was done by polymerase chain reaction followed by restriction fragment length polymorphism. Chi-square test and logistic regression analysis were performed for association analysis.
Results: By the individual genotype analysis, mutant genotypes (GA and AA) of COX2 -1195 showed more than a two fold risk (odds ratio [OR]>2) and COX2 8473 (TC and CC) showed a reduced risk for the disease, but the findings were not statistically significant. Haplotype analysis showed that the frequency of the haplotype AT was higher in the case group and a significant association was found for haplotype AT (OR, 1.79; 95% confidence interval, 1.03 to 3.11; P=0.0370) indicating an association between the AT haplotype of *COX2* gene SNPs and chronic periodontitis.
Conclusions: Individual genotypes of both the SNPs were not associated while haplotype AT was found to be associated with chronic periodontitis in North Indians.

Keywords: Chronic periodontitis, Cyclooxygenase 2, Single nucleotide polymorphism.

INTRODUCTION

Chronic periodontitis is an inflammatory disease of the tooth supporting tissues and the alveolar bone. While presence of pathogenic bacteria is essential for the initiation of this disease, environmental and genetic factors are instrumental in modifying the disease process [1]. Human genetic variants that occur most frequently in the form of single nucleotide polymorphisms (SNPs) contribute to individual variations in susceptibility to chronic periodontitis and its severity [2]. SNPs in the genes of certain inflammatory mediators and enzymes have shown strong association with chronic periodontitis in previous studies [3-10].

Cyclooxygenase (COX), also known as prostaglandin endoperoxide synthetase (PTGS) converts arachidonic acid to prostaglandins (PGs) and plays a pivotal role in the inflammatory process [11]. The two isoforms of COX designated as COX1 and COX2 are encoded on distinct genes but essentially catalyse the same reaction. COX1 is constitutively expressed in many tissues and COX2 is inducible in inflammatory con-

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ditions including periodontitis. Release of proinflammatory cytokines (interleukin [IL]-1, IL-6, tumor necrosis factor-α), growth factors, and lipopolysaccharides in periodontitis activate a variety of cells of periodontal tissues to produce COX2-mediated prostaglandin E_2 (PGE_2) [12-14]. COX2 expression and the abundance of its enzymatic product PGE_2 play key roles in influencing tissue destruction in periodontitis [15,16]. Currently, there are very few reports on the role of *COX2* gene polymorphism in chronic periodontitis [13,17,18]. The present study aimed to evaluate the association of two common SNPs, -1195G>A (rs689466) and 8473T>C (rs5275) in the *COX2* gene, with chronic periodontitis in a North Indian population as the *COX2* gene polymorphism might play a role in modifying the risk of the disease.

MATERIALS AND METHODS

Selection of subjects and study design

One hundred sixteen subjects were recruited from the outpatient wing of the Department of Periodontics, Faculty of Dental Sciences, C.S.M.M.U, Lucknow, Uttar Pradesh, India for the present case-control study. All the subjects who participated in the study were from North Indian subpopulations, belonging to the state of Uttar Pradesh. Written informed consent was obtained from each subject as per human ethics guidelines. The study protocol was reviewed and approved by the institution's ethical review board (CDRI/IEC/CEM/07-07-10). The medical and dental histories of the subjects were recorded by means of a questionnaire.

A complete periodontal examination was carried out by a single examiner using a UNC-15 probe. Clinical periodontal parameters including probing pocket depth, clinical attachment level, plaque index [19], calculus index [20], and gingival index [21] were recorded. The diagnosis of the subjects was established based on the clinical criteria proposed by the 1999 International Workshop for Classification of Periodontal Diseases and Conditions [22]. A radiographic analysis was performed using full mouth intraoral periapical radiographs to assess the bone level and confirm the clinical diagnosis. The subjects were categorized into two groups: a generalized moderate to severe chronic periodontitis group (n=56), which consisted of subjects exhibiting clinical attachment loss ≥3 mm at >30% of the sites; and the control group (n=60), which consisted of subjects showing no signs of periodontitis as determined by absence of clinical attachment loss and no sites with a probing pocket depth ≥3 mm. Exclusion criteria included the following: systemic diseases like diabetes, hepatitis, immunosuppressive disorders, and bleeding disorders; history of any disease known to severely compromise immune function; current pregnancy or lactation; smoking tobacco or using smokeless tobacco; and currently taking anti-inflammatory drugs or having taken them in the previous 3 months.

Blood sample collection and DNA isolation

Two mL blood was collected from each subject in ethylenediaminetetraacetic acid tubes by venipuncture at the antecubital fossa. The tubes were maintained in ice and transported to the Genotoxicity Laboratory, CSIR-Central Drug Research Institute for isolation of genomic DNA and SNP analysis. DNA was isolated from the blood samples using a kit (Gene Elute Blood Genomic DNA kit, NA2010, Sigma-Aldrich Co., St. Louis, MO, USA) following the manufacturer's protocol, quantified using a spectrophotometer (Genequant, Biochrom Ltd., Cambridge, UK) and stored at -20°C until further processing.

Selection of SNPs

Two SNPs, rs689466, G>A (chromosome 1, promoter region) and rs5275, T>C (chromosome 1, 3'UTR), in the prostaglandin endoperoxidase synthetase 2 (*PTGS2* or *COX2*) gene were selected for the study based on the available literature.

Polymerase chain reaction (PCR)

A gradient PCR reaction was performed initially for standardization of DNA amplification conditions and optimization of the annealing temperature for the primer sets. Subsequently, PCR reactions were performed using the optimized annealing temperatures. All PCR reactions were performed in 200 μL transparent PCR tubes on a Peltier-based thermal cycler (MJ Research PTC-100 Thermal Cycler, Watertown, MA, USA) using reagents from Fermentas Life Sciences (Fermentas Life Sciences, Burlington, ON, Canada) in a total reaction volume of 20 μL containing nearly 100 ng genomic DNA, 1.5 U Taq polymerase in 1× PCR buffer, 1.5 mM $MgCl_2$, 10 pmol of each primer and 10 mM dNTPs. The primers used are shown in Table 1 [23,24]. Thermal cycling conditions were as follows: initial denaturation step at 95°C for 7 minutes, 31 cycles of PCR consisting of denaturation at 94°C for 30 seconds, annealing at 61°C and 49°C (COX2 -1195 and COX2 8473, respectively) for 30 seconds and extension at 72°C for 30 seconds, followed by a final extension step at 72°C for 7 minutes. The reaction was held at 4°C. The PCR products were visualized by electrophoresis on 1.2% agarose gel. For gel electrophoresis, 5 μL of the amplified product was mixed with 1 μL of 6× gel loading buffer (analytical grade water containing 30% glycerol, 0.25% bromophenol blue, 0.25% xylene cynole) and resolved on 1.2% agarose gel in TAE buffer at 80 volts for 2 hours. 50 bp DNA markers (Gene Ruler, SM0371, Fermentas Inc., a subsidiary of Thermo Fisher Scientific Inc., Rockford,

Table 1. Polymerase chain reaction primers, restriction enzymes and lengths of fragments generated upon restriction digestion.

SNPs	Primers	Restriction enzyme	Allele	Fragment size (bp)	Reference
COX2 -1195GA	FP: 5'CCCTGAGCACTACCCATGAT-3'	*PvuII*	G	220+53	Zhang et al. [23] (2005)
	RP:5'-GCCCTTCATAGGAGATACTGG-3'		A	273	
COX2 8473TC	FP:5'GAAATTTTAAAGTACTTTTGAT-3'	*BclI*	T	177	Sanak et al. [24] (2005)
	RP:5'-CTTTTACAGGTGATTCTACCC-3'		C	156+21	

SNPs: single nucleotide polymorphisms, FP: forward primer, RP: reverse primer.

IL, USA) were run with the amplified products as a reference. PCR products were assessed based upon the presence of a 273 base pair (bp) product for COX2 -1195 and a 177 bp product for COX2 8473.

Restriction fragment length polymorphism

COX2 -1195G>A

The 10 μL PCR product was mixed with 2 μL buffer and was digested with 0.5 μL of *PvuII* enzyme. The reaction mixture was incubated at 37°C overnight in a water bath. The resulting digestion products of 220+53 bp (allele G) and 273 bp (allele A) were visualized using 2% agarose gel electrophoresis with ethidium bromide staining (Table 1).

COX2 8473T>C

The 10 μL PCR product was digested with 0.5 μL of *BclII* enzyme. The reaction mixture was incubated at 50°C overnight in a water bath. The resulting digestion products of 177 bp (T allele) and 156+21 bp (C allele) were visualized using 2.5% agarose gel electrophoresis with ethidium bromide staining (Table 1).

Statistical analysis

For the present study, statistical analysis of data was performed using the SPSS ver. 16.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism ver. 5 (GraphPad Software Inc., La Jolla, CA, USA). Hardy-Weinberg equilibrium was tested to compare the observed and expected genotype frequencies in the control group. The distribution of genotype and allele frequency in the chronic periodontitis and control groups were compared using a standard chi-squared test. The risks associated with individual genotypes or alleles were calculated as odds ratios (ORs) with a 95% CI. Age and gender distribution between the diseased and control groups were compared by an independent *t*-test and chi-squared test, respectively. SHEsis software was used for the linkage disequilibrium (LD) index (D' and r2) and haplotype analysis between genetic markers [25,26]. Haplotypes were constructed in the following order: COX2-1195GA/COX2 8473TC. All tests used for the analysis were two sided and a probability value <0.05 was considered statistically significant.

Table 2. Characteristics of control and chronic periodontitis groups.

Characteristic	Controls (n=60)	ChP (n=56)	*P*-value
Age (year)[a]	41.35±11.686	43.30±11.818	0.373
Gender[b]			
Female	24 (40)	26 (46.4)	0.4848
Male	36 (60)	30 (53.6)	

Values are presented as mean±SD or number (%).
ChP: chronic periodontitis group.
[a]Differences between groups was analyzed by an independent *t*-test.
[b]Differences between groups were analyzed by chi-square test ($P<0.5$).

RESULTS

The mean age (±SD) was 43.30 (±11.8) years for chronic periodontitis patients and 41.35 (±11.68) years for control subjects ($P=0.373$). No statistically significant differences were present in age or gender distribution between the study groups (Table 2). Genotype and allelic distribution of COX2 -1195 and COX2 8473 polymorphisms in the control and chronic periodontitis groups are shown in Table 3. In case of COX2 -1195 SNP, distribution of mutant allele A and genotypes containing allele A (GA+AA) were higher in the chronic periodontitis group (58% and 82.1%, respectively) than in the control group (45.8% and 66.7%). The results show that an OR of more than two was found for the mutant genotypes when compared with the wild type (GA/GG: OR, 2.16; $P=0.106$ and AA/GG: OR, 2.53; $P=0.073$). For the COX2 8473 SNP, the prevalence of C (mutant) allele carriers was higher in the controls (35%) than in the diseased subjects (26.8%). When the genotypes were compared for association, an OR of less than one was found for mutant genotypes (TC/TT: OR, 0.617; $P=0.247$ and CC/TT: OR, 0.59; $P=0.349$); however, neither of these two SNPs was found to be significantly associated with chronic periodontitis ($P>0.05$) prior to or after adjustment for age and gender.

Haplotype analysis

To evaluate the extent of LD, D′ and r2 between pairs of polymorphisms were calculated. It was found that neither of the SNPs were in LD (D'-0.134 and r^2-0.008). When both the SNPs were assessed for haplotype analysis, four potential

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Table 3. Distribution of genotype and allele of *COX2* gene polymorphism in control and chronic periodontitis groups.

SNP	Controls	Chronic periodontitis	*P*-value	OR (95% CI, *P*-value)	OR[a] (95% CI, *P*-value)
COX2 -1195G>A					
Genotypes	(n=60)	(n=56)			
GG (WW)	20 (33.3)	10 (17.9)		1	1
GA (WM)	25 (41.7)	27 (48.2)	0.1535	2.16 (0.85-5.50, 0.106)	2.05 (0.79-5.3, 0.139)
AA (MM)	15 (25.0)	19 (33.9)		2.53 (0.92-7.00, 0.073)	2.39 (0.83-6.90, 0.105)
GA (WM)+AA (MM)				2.3 (0.96-5.49, 0.060)	2.173 (0.89-5.32, 0.090)
Alleles	(2n=120)	(2n=112)			
G (W)	65 (54.2)	47 (42.0)			
A (M)	55 (45.8)	65 (58.0)	0.0630	1.63 (0.97-2.75)	
COX2 8473T>C					
Genotypes	(n=60)	(n=56)			
TT (WW)	28 (46.7)	33 (58.9)		1	1
TC (MM)	22 (36.7)	16 (28.6)	0.4166	0.617 (0.27-1.39, 0.247)	0.601 (0.26-1.39, 0.233)
CC (MM)	10 (16.6)	7 (12.5)		0.59 (0.20-1.76, 0.349)	0.587 (0.193-1.785, 0.348)
TC (WM)+CC (MM)				0.61 (0.29-1.27, 0.187)	0.597 (0.280-1.271, 0.181)
Alleles	(2n=120)	(2n=112)			
T (W)	78 (65)	82 (73.2)	0.1766	0.68 (0.39-1.91)	
C (M)	42 (35)	30 (26.8)			

Values are presented as number (%).
P<0.05 is considered to be significant.
SNPs: single nucleotide polymorphisms, OR: odds ratio, CI: confidence interval, W: wild, M: mutant.
[a]Age and gender adjusted odds ratio.

Table 4. Distribution of COX2 haplotype frequencies and odds ratios among chronic periodontitis and control groups.

Haplotype	Controls (2n=120)	Chronic periodontitis (2n=112)	OR (95% CI)	*P*-value
AC	0.1855 (22)	0.1782 (20)	0.9518 (0.4881-1.8562)	0.8848
AT	0.2728 (33)	0.4022 (45)	1.7933 (1.0332-3.1125)	0.0370
GC	0.1645 (20)	0.0897 (10)	0.5004 (0.2230-1.1228)	0.0888
GT	0.3772 (45)	0.3300 (37)	0.8131 (0.4739-1.3951)	0.4524

Haplotypes were constructed in the following order: COX2 -1195GA/COX2 8473TC using SHEsis software.
OR: odds ratio, CI: confidence interval.

haplotypes were formed, as presented in Table 4. There was a predominance of haplotype 'AT' in the chronic periodontitis group and haplotype 'GT' in the control group. Haplotype 'AT' was at significantly higher risk for chronic periodontitis (OR, 1.79; 95% CI, 1.03 to 3.11; *P*=0.0370), indicating that the A allele at the -1195 locus increases the risk.

DISCUSSION

In inflamed periodontal tissues, activity of proinflammatory molecules leads to the production of COX2 mediated PGE_2, which plays an important role in tissue destruction and bone resorption. COX2 is the crucial enzyme involved in conversion of arachidonic acid to PGs. COX2 dependent PGE_2 acts on osteoblasts to increase the expression of receptor activation of NF-*k*B ligand on their surfaces and thus enhances osteoclastogenesis [27]. Polymorphisms within the *COX2* gene therefore influence the inflammatory disease processes as suggested by previous studies [24,28]. This report explored the probable association of two SNPs in the *COX2* gene with chronic periodontitis in North Indians.

All of the subjects inducted into this study were recruited strictly from the North Indian subpopulation of Uttar Pradesh to maintain homogeneity within the ethnic group constituting mostly the Caucasian morphological subtype of the Indo-European linguistic group [29]. Smokers and smokeless tobacco users were excluded from the study as tobacco is a major risk factor in the development of periodontal diseases and previous studies have found that genetic association of chronic periodontitis was more evident when smokers were excluded [4-6, 30].

The human *COX2* gene mapped to chromosome 1q25.2-q25.3 is about 8.3 kbp in size and consists of 10 exons [31]. Few SNPs in this gene have been studied for their effect in altering its expression or function although many SNPs have been identified. This study deals with COX2 -1195G>A, located in the promoter region and 8473T>C, located in the 3'UTR region. No association was found between COX2 -1195G>A

and chronic periodontitis (Table 3), even after adjustment of age and gender, indicating that age and gender were not additive to the risk of this SNP. Similar results were observed by Schaefer et al. [17] in Europeans. However, in a Chinese population, allele A was significantly associated with the risk of chronic periodontitis (OR, 1.46) [9]. A similar trend with a higher frequency of allele A in a chronic periodontitis group (58%) than in a control group (45.8%) was observed in the present study, nearing statistical significance (OR, 1.62; P= 0.0630).

The exact mechanism by which the variant alleles affect the *COX2* gene function remains unclear. However, a few possible mechanisms have been suggested in previous studies. As 1195G>A is in the 5' flanking region of the *COX2* gene and many putative transcription factor binding sites exist in the promoter region of the *COX2* gene [32], it is likely that variant alleles could influence the gene function by interfering with the specific binding between the transcription factors and the promoter sequences. A previous report indicated that the A allele of SNP COX2 1195 resulted in a heightened gene expression and thus an increased enzymatic activity conferring an increased disease susceptibility to the host [33]. The same may be true in the present study as an increased prevalence of the A allele was observed in the chronic periodontitis group.

An association could not be observed for the allelic and genotypic distribution of COX2 8473T>C with chronic periodontitis in the current study (Table 3). A higher carriage of allele C was observed in the control group (35%) than in the chronic periodontitis group (26.8%), similar to the findings of Xie et al. [9] in a Chinese population where the 8473C allele was associated with a decreased risk for chronic periodontitis. The possible explanatory mechanism is that adenine-uracil rich motifs are present in the mRNA of the 3'UTR region of the *COX2* gene, which are involved in regulation of COX2 production by acting as a message instability determinant and a translation inhibitory element [34,35]. The 8473T>C polymorphism changes these motifs to cause degradation of COX2 transcripts and thus a differential COX2 expression.

Haplotype analysis is thought to be more potent than analysing individual SNPs for detecting associations between genotype and phenotype. A haplotype model is better at detecting LD than are individual SNP markers [36,37]. Statistically, low D' and r^2 values predict that neither of the SNPs were in LD and the two loci tend to be inherited in a random manner for the present endeavour. Further haplotype analysis revealed that the 1195A/8473T haplotype was significantly associated with increased risk (OR, 1.79) for chronic periodontitis (Table 4), while the -1195G/8473C haplotype showed a reduced risk (OR, 0.501). These findings are consistent with a report from a Chinese population [9] where the AT haplotype was a strong predictor for chronic periodontitis.

The present study adds to the understanding of the role of genetic variants in the development of chronic periodontitis. New insights into the COX2 genetic polymorphism provide an altered paradigm of periodontal disease that emphasizes the variation of the host response. The small sample size is a limitation of the current study but randomness needed to be taken into account. In order to determine the exact direct involvement of the *COX2* gene in periodontitis, studies that simultaneously examine the distribution and dynamics of genetic variants at many loci on the *COX2* gene are warranted. Studies evaluating local and circulating levels of COX2 mRNA and protein could be undertaken to further support the findings.

The results of this pilot study suggest that SNPs COX2 -1195G>A and COX2 8473T>C are not individually associated with chronic periodontitis; however, haplotype AT significantly increased the risk of chronic periodontitis in a North Indian population. Further studies with a larger sample size and more elaborate study designs are needed for validation of the above findings.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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