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Common single nucleotide polymorphisms in cyclooxygenase-2 and risk of severe chronic periodontitis in a Chinese population

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Abstract

Aim: Several common single nucleotide polymorphisms (SNPs) of the cyclooxygenase-2 (COX-2) gene have been reported to be functional. The association between −1195GA, −765GC and 8473TC of COX-2, and severe chronic periodontitis (CP) in a Chinese population was investigated.

Material and Methods: 148 cases of healthy controls (control group) and 146 cases of severe CP were recruited in this study. Genotypes of −1195GA, −765GC and 8473TC were determined by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP). The distributions of genotypes and haplotypes were compared by χ^2 test and the odds ratios (ORs) were calculated by logistic regression analysis.

Results: The prevalence of the −1195A was more prevalent in CP group (60.62%) than control group (51.35%), and the distributions of the −765C and 8473C were higher in control group (6.76% and 21.96%) compared with CP group (3.08% and 15.07%). Only genotype distribution of −1195GA was significant when p-value was corrected for multiple testing ($p_c = 0.033$). The adjusted ORs for the −1195AA/GA, −765GC and 8473CC/TC were 2.49 (95% CI = 1.33–4.69, $p = 0.005$), 0.45 (95% CI = 0.20–1.04, $p = 0.061$) and 0.67 (95% CI = 0.41–1.11, $p = 0.118$). Subjects with the haplotype AGT had a significantly higher risk of periodontitis than those with the most common haplotype GGT (OR = 1.91, 95% CI = 1.32–2.76, $p_c < 0.001$).

Conclusions: It suggests the −1195A variant is associated with an increased risk for severe CP.

Key words: Chinese; COX-2; genotype; haplotype; polymorphism; risk of periodontitis

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Chronic periodontitis (CP) is a multifactorial disease in which dental plaque is the initial factor; however, accumulating studies show a strong relationship between the single nucleotide polymorphisms (SNPs) of certain cytokines and the susceptibility to periodontitis (Yoshie et al. 2007).

Cyclooxygenase (COX) is a key enzyme to convert arachidonic acid to prostaglandins (PGs), and it plays a very important role in the progress of inflammation and carcinogenesis (Noguchi & Ishikawa 2007). It has at least two subtypes: COX-1 and COX-2. COX-1 is constitutively expressed in most cell types, while COX-2 is an induced isoform, which is higher expressed in pathological conditions. Elevation of the COX-2 expression is reported in periodontitis (Lohinai et al. 2001, Cai et al. 2008) and COX-2 mediated PGs synthesis is associated with the bone resorption in periodontal disease, which can be reduced by the selective COX-2 inhibitor

Conflict of interest and source of funding statement

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medicine (Vardar et al. 2003, Yen et al. 2008).

Several common SNPs of the COX-2 gene have been found to be related to breast cancer (Gao et al. 2007), cardiovascular disease (Orbe et al. 2006), diabetes mellitus (Konheim & Wolford 2003), asthma (Sanak et al. 2005), esophageal adenocarcinoma (Ferguson et al. 2008), lung cancer (Park et al. 2006) and so on. Few studies are known about a role of the COX-2 gene polymorphisms in periodontitis, except for the −765C variant which is considered to be a protective factor for periodontitis in a recent report (Ho et al. 2008)

The present study was motivated by the hypothesis that the gene variant of COX-2 may modify the individual susceptibility to periodontitis. Three common SNPs, −1195GA (rs689466), −765GC (rs20417), and 8473TC (rs5275), were identified and a case-control analysis was conducted to investigate more about the relationship between the SNPs and severe CP.

Material and Methods

Subjects

Periodontitis patients and healthy controls were selected from the periodontal department of Guangdong Provincial Stomatology Hospital. The subjects recruited were age and gender frequency matched with a total of 146 patients and 148 controls.

Clinical periodontal parameters of the six index teeth, including PD (probe depth), AL (attachment loss), PL (plaque index) and BI (bleeding index) were recorded at six sites for each tooth. The diagnoses were made on the basis of the classification defined by the American Academy of Periodontology (1999). In general, the patients with severe CP presented with a mean AL more than 5 mm and at least two molars with PD more than 6 mm; individuals with PD less than 3 mm or no obvious AL were defined as healthy controls. All the subjects were of Han ethnicity, with patients older than 35 years and healthy controls older than 40 years.

The excluded criteria included those risk factors that may influence the periodontal conditions, for instance, diabetes mellitus, cardiovascular disease, women with pregnancy and antibiotic medicine taken in previous 3 months. Those personal data were obtained via a questionnaire interviewed by a trained interviewer, including gender, age, education background, medical welfare, smoking status, tooth-brushing habit and so on. Smokers were defined as having over 100 cigarettes in lifetime according to the epidemiological criteria (Hu et al. 2005).

This study was approved by the Ethic Committee of Southern Medical University and an informed consent was obtained from each individual.

Sampling and DNA extraction

Buccal swab was obtained from each subject. DNA was extracted by chelex-100 (Sigma-Aldrich Corporation, St. Louis, Missouri, USA) as previously described (Duan et al. 2001). The DNA concentration was determined by ultraviolet (UV) spectrophotometry. The final preparation was stored at −20°C until further analysis.

Polymerase chain reaction

Genotyping was performed by the polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) method. The primers were as follows: F:5′-ccctgagcactacccatgat-3′, R:5′-gcccttcataggagatactgg-3′ for −1195GA (Zhang et al. 2005); F:5′-ccgcttcctttgtccatcag-3′, R:5′ggctgtatatct gctctatatgc-3′ for −765GC (Orbe et al. 2006); F:5′-gaaattttaaagtacttttgat-3′; R:5′-cttttacaggtgattctaccc-3′ for 8473TC (Sanak et al. 2005). All the primer sequences could be referred to the human COX-2 gene sequence (GeneBank accession no. D28235.1). PCR was carried out in a total of 25 μl reaction mixture, containing 100 ng genomic DNA, 10 mM Tris–HCL (pH 8.3), 50 mM KCL, 1.5 mM $MgCL_2$, 0.2 mM dNTPs, 0.4 μM of each primer and 1.0 U Taq DNA polymerase (TaKaRa, Dalian, China). The PCR cycling conditions for the −1195GA and −765GC were as follows: 5 min at 95°C followed by 32 cycles of 30 s at 94°C, 30 s at 58°C, and 40 s at 72°C, with a final extension of 72°C for 6 min. The PCR procedure for the 8473TC was as follows: 94°C for 5 min, 32 cycles of 30 s at 94°C, 30 s at 46°C, and 20 s at 72°C followed by a final extension of 72°C for 6 min. All the PCR procedures were carried with a negative control. A 273 bp PCR product for the −1195GA, a 306 bp PCR product for the −765GC and a 177 bp PCR product for the 8473TC were proven by the 1.5% agarose gel electrophoresis.

Restriction endonuclease cleavage

A total of 8 μl of the PCR product was restricted by PvuII (for −1195GA), AciI (for −765GC) and BclI (for 8473TC) according to the manufacturer instructions (New England Biolabs Ltd., Beijing, China), respectively. The reaction took place at 37°C (for −1195GA and −765GC) and 50°C (for 8473TC) overnight with a negative control.

PvuII could cut the wild allele G at the position −1195, and the fragment presence of 220+53 bp indicated a GG homozygosity while the smallest fragment of 53 bp could not be recognized. A fragment presence of 273+220 bp showed a GA heterozygosity. For −765GC, a 306 bp fragment could be cut into 188+118 bp fragments by AciI when the allele was G, and it indicated a GG homozygosity whereas the presence of all the three bands indicated a GC heterozygosity. The uncut fragment of 177 bp for 8473TC showed a TT homozygosity, and it could present two fragments of 177+156 bp when it was a TC heterozygosity.

Gel electrophoresis

The digested fragments were electrophoretically separated on the 3% agarose gel (Spain) containing gold-view (Dingguo, Beijing, China) and observed under the UV light.

Statistical analyses

All the analyses were performed with Statistical Analysis System software (V.13.0; SAS Institute, SPSS Inc., CH, USA). Hardy–Weinberg equilibrium was tested to compare the observed and expected genotype frequencies in control groups. The demography characters and genotype frequencies were compared with *t*-test (for continuous variables) and χ^2 test (for categorical variables). The linkage disequilibria (LD) and the haplotype frequencies were calculated with SHEsis (available online http://www.analysis.-bio-x.cn/myAnalysis.php, Shanghai, China) and PHASE 2.0 (Seattle, WA, USA) software. Bonferonni correction was used for multiple testing and the statistical power was calculated with QUANTO 1.2 (Los Angeles, CA, USA) software. The risk of genotypes with CP were estimated by computing the odds ratios (ORs) and 95% CIs from both univariate and multivariate logistic

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regression analysis, adjusting for age, gender, education background, insurance, smoking status and tooth-brushing habit. The criterion for significance was set at $p < 0.05$.

Results

The general characters of the subjects were shown in Table 1. The gender and age distributions were similar between the two groups. There was a significant difference ($p = 0.023$) in smoking status. Education years and insurance had no significant effects on the disease ($p > 0.05$). Tooth-brushing habit was found significantly different between the two groups ($p = 0.024$).

The SNPs distributions and the allele frequencies were shown in Table 2. The genotype distributions in control groups all demonstrated Hardy–Weinberg equilibrium (data not shown). Our sample size provided the power of 0.92, 0.73 and 0.80 to detect an effect size of OR 2.5 for the −1195GA, −765GC and 8473TC, respectively.

For −1195GA, the distribution of the A allele frequency was higher in CP group (60.62%) than in control group (51.35%), and there was a higher risk for the AA/GA genotype compared with the GG genotype ($OR^a = 2.49$, 95% CI = 1.33–4.69, $p = 0.005$). The adjusted OR indicated smoking status and tooth-brushing habit might be additive to the risk of the −1195A. The GC genotype for the −765 site was more prevalent in control group than in CP group (13.51% and 6.16%), and this protective effect was minor changed after the OR was adjusted for smoking and tooth-brushing habit ($OR^a = 0.45$, 95% CI = 0.20–1.04, $p = 0.061$). The prevalence of the C carriers for 8473TC was higher in control group than in CP group (21.96% and 15.07%). The risk for the CC/TC genotype was not significant ($OR^a = 0.67$, 95% CI = 0.41–1.11, $p = 0.118$). After the correction for multiple testing (Table 2), only the genotype distribution of −1195GA was significant ($p_c = 0.033$), while the other differences between groups were non-significant ($p_c > 0.05$).

Table 1. General characteristics in healthy controls and severe chronic periodontitis group

	Controls ($n = 148$)	CP ($n = 146$)	*p*-value
Age	50.84 ± 10.00	49.50 ± 9.74	0.244
Gender			0.568
F	92 (62.16)	86 (58.90)	
M	56 (37.84)	60 (41.10)	
Education			0.950
⩽12 years	109 (73.65)	108 (74.00)	
>12 years	39 (26.35)	38 (26.00)	
Medical welfare			0.649
Insurance	85 (57.40)	80 (54.80)	
No insurance	63 (42.60)	66 (45.20)	
Smoking			0.023
Yes (⩾100)	26 (17.57)	42 (28.77)	
No (<100)	122 (82.43)	104 (71.23)	
Tooth brushing			0.024
⩾Twice/day	114 (77.00)	95 (65.10)	
⩽Once/day	34 (23.00)	51 (34.90)	

Values represented as numbers (%) of subjects or mean ± SD.
CP, clinical periodontitis.

Table 2. COX-2 genotype distributions and odds ratios for severe chronic periodontitis

	Controls ($n = 148$)	CP ($n = 146$)	*p*-value	p_c-value	OR (95%CI, *p*-value)	OR* (95%CI, *p*-value)
−1195GA			0.011	0.033		
GG	40 (27.03)	19 (13.01)			1	1
GA	64 (43.24)	77 (52.74)			2.53 (1.34–4.80, 0.004)	2.60 (1.34–5.07, 0.005)
AA	44 (29.73)	50 (34.25)			2.39 (1.21–4.72, 0.012)	2.57 (1.25–5.28, 0.010)
AA+GA					2.48 (1.35–4.53, 0.003)	2.49 (1.33–4.69, 0.005)
Allele			0.024	0.072		
G	144 (48.65)	115 (39.38)				
A	152 (51.35)	177 (60.62)				
−765GC			0.035	0.105		
GG	128 (86.49)	137 (93.84)			1	1
GC	20 (13.51)	9 (6.16)			0.42 (0.19–0.96, 0.039)	0.45 (0.20–1.04, 0.061)
Allele			0.040	0.120		
G	276 (93.24)	283 (96.92)				
C	20 (6.76)	9 (3.08)				
8473TC			0.044	0.132		
TT	93 (62.83)	104 (71.23)			1	1
TC	45 (30.41)	40 (27.40)			0.79 (0.48–1.32, 0.377)	0.80 (0.48–1.35, 0.402)
CC	10 (6.76)	2 (1.37)			0.18 (0.04–0.84, 0.029)	0.17 (0.04–0.84, 0.029)
TC+CC					0.68 (0.42–1.11, 0.127)	0.67 (0.41–1.11, 0.118)
Allele			0.032	0.096		
T	231 (78.04)	248 (84.93)				
C	65 (21.96)	44 (15.07)				

*Adjusted for gender, age, smoking status, education years, insurance and tooth-brushing habit.
p_c-value, *p*-value corrected for multiple testing.
CI, confidence interval.
Values represented as numbers (%).
CP, clinical periodontitis; COX-2, cyclooxygenase-2; OR, odds ratio.

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Table 3. Distribution of COX-2 haplotype frequencies and odds ratios for severe chronic periodontitis

Haplotype	Control ($n = 148$)	CP ($n = 146$)	OR (95%CI)	p-value	p_c-value
Total	296	292			
G–G–T	144 (48.65)	115 (39.38)	1		
A–G–T	87 (29.40)	133 (45.55)	1.91 (1.32–2.76)	<0.001	<0.001
A–G–C	45 (15.20)	35 (11.99)	0.97 (0.59–1.61)	0.918	2.754
A–C–C	20 (6.75)	9 (3.08)	0.56 (0.25–1.29)	0.172	0.516

Haplotype shows single nucleotide polymorphisms in the following sequence: −1195GA, −765GC and 8473TC.

p_c-value, p-value corrected for multiple testing.

CI, confidence interval; CP, clinical periodontitis; COX-2, cyclooxygenase-2; OR, odds ratio.

Values represented as numbers (%).

No pairs of these three variants showed a complete LD ($0.85 < D' < 1$, $r^2 < 0.33$). The haplotype analysis documented there were four distinct haplotypes among these three SNPs (Table 3). It showed the AGT haplotype was nearly two times higher risk compared with the most common haplotype GGT (OR = 1.91, 95% CI = 1.32–2.76, $p_c < 0.001$), indicating the A allele at the −1195 position significantly increased the risk, and it was consistent with the above genotype distributions. There was no significance in the other haplotypes.

Discussion

Periodontitis is a multifactorial disease in which bacteria plaque, genetic factor and environmental influence are all interactional factors for the progress of periodontitis. Genetic factors influence the susceptibility of individuals and studies show that SNPs in most cytokines and enzymes including COX-2 gene have many functional changes (Yoshie et al. 2007). In this study, it was found that the −1195A was a meaningful indicator for severe CP, and the haplotype AGT was significantly associated with the risk of severe CP.

There are many literatures about the SNPs in the COX-2 gene but the conclusions are conflicting. Even the same author had different conclusions on the same disease and the same population. For instance, Campa et al. (2004) reported the 8473C was associated with the risk of non small cell cancer, but he rejected the conclusion the next year (Campa et al. 2005). Papafili et al. (2002) found that the −765C allele had significantly lower promoter activity compared with the −765G, while another study showed the −765CC genotype increased the risk of asthma and the PGE2 production was 10 times higher than the GG group (Szczeklik et al. 2004). These contradictory conclusions between the −765C allele and risk of inflammation also exist in other papers (Colaizzo et al. 2006, Orbe et al. 2006, Pereira et al. 2006, Ueda et al. 2008). The 8473C allele was protective to lung cancer in Chinese and Korean populations (Hu et al. 2005, Park et al. 2006). As there were different conclusions, Sanak et al. (2005) doubted whether the linkage between the genotypes of 8473CC and −765CC was associated with asthma, and an increased risk resulted from the −765C8473C haplotype was found. The 8473CC genotype was associated with breast cancer in different populations (Langsenlehner et al. 2006, Shen et al. 2006, Cox et al. 2007), and in contrast, Gao et al. (2007) detected no significant association between the 8473C and breast cancer, only finding the haplotype of −1195A −765C8473T increased the risk. The discrepancy could be explained as that different conclusions are made on different ethnic background, regions, sample size, diagnose criteria, laboratory techniques and so on (Yoshie et al. 2007); however, the exact mechanism about how the variant alleles affect the gene function remains unclear.

Some possible mechanisms are provided in previous studies. The −1195GA and −765GC are in the 5′ flanking region, and there are many putative transcription factor binding sites in the promoter region of the COX-2 gene (Papafili et al. 2002). It is likely that the variant allele could influence the gene function by changing these specific binding elements. The possible mechanism is that the −765C allele could disrupt the stimulatory protein-1 (SP1) binding site, which is considered to activate the transcription by binding the GC box. The C allele is also located in the stimulatory protein-3 (SP3) region which could increase or repress the SP1 function (Ho et al. 2008). The C allele is demonstrated to have a 30% lower promoter activity with a decrease of C-reaction-protein (CRP), compared with the wild G allele (Papafili et al. 2002), and this protective effect of the C allele on the outcome of diseases has been proven in recent reports (Orbe et al. 2006, Ho et al. 2008). It is similar with our study that the C allele frequency was more prevalent in the control group. However, our findings were not as dramatically as that in Ho's study. It is postulated that the frequency of the C allele was much lower in current studied population, and no CC homozygosity was found in both case group and control group. The A allele at the −1195 site creates a C-MYB binding site, and this element is considered to up-regulate the gene expression (Zhang et al. 2005). Most of the reports indicate that the A allele is a risk factor by increasing the promoter activity (Guo et al. 2007), and in the current study, the −1195G allele carriers were found to be less susceptible to inflammation which is consistent with former studies. Furthermore, the 8473C allele contributes to a decreased risk of CP in present study, and the explanatory mechanism is that the 8473C allele variant in 3′UTR region is considered to disrupt the mRNA stabilization, for the adenine-uracil-rich motif was changed and caused a degradation of COX-2 transcript. Therefore, a reduced risk of breast cancer and lung cancer for the C allele was reported (Hu et al. 2005, Shen et al. 2006). Additionally, our haplotype analysis revealed the −1195A-765G8473T haplotype was a strong predictor for CP ($p_c < 0.001$), and it is consistent with a study about breast cancer in the same ethnicity (Gao et al. 2007), although the author detected no significant association with either of these three SNPs. However, as our study did not cover all the functional SNPs, the conclusions might be different in other haplotypes.

It was noted that the conclusion for the −1195GA was reasonable as the p-value was corrected ($p_c = 0.033$), as well as for the haplotype AGT, which were the most significant results in this study. For the other two SNPs, either allelic or genotype test, the association were not statistically significant ($p_c > 0.05$). It suggests that the association

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between −1195GA and severe CP is confident, and the most significant role of the presented risk haplotype AGT is that of the −1195A variant. Although no complete LD was found between these three SNP pairs, it could not be excluded that the role of the −1195A variant may be in LD with other potential functional SNPs.

The present study, for the first time, reported the distributions of −1195GA, −765GC and 8473TC in a Chinese population with sever CP. In this study, environmental factors such as education background, medical welfare, smoking status and tooth-brushing habit were also considered. It was found that smokers were more prevalent in CP groups ($p < 0.05$) and tooth-brushing habit was also associated with the risk of CP ($p < 0.05$). After adjustment for these environmental factors, the ORs were minor modified, suggesting genetic factor was the principal determinant of disease expression rather than bacterial plaque and environmental factors (Yoshie et al. 2007). However, it was important to note that the variant allele frequencies were not dramatically different between the two groups, and the sample size was not sufficiently large, in that the variant allele C of the −765GC site was rare; thus, the observation might be due to a chance effect. Furthermore, various genes may be involved in the susceptibility to periodontitis, so it is more reasonable to make conclusions on more related genes, although the complexity of gene interaction is not clear.

In conclusion, among these three SNPs, we found that only the −1195A of the COX-2 gene is associated with a higher risk of severe CP. However, validation of these findings with larger sample and more rigorous design studies are needed.

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Clinical Relevance

Scientific rationale for the study: The polymorphisms of cytokines modify individual susceptibility to CP. COX-2 plays an important role in inflammation process including CP. The −1195GA, −765GC and 8473TC SNPs are hypothesized to be associated with risk of severe CP in a Chinese population.

Principal findings: Among the three SNPs, only the −1195A was associated with an increased risk to CP.

Practical implications: The COX-2 −1195A might be a risk diagnostic predictor for severe CP in Chinese.

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