Combined Association of CCR2-V64I and MCP-1-2518A/G Polymorphisms with Generalised Aggressive Periodontitis in Chinese

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Objective: *To examine the possible association of CCR2-V64I and MCP-1-2518A/G polymorphisms with generalised aggressive periodontitis (GAgP) in the Chinese population.*
Methods: *One hundred and twenty-four GAgP patients and 94 healthy subjects were included in the study. A peripheral blood sample was obtained from each subject and genomic DNA was isolated. Gene polymorphisms of CCR2-V64I and MCP-1-2518A/G were analysed by standard polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay.*
Results: *A possible combined effect of CCR2-V64I and MCP-1-2518A/G was observed in the female GAgP patients, as the odds ratio for VV genotype (CCR2) and G^+ genotype (MCP-1) was 0.2 (*P *= 0.023). Individuals carrying VV genotype and G^+ genotype were at reduced risk for GAgP. A possible combined effect of genotype and smoking was observed in the male GAgP patients, as the odds ratio for VV genotype (CCR2) and smoking, or G^+ genotype (MCP-1) and smoking were 7.4 (*P *= 0.022) and 4.9 (*P *= 0.030), respectively.*
Conclusion: *The combined association of CCR2-V64I and MCP-1-2518A/G polymorphisms may play an important role in determining GAgP susceptibility in Chinese females. A possible combined effect of genotype and smoking on GAgP susceptibility was suggested in males.*

Key words: *aggressive periodontitis, CCR2, MCP-1, polymorphism*

Aggressive periodontitis (AP) is a subgroup of periodontal diseases characterised by significant and relatively rapid destruction of the periodontal supporting tissues in otherwise healthy adolescents and young adults. The results of population and familial studies in AP[1], as well as a twin study in chronic periodontitis[2], indicate that genetic factors seem to have a strong influence on susceptibility to periodontitis.

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Chemokines and their receptors play a major role in the inflammatory and immune responses. Monocyte chemoattractant protein-1 (MCP-1), a potent mediator of both monocyte recruitment and activation, is expressed in the chronic inflamed gingival tissues. The number of cells expressing MCP-1 is related to the degree of inflammation[3-5]. Chronic periodontitis (CP) and AP patients have significantly higher MCP-1 levels in the gingival crevicular fluid (GCF) compared with healthy groups, and the MCP-1 level in GCF is positively correlated with both probing depth and clinical attachment loss[6,7]. All these studies suggest that MCP-1 plays an important role in the recruitment of monocytes and amplification of inflammatory signals in bacterially induced periodontitis.

Recently, a biallelic A/G polymorphism was found in the MCP-1 distal gene regulatory region at position

Table 1 Study population characteristics

Characteristic	GAgP patients (n = 124)	Healthy controls (n = 94)
Age (years; mean ± SD)	28.9 ± 6.7	30.7 ± 8.2
Gender		
Male	58 (46.8%)	45 (47.9%)
Female	66 (53.2%)	49 (52.1%)
Smoking status*		
Non-smoker	101 (81.5%)	89 (94.7%)
Current smoker	23 (18.5%)	5 (5.3%)

* Significantly different distribution between GAgP and control: $\chi^2 = 8.36$, $P = 0.004$

Table 2 Genotype distributions of CCR2-V64I and MCP-1-2518A/G polymorphisms in GAgP patients and healthy controls

Genotype*	GAgP patients (n = 124)	Healthy controls (n = 94)	χ^2
CCR2-V64I			
V/V	74 (59.7%)	61 (64.9%)	
I+	50 (40.3%)	33 (35.1%)	0.62
MCP-1-2518A/G			
A/A	32 (25.8%)	18 (19.1%)	
G+	92 (74.2%)	76 (80.9%)	1.34

* There was no significant difference in the genotype distributions ($P > 0.05$)

-2518 (number indicates nucleotide positions relative to the major transcription start site), that affects the level of MCP-1 expression in response to an inflammatory stimulus. Monocytes from individuals carrying a G allele at -2518 produce more MCP-1 after treatment with interleukin 1β (IL-1β) than monocytes from A/A homozygous subjects[8]. The MCP-1-2518A/G polymorphism has been implicated as a risk or susceptibility factor for a variety of autoimmune conditions and inflammatory diseases, including asthma[9], rheumatoid arthritis[10], coronary artery disease[11], and systemic lupus erythematosus[12].

Chemokine (C-C motif) receptor 2 (CCR2) is a prominent receptor for MCP-1. The important role of the receptor in the host defence mechanism has recently been demonstrated in a CCR2-deficient mouse model. A targeted disruption of the CCR2 gene results in a drastic reduction in MCP-1-induced monocyte chemotaxis, and the mice are unable to combat bacterial infections[13].

In the CCR2 gene, a G to A nucleotide substitution was detected at position 190 leading to an amino acid substitution of valine (V) for isoleucine (I) at position 64 in the receptor[14]. It is not yet proven whether the 64I mutation impairs the function of this receptor or is just a neutral polymorphism[15], but its association in HIV-infected patients with a 2–4 year delay in the progression to AIDS suggests just such an effect[16]. Some subsequent reports also suggest CCR2-64I may be related to the reduced function of CCR2 and may play a protective role for the host[17-20].

The combined effect of CCR2-V64I and MCP-1-2518A/G polymorphisms on periodontitis remains unknown. The present study aimed to explore the combined association of CCR2-V64I and MCP-1-2518A/G polymorphisms with generalised aggressive periodontitis (GAgP) in the Chinese population.

Materials and methods

Study population

One hundred and twenty-four GAgP patients were recruited from the periodontal clinics at Peking University Hospital of Stomatology. The diagnostic criteria for GAgP were based on the 1999 International Classification of Periodontal Diseases and Conditions[21]. Diagnoses were made by periodontal examination and full-mouth periapical radiographs. The following clinical criteria were used:

- patients were under 35 years old
- at least six teeth (at least three of which were not first molars or incisors) had a probing depth ≥ 5 mm and clinical attachment ≥ 3 mm
- no periodontal treatments within the past 12 months
- female patients were not pregnant or lactating
- except for the presence of periodontitis, the patients were clinically healthy.

Ninety-four healthy subjects were included, some voluntarily from the staff and students of the hospital, and the rest voluntarily from patients attending regular dental check-ups. None of the healthy subjects had previous or existing clinical evidence of periodontitis (probing depth ≤ 3mm; clinical attachment levels ≤ 1 mm from the cementoenamel junction; percentage of sites with bleeding index (BI) ≥ 2 less than 10%). None had a familial history of severe periodontitis or a known systemic dis-

order that could affect the periodontal conditions. All the clinical examinations and diagnoses were performed by two skilled practitioners with good calibration. Smoking status was also recorded. All the subjects were of Chinese descent and were unrelated. The study protocol was approved by the Ethics Committee of Peking University Health Science Center. Informed consent was obtained from all the participants in accordance with the Helsinki declaration.

Isolation of genomic DNA

A 2 ml ethylenediaminetetraacetic acid (EDTA) anticoagulated peripheral blood sample was obtained from each subject by venipuncture. Genomic DNA was isolated from each sample with a blood DNA mini kit (Watson Biotechnologies, Shanghai, P.R. China) following the manufacturer's instructions. DNA integrity was checked and quantitated using agarose gel electrophoresis.

CCR2-V64I and MCP-1-2518A/G genotyping

Standard polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay was utilised for CCR2-V64I and MCP-1-2518A/G genotyping as described previously[11,14]. Briefly, PCR reactions included 100–500 ng DNA, 2× Taq PCR MasterMix (TianGen Biotech, Beijing, P.R. China), 1.0 μmol/l each of forward and reverse primers in a final volume of 25 μl. All PCR reactions were performed at an optimum annealing temperature in the thermocycler (PTC-200, MJ Research, Watertown, MA, USA). PCR products were checked by 3% (w/v) agarose gel electrophoresis and the target fragment was digested by corresponding restriction endonuclease (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's instructions. Digested products were detected by gel electrophoresis.

CCR2-V64I

The CCR2 gene contains a guanine (G) to adenine (A) substitution at position 190. The A allele is completing a *BsaB*I site. Digestion of the PCR products with *BsaB*I yielded fragments of 149 bp and 24 bp for A allele, or 173 bp for G allele.

MCP-1-2518A/G

The MCP-1 gene has an adenine (A) to guanine (G) substitution at position -2518, and the G allele is completing a *Pvu*II site. Digestion of the PCR products (234 bp) with *Pvu*II yielded fragments of 159 bp and 75 bp when G is at position -2518.

Statistical analysis

Quantitative value for age was expressed as mean ± standard deviation (SD) and categorical values (gender and smoking status) were presented as counts and percentages. These values were tested with *t* or chi-square (χ^2) tests of parametric analysis between the patients and controls.

A logistic regression analysis was performed to test the combined association of CCR2-V64I and MCP-1-2518A/G genotypes with GAgP, and the combined effect of genotype and smoking status with GAgP was also examined.

All *P* values were two-sided and defined as $P = 0.05$ for statistical significance. The strength of the associations was determined using an odds ratio (OR) calculation and 95% confidence interval (CI). Statistical software (SAS Institute, Cary, NC, USA) was used for all analyses.

Results

The characteristics of the study population are shown in Table 1. The age and gender distribution was reasonably well balanced in the patients and controls. The mean ages of GAgP patients and control subjects were 28.9 and 30.7 years, respectively. The smoking rate was higher in the patients than in the controls (18.5% vs. 5.3%, $\chi^2 = 8.36$, $P = 0.004$).

The genotype distributions of CCR2-V64I and MCP-1-2518A/G polymorphisms in GAgP patients and healthy controls are shown in Table 2. For the CCR2 gene, there was a deviation from the Hardy–Weinberg equilibrium ($\chi^2 = 4.56$, $P < 0.05$) in controls. There was no significant difference in the genotype distributions for different polymorphic loci when the GAgP patients as a whole were compared with the control, even after adjusting for age, gender and smoking status. However, a significant decrease in the frequency of G^+ genotype at MCP-1-2518A/G was found in the female GAgP patients compared with the female healthy controls (data not shown).

Using multivariate logistic regression (adjusted for age and smoking status), the combined effect of CCR2-V64I and MCP-1-2518A/G was shown to be significantly associated with the risk of GAgP in the females. The adjusted OR of GAgP for VV genotype (CCR2) and G^+ genotype (MCP-1) was 0.2 (95%CI: 0.0–0.8, $P = 0.023$; Table 3). A possible combined effect of genotype and smoking was observed in the male GAgP patients, as the OR for VV genotype (CCR2) and smoking, or G^+ genotype (MCP-1) and

Table 3 Combined associations of CCR2-V64I and MCP-1-2518A/G genotype in GAgP patients

		Male*					Female*				
Genotype		Control	GAgP	GAgP versus control			Control	GAgP	GAgP versus control		
CCR2	MCP-1	n (%)	n (%)	OR	95%CI	P	n (%)	n (%)	OR	95%CI	P
I^+	AA	5 (11.1)	4 (7.0)	1			3 (6.1)	11 (16.7)	1		
VV	AA	6 (13.3)	9 (15.5)	2.3	0.4–13.1	0.358	4 (8.2)	8 (12.1)	0.4	0.1–2.5	0.312
I^+	G^+	11 (24.4)	17 (29.3)	1.6	0.3–7.9	0.580	14 (28.6)	18 (27.3)	0.3	0.1–1.3	0.095
VV	G^+	23 (51.1)	28 (48.3)	1.6	0.4–7.2	0.547	28 (57.1)	29 (43.9)	0.2	0.0–0.8	0.023

* Multiple logistic regression model adjusted for age (tertile), smoking status (no, yes)

Table 4 Combined associations of CCR2-V64I, MCP-1-2518A/G genotype and smoking status with GAgP*

CCR2 genotype	Smoking	GAgP vs. healthy controls			MCP-1 genotype	Smoking	GAgP vs. healthy controls		
		OR	95%CI	P			OR	95%CI	P
I^+	No	1			AA	No	1		
I^+	Yes	3.7	0.8–18.0	0.100	AA	Yes	3.0	0.3–33.7	0.382
VV	No	1.1	0.4–2.9	0.849	G^+	No	0.9	0.3–2.4	0.788
VV	Yes	7.4	1.3–41.1	0.022	G^+	Yes	4.9	1.1–20.3	0.030

* Multiple logistic model adjusted by age (tertile), gender (male, female)

smoking were 7.4 ($P = 0.022$) and 4.9 ($P = 0.030$), respectively (Table 4).

Discussion

In the present study, the gene polymorphisms of MCP-1 and its major receptor CCR2 were analysed. Genotype frequencies of MCP-1-2518A/G were similar to those of previously reported studies in Chinese healthy subjects[22,23], and those in Japanese[24] and Korean[25] subjects. The genotype frequencies in Chinese subjects were obviously different from those of African American, Caucasian and Hispanic subjects[8,11,26]. The genotype frequencies of CCR2-V64I detected in this study were also consistent with the frequencies reported previously[22,27,28]. The G allele frequency was higher in Chinese than in African American and Caucasian subjects[11,14]. For the CCR2 gene, there was a deviation from the Hardy–Weinberg equilibrium in the control group. This deviation may be due to the random fluctuations in the present relatively small sample size.

The present study was the first report to explore the combined effect of CCR2-V64I and MCP-1-2518A/G polymorphisms. A possible combined effect of CCR2-V64I and MCP-1-2518A/G was observed in the female GAgP patients, as the OR for VV genotype (CCR2) and G^+ genotype (MCP-1) was 0.2 ($P = 0.023$). VV genotype (CCR2) and G^+ genotype (MCP-1) was significantly associated with the risk of GAgP. Individuals carrying VV genotype and G^+ genotype were at reduced risk for GAgP in females.

MCP-1 and CCR2 play important roles in the recruitment of monocytes and amplification of inflammatory signals in bacterially induced periodontitis. Studies demonstrate that pro-inflammatory cytokines, including MCP-1 itself, rapidly down-regulate the expression of CCR2, which may aid the retention of monocytes at sites of inflammation after their recruitment from the circulation. These cytokines also induce the secretion of MCP-1 by monocytes and initiate the switch from the MCP-1-responsive state to the MCP-1-unresponsive state, which coincides with the loss of CCR2[29]. Many chemokines are involved in the immunopathogenesis of periodontal diseases, making the monocyte recruitment and activation mediated by MCP-1/CCR2 more complex. The exact combined association of CCR2-

V64I and MCP-1-2518A/G polymorphisms with GAgP needs to be further investigated in larger samples and other populations.

Cigarette smoking is a well-documented risk factor for periodontitis, with a dose-dependent effect. It is associated with an increased risk of gingivitis[30], clinical attachment loss, alveolar bone loss, tooth loss[31], disease severity and treatment response[32]. In the present study, a possible combined effect was observed in male Chinese with the OR 7.4 for V/V genotype (CCR2) and smoking, and OR 4.9 for G^+ genotype (MCP-1) and smoking. The interaction between smoking and the genotype on periodontitis has been demonstrated previously[33-35].

The present study indicates that the combined association of CCR2-V64I and MCP-1-2518A/G polymorphisms may play an important role in determining GAgP susceptibility in Chinese females. This marker might be used to identify the subgroup at higher risk of AP in Chinese patients.

Acknowledgments

The authors thank Dr Dafang Chen, Department of Epidemiology and Biostatistics, School of Public Health, Peking University Health Science Center, for his help in statistical analysis.

The present study was supported in part by the National Natural Science Foundation of China (#30271411, #30471882), by the National Key Project of Scientific and Technical Supporting Programs of China (2007BAZ18B02), and by the National High Tech Program ('863' Program) (2002AA217091).

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