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Correlation between single nucleotide polymorphisms in a calprotectin subunit gene and risk of periodontitis in a Chinese population

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Summary

S100A8, the light subunit of calprotectin, has been known to be associated with periodontal inflammation. The present study looked to detect whether three polymorphisms in the upstream region of the *S100A8* gene are correlated with periodontitis. Three hundred and twenty one subjects, including chronic periodontitis (CP) patients, aggressive periodontitis (AgP) patients and periodontally healthy controls, were recruited. The SNPs rs3795391, rs3806232 and rs3885688 were analyzed by PCR-RFLP analysis. No person carried the rs3885688 polymorphism in this cohort. For the other two polymorphisms, the combined effects of genotype/allele and gender were shown to be associated with the risk of periodontitis using multivariate logistic regression analysis. The G^+ genotype/G allele may be considered to exert a significant protective effect in males against AgP (Genotype: rs3795391: $P = 0.032$, rs3806232: $P = 0.017$; Allele: rs3795391: $P = 0.024$, rs3806232: $P = 0.013$). Although the combined effects of genotype and gender on CP susceptibility were not observed for these two polymorphisms, there does seem to be increased risk of CP in males with allele A compared to females with allele A (rs3795391: $P = 0.008$; rs3806232: $P = 0.009$). Hence we found an important association between polymorphisms in the *S100A8* gene and periodontitis in a Chinese population.

Keywords: Calprotectin/S100A8, Periodontitis, Single nucleotide polymorphism, Susceptibility, Chinese

Introduction

Calprotectin composes about half of the cytoplasmic protein in neutrophils. Although the physiological function of this protein is not clear, its levels in plasma, urine and faeces are known to increase markedly in many pathological conditions, including rheumatoid arthritis and systemic lupus erythematosus *et al.* These findings indicate that calprotectin could be a possible marker for these diseases. Using the new S100 protein nomenclature, calprotectin is designated as the S100A8/9 complex (Schafer *et al.* 1995).

Previous results from our laboratory have demonstrated that S100A8, the light subunit of calprotectin, exists prominently in gingival crevicular fluid (GCF). Its level is significantly correlated with periodontal inflammation (Zhou *et al.* 1998; Li *et al.* 1998, 2003; Wang *et al.* 2002). Other researchers have also found that the concentration of calprotectin in GCF correlates not

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only with clinical indicators, including probing depth, bleeding index, GCF volume and gingival index, but also with levels of current biochemical markers, such as interleukin-1 β (IL-1 β), PGE_2, collagenase and aspartate aminotransferase (Nakamura *et al*. 2000). Hence it could be useful for evaluating the extent of periodontal inflammation as a disease marker.

Until now there has been no published data on single nucleotide polymorphisms (SNPs) in the calprotectin gene in relation to inflammatory disease. However, this genetic susceptibility is functionally plausible. Firstly, calprotectin *in vitro* has bacteriostatic and bactericidal activity against the genus *Capnocytophaga*, which are pathogens of periodontal origin (Miyasaki *et al*. 1993). Cells expressing calprotectin resist invasion by *Listeria monocytogenes* and *Salmonella enterica* serovars Typhimurium. The gingival epithelium expressing calprotectin also confers resistance *in vitro* to *P. gingivalis*, a putative pathogen in periodontal disease, and is more resistant to detachment mediated by Arg-gingipain and Lys-gingipain (Nisapakultorn *et al*. 2001a, 2001b). In summary, calprotectin may augment both the barrier protection and innate immune functions of the gingival epithelium. Secondly, calprotectin has a broad apoptosis-inducing activity against normal fibroblasts (Yui *et al*. 1997), various tumour cells (Yui *et al*. 1995) and myoblasts (Seeliger *et al*. 2003). Fibroblasts are the preponderant cell type in the periodontal tissue, and play an important role in its function and recovery. Therefore, calprotectin may exert a regulatory activity in inflammatory processes through its effect on the survival or growth states of cells participating in the inflammatory reaction. It is possible that calprotectin, at a high concentration for a long period of time, might cause tissue destruction in severe inflammatory conditions, and influence the recovery of inflammatory tissue when it is released into extracellular spaces (Yui *et al*. 2003). Thirdly, calprotectin plays a prominent role in leukocyte trafficking and arachidonic acid metabolism. When calprotectin is released by primed phagocytes under inflammatory conditions it may promote further recruitment of leukocytes to inflammatory sites by a positive feedback mechanism (Rammes *et al*. 1997). S100A9 regulates neutrophil adhesion to fibrinogen by affecting Mac-1 affinity (Eue *et al*. 2000). Polymorphonuclear leukocytes expressing calprotectin are the predominant cell type in areas of acute inflammation and could release high amounts of tumour necrosis factor alpha and IL-1β (Bhardwaj *et al*. 1992). Calprotectin may also serve as a transport protein to move arachidonic acid to its target cells (Roulin *et al*. 1999). All this evidence leads to a propagating role for calprotectin in inflammatory responses. Finally, the chemotactic activity of S100 proteins, including S100A2, S100A7, S100A12 and murine S100A8, has been demonstrated in many studies (Komada *et al*. 1996; Jinquan *et al*. 1996; Yang *et al*. 2001; Lackmann *et al*. 1993). Murine S100A8 has been shown to be chemotactic for myeloid cells, and is a more potent chemoattractant than most chemokines (Yang *et al*. 2001; Lackmann *et al*. 1993). Recently, Tessier and colleagues presented remarkable data which indicated direct chemotactic activity of both murine and human S100A8 and S100A9 (Ryckman *et al*. 2003; Vandal *et al*. 2003), although this *in vitro* effect needs further study *in vivo* (Roth *et al*. 2003). Nishimura reported that S100A8 is chemotactic for human periodontal ligament (PDL) cells but not for gingival fibroblasts (GF). It has been suggested that selective recruitment of PDL cells to a previously exposed root surface enhances periodontal regeneration, but the competition from GF may reduce the potential for periodontal regeneration (Nishimura & Terranova, 1996; Nishimura *et al*. 1999). S100 proteins might possess the same functions as cytokines and represent a new class of chemotactic factors contributing to neutrophil migration to inflammatory sites.

Based on the above knowledge it is natural to consider that, in addition to cytokines and enzymes, calprotectin is a candidate for being a novel inflammatory mediator released by neutrophils. This complex or its individual subunits are not only useful markers of inflammatory states, but also important mediators with multiple regulatory functions in inflammatory reactions. Whether or not calprotectin has a beneficial or a harmful role in periodontitis needs further elucidation.

While mutations in protein coding regions affect the structure of gene products, polymorphisms outside these regions may cause quantitative differences in gene expression. In this study, we hypothesized that *S100A8* gene polymorphisms, perhaps involved in overexpression of S100A8, might be related to periodontitis. To verify this hypothesis three SNPs upstream from the ATG start codon of the *S100A8* gene were selected, and

	Healthy controls N = 101 (%)	CP patients N = 70 (%)	AgP patients N = 150 (%)	Periodontitis patients N = 220 (%)
Age (years)				
Means ± SD	30.9 ± 7.9	46.3 ± 8.8	28.9 ± 5.9	34.2 ± 10.5
Gender				
male	40 (39.6)	25 (35.7)	51 (34.0)	76 (34.5)
female	61 (60.4)	45 (64.3)	99 (66.0)	144 (65.5)
rs3795391 Genotype				
AA	77 (76.2)	54 (77.1)	119 (79.3)	173 (78.6)
AG	23 (22.8)	16 (22.9)	30 (20.0)	46 (20.9)
GG	1 (1.0)	0 (0)	1 (0.7)	1 (0.5)
G^+ (AG+GG)*	24 (23.8)	16 (22.9)	31 (20.7)	47 (21.4)
rs3795391 Allele				
A	177 (87.6)	124 (88.6)	268 (89.3)	392 (89.1)
G	25 (12.4)	16 (11.4)	32 (10.7)	48 (10.9)
rs3806232 Genotype				
AA	76 (75.2)	54 (77.1)	120 (80.0)	174 (79.1)
AG	24 (23.8)	16 (22.9)	28 (18.7)	44 (20.0)
GG	1 (1.0)	0 (0)	2 (1.3)	2 (0.9)
G^+ (AG+GG)*	25 (24.8)	16 (22.9)	30 (20.0)	46 (20.9)
rs3806232 Allele				
A	176 (87.1)	124 (88.6)	268 (89.3)	392 (89.1)
G	26 (12.9)	16 (11.4)	32 (10.7)	48 (10.9)

*Because of the low frequency of the homozygous polymorphic genotype, the GG genotype was combined with heterozygous genotype (AG) as the G positive genotype (G^+).

Table 1 Study Population Characteristics and Genotype/Allele Distributions in Patients and Healthy Controls

the relationship between the SNPs and susceptibility to periodontitis examined by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) in a relatively large Chinese cohort.

Materials and Methods

Study population

The study population characteristics are shown in Table 1. Three hundred and twenty one subjects were studied for the SNPs rs3806232 and rs3885688. From the total, two hundred and fifty four were studied for SNP rs3795391. The periodontitis subjects came from the Department of Periodontology at Peking University School of Stomatology. For each patient, a periodontal examination and a set of full-mouth periapical radiographs were taken. Further, patients were divided into two groups, chronic periodontitis (CP) or aggressive periodontitis (AgP), according to the 1999 international classification of periodontal diseases (Armitage, 1999). The diagnostic criteria of CP were: 1) patients were more than 35 years old; 2) the amount of periodontal destruction was consistent with the presence of local factors such as plaque and calculus; 3) at least two sites had probing depth ≥ 5 mm and clinical attachment loss ≥ 1 mm in every quadrant; the number of the teeth with alveolar bone absorption more than $\frac{2}{3}$ root length was less than eight. The clinical criteria of AgP were: 1) patients were under 35 years old; 2) at least six teeth, at least three of which were not first molars or incisors, had a probing depth ≥ 5 mm and clinical attachment loss ≥ 3 mm. All patients had not received periodontal treatment within the past 12 months. Except for the presence of periodontitis the patients were clinically healthy. All female patients were not pregnant or lactating. Some healthy control subjects were selected from staff and students at the School of Stomatology, and others were volunteers who visited the School of Stomatology for regular dental check-ups. None of the healthy subjects had previous or existing clinical evidence of periodontitis (probing depth ≤ 3 mm; the percentage of sites with bleeding index (BI) ≥ 2 less than 10%; no site with BI ≥ 4). None had a familial history of severe

periodontitis or a known systemic disorder that could affect the periodontal conditions. All subjects were non-smokers, members of the Chinese Han race, and unrelated. The present study was conducted with informed consent of all subjects, and approved by the Ethics Committee of the Peking University Health Science Center.

Isolation of Genomic DNA

Two ml of EDTA anti-coagulated peripheral blood was sampled from each subject by venipuncture. Genomic DNA was extracted from each sample with a Blood DNA Mini Kit (Watson Biotechnologies, Inc., Shanghai, China) following the manufacturer's instructions. DNA integrity was checked and DNA quantitated using agarose gel electrophoresis.

S100A8 genotyping

A standard PCR-RFLP assay was utilized for *S100A8* genotyping. PCR reactions includes 100 to 500 ng DNA, 10× reaction buffer (containing 20 mM $MgCl_2$), 0.25 mM dNTP, 1.0 U *Taq* polymerase, and 1.0 μM each of the forward and reverse primers (Table 2) in a 25 μl final volume. All PCR reactions were performed in a thermocycler (PTC-200, MJ Research, Inc., Watertown, MA, USA.). Amplification was performed for one cycle at 94°C for 5 minutes, 35 cycles each at 94°C for 30 seconds, at 30 seconds an optimum annealing temperature (Table 2) and 72°C for 30 seconds, and one cycle at 72°C for 7 minutes. PCR products were checked by 1% (w/v) agarose gel electrophoresis. Then, 10 μl of a different target fragment was digested by 1 to 3 units of the corresponding restriction endonuclease (New England Biolabs, Beverly, MA, USA) (Table 2) in a volume of 20 μl according to the manufacturer's instructions and incubated overnight. Digested products were detected by 15% polyacrylamide gel electrophoresis and ethidium bromide staining.

rs3795391

rs3795391 is an adenine (allele A) to guanine (allele G) substitution in the *S100A8* gene, and allele A completes a *Tfi*I site. The target fragment contains one obligate *Tfi*I site, and a second potential *Tfi*I site depending on the genotype: allele A yields products of 147bp, 54bp and 47bp; allele G yields products of 194bp and 54bp. Heterozygous individuals were identified by the presence of an additional band, and the constant 54bp band also served as a restriction control site.

rs3806232

rs3806232 is also an adenine (allele A) to guanine (allele G) substitution in the 5′ flanking region of *S100A8*, and allele A completes an *Hpy*CH4 V site. Allele A yields products of 188bp and 21bp; allele G yields a product of 209bp. Three bands were found in heterozygous individuals.

rs3885688

rs3885688 is a guanine (allele G) to adenine (allele A) substitution in *S100A8* and the allele G completes a *Bsp*1286 I site. Allele G yields products of 268bp and

Table 2 Genotyping of the three Polymorphic Variants in the *S100A8* Gene

Polymorphic Variants	Primer sequences (5′→3′)	PCR		Digestion	
		Size (bp)	Tm†	Enzyme	T‡
rs3795391 (A→G)	Fw: GTGTGCACATGTCTCTGTGTG Rv: CAACATGATGCCCACGGAACTTGC	248bp	58°C	*Tfi I*; A = *Tfi I* (+), G = *Tfi I* (−)	65°C
rs3806232 (A→G)	Fw: AGGAATGGATATAGCCCTTTGC Rv: CACTCAGTGAGAACATTCCTCC	209bp	57°C	*HpyCH4* V; A = *HpyCH4* V (+), G = *HpyCH4* V (−)	37°C
rs3885688 (G→A)	Fw: CTGTCACATCACATCATGAGTCAC Rv: AATCCAGCTCTCTCTGGATGTG	312bp	60°C	*Bsp1286 I*; G = *Bsp1286 I* (+), A = *Bsp1286 I* (−)	30°C

†Tm : Annealing temperature.
‡T : Incubation temperature.

44bp; allele A yields a product of 312bp. Heterozygous individuals were again identified by the presence of three bands.

Quality control

To check the genotyping results direct sequencing of PCR products was performed in a 20% random sample using the Forward or Reverse primer. PCR fragments were gel purified by E.Z.N.A.®Gel Extraction kit (Omega Bio-tek, Inc, GA, USA) according to the manufacturer's protocol. Sequencing analyses were performed using ABI Big Dye terminator reagents 2.0 (Applied Biosystems, CA, USA) on an ABI PRISM 3100 Genetic Analyzer, and the results analyzed with the sequencing software provided.

Statistical analysis

A logistic regression analysis was performed to analyze the association of *S100A8* genotype/alleles between the different two groups and to test the combined associations of *S100A8* genotype/alleles and gender between the control group, and periodontitis group. The CP and AgP subgroups were combined as one periodontitis group, which was also tested using the same statistical analysis. It should be emphasized that the association was explored between the patient subgroup (CP and AgP) without age in the model, as age was one of the criteria used to discriminate between CP and AgP (Loos *et al*. 2003). All P values were 2-sided and defined as $P = 0.05$ for statistical significance. The strength of the associations was determined using an OR calculation and 95% CI. Statistical software SAS (SAS Institute Inc, Cary, NC) was used for all data analyses.

Results

The gender distribution was reasonably well balanced in patients and controls. No individual carried the rs3885688 polymorphism in the present study. The genotype and allele distributions of the other two *S100A8* polymorphisms in the different groups are presented in Table 1.

There was no statistically significant difference in the distributions of the genotype/alleles for rs3795391 and rs3806232 in the different groups (Table 1), even after adjusting for age and gender (data not shown). When the cases were stratified according to gender, there was no significant difference in the distributions of the genotype/alleles for the two polymorphic loci in the different groups in males or females (Table 3 and 4). However, a decrease in the frequency of the G^+ genotype/G allele at rs3806232 was found in male AgP patients compared to male healthy controls, despite there being no significant difference (genotype: 9.8% vs 25.0%, adjusted OR = 0.3, 95% CI = 0.1-1.1, $P = 0.067$; allele: 4.9% vs 12.5%, adjusted OR = 0.4, 95% CI = 0.1-1.1, $P = 0.083$)(Table 3). Further, the combined effect of *S100A8* genotype/allele and gender on susceptibility of periodontitis was explored and demonstrated using multivariate logistic regression (Tables 5 and 6). For two polymorphic loci a significant combined effect between the G^+ genotype and being male was found to be associated with AgP, compared to females with the AA genotype (rs3795391: OR = 0.3, 95%CI = 0.1-0.9, $P = 0.032$; rs3806232: OR = 0.2, 95%CI = 0.1-0.8, $P = 0.017$). Also, a significantly combined effect between allele G and male was observed (rs3795391: OR = 0.3, 95%CI = 0.1-0.9, $P = 0.024$; rs3806232: OR = 0.2, 95%CI = 0.1-0.7, $P = 0.013$). Although combined effects of genotype and gender on CP susceptibility were not observed for these two polymorphisms, there was an increased risk of CP for males with allele A compared females with allele A (rs3795391: OR = 2.5, 95%CI = 1.3-5.0, $P = 0.008$; rs3806232: OR = 2.5, 95%CI = 1.3-5.0, $P = 0.009$). There was no significant combined effect of *S100A8* genotype/allele and gender for the patients as a whole group were compared to the controls, and between the two subgroups of periodontitis patients (Table 5 and 6).

In addition, the results of direct sequencing were coincident with those found by restriction enzyme digestion in all selected subjects. The coincidence rate was 100%.

Discussion

Periodontitis is an inflammatory disorder characterized by connective tissue and alveolar bone destruction.

Table 3 Associations Between *S100A8* Genotype/Allele and Periodontitis in Males

	control	CP vs control*				AgP vs control*				Periodontitis vs control*				AgP vs CP		
loci	N (%)	N (%)	OR	95%CI	*P*	N (%)	OR	95%CI	*P*	N (%)	OR	95%CI	*P*	OR	95%CI	*P*
rs3795391																
Genotype																
AA	30(75.0)	19(76.0)	1			45(88.2)	1			64(84.2)	1			1		
G^+	10(25.0)	6(24.0)	1.1	0.2-7.4	0.919	6(11.8)	0.4	0.1-1.3	0.119	12(15.8)	0.5	0.2-1.5	0.233	0.4	0.1-1.5	0.177
Allele																
A	70(87.5)	44(88.0)	1			96(94.1)	1			140(92.1)	1			1		
G	10(12.5)	6(12.0)	1.1	0.2-6.2	0.926	6(5.9)	0.4	0.2-1.3	0.140	12(7.9)	0.6	0.2-1.5	0.260	0.5	0.1-1.5	0.197
rs3806232																
Genotype																
AA	30(75.0)	19(76.0)	1			46(90.2)	1			65(85.5)	1			1		
G^+	10(25.0)	6(24.0)	1.1	0.2-7.4	0.919	5(9.8)	0.3	0.1-1.1	0.067	11(14.5)	0.5	0.2-1.3	0.155	0.3	0.1-1.3	0.108
Allele																
A	70(87.5)	44(88.0)	1			97(95.1)	1			141(92.8)	1			1		
G	10(12.5)	6(12.0)	1.1	0.2-6.2	0.926	5(4.9)	0.4	0.1-1.1	0.083	11(7.2)	0.5	0.2-1.4	0.177	0.4	0.1-1.3	0.124

*Multiple logistic regression model adjusted for age (tertile).

Table 4 Associations Between *S100A8* Genotype/Allele and Periodontitis in Females

	control	CP vs control*				AgP vs control*				Periodontitis vs control*				AgP vs CP		
loci	N (%)	N (%)	OR	95%CI	*P*	N (%)	OR	95%CI	*P*	N (%)	OR	95%CI	*P*	OR	95%CI	*P*
rs3795391																
Genotype																
AA	47(77.0)	35(77.8)	1			74(74.7)	1			109(75.7)	1			1		
G^+	14(23.0)	10(22.2)	0.8	0.2-2.5	0.691	25(25.3)	1.1	0.5-2.4	0.802	35(24.3)	1.1	0.5-2.2	0.798	1.2	0.5-2.7	0.695
Allele																
A	107(87.7)	80(88.9)	1			172(86.9)	1			252(87.5)	1			1		
G	15(12.3)	10(11.1)	0.8	0.3-2.2	0.603	26(13.1)	1.1	0.5-2.2	0.833	36(12.5)	1.0	0.5-2.0	0.923	1.2	0.6-2.6	0.631
rs3806232																
Genotype																
AA	46(75.4)	35(77.8)	1			74(74.7)	1			109(75.7)	1			1		
G^+	15(24.6)	10(22.2)	0.7	0.2-2.2	0.518	25(25.3)	1.0	0.5-2.2	0.945	35(24.3)	1.0	0.5-2.0	0.998	1.2	0.5-2.7	0.695
Allele																
A	106(86.9)	80(88.9)	1			171(86.4)	1			251(87.2)	1			1		
G	16(13.1)	10(11.1)	0.7	0.2-1.9	0.460	27(13.6)	1.1	0.5-2.1	0.877	37(12.8)	1.0	0.5-1.9	0.973	1.3	0.6-2.7	0.553

*Multiple logistic regression model adjusted for age (tertile).

Table 5 Combined Associations of *S100A8* Genotype and Gender with Periodontitis

				CP vs control*				AgP vs control*				Periodontitis vs control*				AgP vs CP		
loci	gender	genotype	control N (%)	N (%)	OR	95%CI	*P*	N (%)	OR	95%CI	*P*	N (%)	OR	95%CI	*P*	OR	95%CI	*P*
rs3795391																		
	Female	AA	47(46.5)	35(50.0)	1			74(49.3)	1			109(49.5)	1			1		
	Female	G^+	14(13.9)	10(14.3)	0.7	0.2-2.6	0.623	25(16.7)	1.1	0.5-2.4	0.784	35(15.9)	1.1	0.5-2.3	0.744	1.2	0.5-2.7	0.695
	Male	AA	30(29.7)	19(27.1)	2.5	0.9-6.9	0.086	45(30.0)	0.8	0.4-1.5	0.504	64(29.1)	1.0	0.6-1.8	0.887	1.1	0.6-2.2	0.740
	Male	G^+	10(9.9)	6(8.6)	2.4	0.5-11.0	0.256	6(4.0)	0.3	0.1-0.9	0.032	12(5.5)	0.6	0.2-1.4	0.232	0.5	0.1-1.6	0.222
rs3806232																		
	Female	AA	46(45.5)	35(50.0)	1			74(49.3)	1			109(49.5)	1			1		
	Female	G^+	15(14.9)	10(14.3)	0.6	0.2-2.1	0.448	25(16.7)	1.0	0.5-2.2	0.939	35(15.9)	1.0	0.5-2.1	0.956	1.2	0.5-2.7	0.695
	Male	AA	30(29.7)	19(27.1)	2.4	0.8-6.7	0.102	46(30.7)	0.8	0.4-1.5	0.508	65(29.6)	1.0	0.6-1.8	0.906	1.1	0.6-2.2	0.691
	Male	G^+	10(9.9)	6(8.6)	2.3	0.5-10.6	0.280	5(3.3)	0.2	0.1-0.8	0.017	11(5.0)	0.5	0.2-1.3	0.155	0.4	0.1-1.4	0.145

*Multiple logistic regression model adjusted for age (tertile).

Table 6 Combined Associations of *S100A8* Allele and Gender with Periodontitis

				CP vs control*				AgP vs control*				Periodontitis vs control*				AgP vs CP		
loci	gender	allele	control N (%)	N (%)	OR	95%CI	*P*	N (%)	OR	95%CI	*P*	N (%)	OR	95%CI	*P*	OR	95%CI	*P*
rs3795391																		
	Female	A	107(53.0)	80(57.1)	1			172(57.3)	1			252(57.3)	1			1		
	Female	G	15(7.4)	10(7.2)	0.7	0.2–2.2	0.549	26(8.7)	1.1	0.5–2.2	0.832	36(8.2)	1.1	0.5–2.0	0.881	1.2	0.6–2.6	0.631
	Male	A	70(34.6)	44(31.4)	2.5	1.3–5.0	0.008	96(32.0)	0.7	0.5–1.1	0.125	140(31.8)	1.0	0.7–1.4	0.798	1.0	0.7–1.6	0.948
	Male	G	10(5.0)	6(4.3)	2.5	0.6–10.4	0.213	6(2.0)	0.3	0.1–0.9	0.024	12(2.7)	0.6	0.2–1.3	0.195	0.5	0.1–1.5	0.197
rs3806232																		
	Female	A	106(52.5)	80(57.1)	1			171(57.0)	1			251(57.0)	1			1		
	Female	G	16(7.9)	10(7.2)	0.6	0.2–1.9	0.405	27(9.0)	1.1	0.5–2.1	0.884	37(8.5)	1.0	0.5–1.9	0.986	1.3	0.6–2.7	0.553
	Male	A	70(34.6)	44(31.4)	2.5	1.3–5.0	0.009	97(32.3)	0.7	0.5–1.1	0.134	141(32.0)	1.0	0.7–1.4	0.808	1.0	0.7–1.6	0.892
	Male	G	10(5.0)	6(4.3)	2.4	0.6–10.2	0.222	5(1.7)	0.2	0.1–0.7	0.013	11(2.5)	0.5	0.2–1.2	0.136	0.4	0.1–1.3	0.129

*Multiple logistic regression model adjusted for age (tertile).

Neutrophils are believed to play an important role in controlling the periodontal microbiota. They are the first leukocytes to arrive at the site of inflammation, which is initiated by the bacteria, and they secrete many proteins including enzymes, antibacterial proteins and cytokines. Calprotectin, simply by its abundance in neutrophils, is implied to have an important role in the effector functions of neutrophils, and in relation to periodontitis. On the other hand, it is widely believed that periodontitis is a complex multi-factorial disease with the involvement of several genes. Encountering bacterial challenge, the host response can be not only protective but also destructive, especially in a susceptible host with hyper-response. The genetic variance in periodontitis may be due not to classical mutations of a single gene (of major effect), but to the combined effects of multiple, common, functional polymorphisms of genes. Emerging scientific evidence suggests that certain polymorphisms in some genes such as *IL-1B* and tumour necrosis factor-α (*TNF*), may determine the degree of the individual's immune response, and the severity of the disease (Kornman *et al*. 1997; Graves & Cochran, 2003).

Despite clear associations with inflammatory diseases, the regulation of calprotectin overexpression in inflammation is unclear. Studies have indicated that the patterns of metabolism of calprotectin do not change during up- or down-regulation. By Northern-blot analysis it has been shown that calprotectin is regulated at the transcriptional level, rather than by biochemical modification of the complex (Roth *et al*. 1994). Regulation of gene expression is often associated with the binding of transcription factors in the 5′-upstream region of a gene. Based on progress in locating the regulatory elements and the corresponding transcription factors of the *S100A8* gene (Melkonya *et al*. 1998; Passey *et al*. 1999; Klempt *et al*. 1999; Nacken *et al*. 2001), three SNPs in the upstream region of *S100A8* were selected from the GenBank database for the present study. They are rs3795391, rs3806232 and rs3885688.

In our pilot study rs3795391, 94bp upstream from the ATG start codon in *S100A8*, was examined in a Chinese group (Li *et al*. 2004). This SNP is found in a cis-acting element, the gamma-interferon response element (γ-IRE) (CWKKANNY), and corresponds to the *N*. Thus, it could be deduced that this polymorphism may not alter transcription factor binding and transcriptional activity through this γ-IRE. Considering that periodontitis is a multi-factorial disease, a multivariate logistic regression model was used to further analyze the data in the present study. The combined effects of genotype/allele and gender on periodontitis were explored and observed. The adjusted OR was 0.3 (95%CI = 0.1-0.9, $P = 0.032$) for the G^+ genotype in the male group for AgP susceptibility, compared to the AA genotype in the female group. The adjusted OR was 0.3 (95%CI = 0.1-0.9, $P = 0.024$) for the allele G in the male group, compared to allele A in the female group. Because the OR value is lower than 1, the G^+ genotype and G allele may be considered to exert a significant protective effect in males against AgP. While no combined association of genotype and gender on CP susceptibility was demonstrated for rs3795391, there was an increasing risk of CP in males with allele A compared to females with allele A (OR = 2.5, 95%CI = 1.3-5.0, $P = 0.008$). Though no direct effect of this SNP was deduced, as discussed above, another possibility is that the effect of this SNP may be mediated by linkage disequilibrium with an as yet unidentified neighbouring polymorphism. For rs3806232 similar results were confirmed. The G^+ genotype and G allele exerted a significant protective effect in males against AgP (genotype: OR = 0.2, 95% CI = 0.1-0.8, $P = 0.017$; allele: OR = 0.2, 95% CI = 0.1-0.7, $P = 0.013$). Allele A also increased the risk in males for CP (OR = 2.5, 95%CI = 1.3-5.0, $P = 0.009$). In the present study, no person carried the rs3885688 polymorphism from the 254 recruited subjects. We also combined CP patients and AgP patients to form a disease group. The same statistical analysis was also used to test the difference between the periodontitis group and healthy controls, but no striking associations were detected. Although AgP shares many pathologic features with CP at the tissue level, different genes appear to be related to host susceptibility for different types of periodontitis. The two periodontal diseases are likely under different genetic controls. Our results also reflected the heterogeneity of the pathogenic mechanisms of periodontitis in the Chinese.

The reasons for the high level of calprotectin in the GCF of periodontitis patients are not clear. Because the amino acid sequences of S100A8 and A9 do not

include a signal or membrane anchor sequence, it is widely accepted that calprotectin is not secreted extracellularly, except as a result of cell disruption or death (Voganatsi *et al*. 2001). Another study has indicated that calprotectin is secreted after activation of protein kinase C, via a novel alternative pathway depending on an intact microtubule network (Rammes *et al*. 1997). As for cytokine release in human neutrophils, calprotectin was induced by the LPS of *P. gingivalis* via the CD14-TLR2-NF-κB signal pathway in the same manner (Kido *et al*. 2003). So the possibility that the protein merely reflects numbers of dying neutrophils is not generally considered to be correct. The level of calprotectin in GCF varied in normal children, showing that expression differences exist in normal individuals (Miyasaki *et al*. 1998). These evidences support the hypothesis that SNPs in the upstream region of the *S100A8* gene might influence protein levels and be associated with disease susceptibility.

The prevalence and sex ratio for periodontitis varies geographically and/or racially in different populations. Gender differences in prevalence and the extent of attachment loss in adult populations have also been reported (Grossi *et al*. 1994, 1995). Investigations of HLA associations with periodontitis have been carried out, and have revealed gender-dependent HLA deviations (Reichert *et al*. 2002). In the Chinese population, the polymorphisms *IL-1A* +4845 and *IL1B* − 511 may play an important role in determining GAgP susceptibility in males (Li *et al*. 2004), and the XX genotype of the estrogen receptor-α gene may be a risk indicator for chronic periodontitis in females (Zhang *et al*. 2004). Gender-dependent genetic susceptibility or resistance factors could exist for periodontitis. Some studies have revealed the combined effects of certain gene polymorphisms and gender on disease susceptibility. The tumour necrosis factor-α -308 polymorphism may contribute to coronary heart disease risk only in women patients with type 2 diabetes (Vendrell *et al*. 2003). The E-selectin S128R polymorphism was found to be significantly associated with the presence of coronary artery calcification in women under 50 years of age (Ellsworth *et al*. 2001). A promoter polymorphism, *IL8* -251, has a protective effect for lung cancer in female subjects (Campa *et al*. 2004). The *IL1A* -889 A1/A1 genotype has been shown to be associated with earlier onset of myasthenia gravis (MG), particularly in males (Sciacca *et al*. 2002). In a Finnish population, the frequency of *IL1B* -511A1/A2 heterozygotes was decreased in male asthmatic patients compared to male controls (Karjalainen *et al*. 2002). Our findings also suggest that gender might play a role in individual predisposition to periodontitis. A combined effect for SNPs in the *S100A8* gene and gender on periodontitis susceptibility has been suggested. Meanwhile, it should be noted that the biological significance of gene polymorphisms should be mainly based on genotype. The exact correlation between CP risk and the combined effect of gender and polymorphisms needs to be further demonstrated.

To date, there have not been any reports of association analysis between the SNPs of *S100A8* and diseases susceptibility, including for periodontitis. Our study has indicated that the combined effect of polymorphisms upstream from the ATG start codon in *S100A8* and gender maybe associated with periodontitis susceptibility in the Chinese. Due to the important role of, and lack of knowledge about, calprotectin, further studies are needed to examine the whole extent of *S100A8* for new and functional polymorphisms, and to explore the association between the severity of periodontitis and polymorphisms. Also, it is necessary to study further whether any polymorphisms result in difference in protein expression levels.

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References

Armitage, G. C. (1999) Development of a classification system for periodontal diseases and conditions. *Ann Periodontol* **4**, 1–6.

Bhardwaj, R. S., Zotz, C., Zwadlo-Klarwasser, G. *et al*. (1992) The calciumbinding proteins MRP8 and MRP14 form a membrane-associated heterodimer in a subset of monocytes/macrophages present in acute but absent in chronic inflammatory lesions. *Eur J Immunol* **22**, 1891–1897.

Campa, D., Zienolddiny, S., Maggini, V. *et al*. (2004) Association of a common polymorphism in the cyclooxygenase 2 gene with risk of non-small cell lung cancer. *Carcinogenesis* **25**, 229–235.

Ellsworth, D. L., Bielak, L. F., Turner, S. T. *et al*. (2001) Gender- and age-dependent relationships between the E-selectin S128R polymorphism and coronary artery calcification. *J Mol Med* **79**, 390–398.

Eue, I., Pietz, B., Storck, J. *et al*. (2000) Transendothelial migration of 27E10$^+$ human monocytes. *Int Immunol* **12**, 1593–1604.

Graves, D. T. & Cochran, D. (2003) The contribution of interleukin-1 and tumor necrosis factor to periodontal tissue destruction. *J Periodontol* **74**, 391–401.

Grossi, S. G., Zambon, J. J., Ho, A. W. *et al*. (1994) Assessment of risk for periodontal disease. I. Risk indicators for attachment loss. *J Periodontol* **65**, 260–267.

Grossi, S. G., Genco, R. J., Machtei, E. E. *et al*. (1995) Assessment of risk for periodontal disease. II. Risk indicators for alveolar bone loss. *J Periodontol* **66**, 23–29.

Jinquan, T., Vorum, H., Larsen, C. G. *et al*. (1996) Psoriasin: a novel chemotactic protein. *J Invest Dermatol* **107**, 5–10.

Karjalainen, J., Nieminen, M. M., Aromaa, A. *et al*. (2002) The IL-1beta genotype carries asthma susceptibility only in men. *J Allergy Clin Immunol* **109**, 514–516.

Kido, J., Kido, R., Suryono, *et al*. (2003) Calprotectin release from human neutrophils is induced by Porphyromonas gingivalis lipopolysaccharide via the CD-14-Toll-like receptor-nuclear factor kappaB pathway. *J Periodontal Res* **38**, 557–563.

Klempt, M., Melkonyan, H., Hofmann, H. A., Sorg, C. (1999) Identification of epithelial and myeloid-specific DNA elements regulating MRP14 gene transcription. *J Cell Biochem* **73**, 49–55.

Komada, T., Araki, R., Nakatani, K. *et al*. (1996) Novel specific chemtactic receptor for S100L protein on guinea pig eosinophils. *Biochem Biophys Res Commun* **220**, 871–874.

Kornman, K. S., Crane, A., Wang, H. Y. *et al*. (1997) The interleukin-1 genotype as a severity factor in adult periodontal disease. *J Clin Periodontol* **24**, 72–77.

Lackmann, M., Rajasekariah, P., Iismaa, S. E. *et al*. (1993) Identification of a chemotactic domain of the pro-inflammatory S100 protein CP-10. *J Immunol* **150**, 2981–2991.

Li, J., Meng, H. & Chen, Z. (1998) Electrophoresis of proteins in gingival crevicular fluid of patients with adult periodontitis before and after treatment. *Zhonghua Kou Qiang Yi Xue Za Zhi* **33**, 276–278. (Chinese)

Li, Q. Y., Zhao, H. S., Meng, H. X. *et al*. (2004) Association Analysis Between Interleukin-1 Family Polymorphisms and Generalized Aggressive Periodontitis in a Chinese Population. *J Periodontol* **75**, 1627–1635.

Li, Q. Y., Zhao, H. S., Meng, H. X. *et al*. (2004) The relationship between a nucleotide substitution of S100A8 gene and aggressive periodontitis. *Zhonghua Kou Qiang Yi Xue Za Zhi* **39**, 181–184. (Chinese)

Loos, B. G., Leppers-Van de Straat, F. G., Van de Winkel, J. G. *et al*. (2003) Fcgamma receptor polymorphisms in relation to periodontitis. *J Clin Periodontol* **30**, 595–602.

Melkonyan, H., Hofmann, H. A., Nacken, W. *et al*. (1998) The gene encoding the myeloid-related protein 14 (MRP14), a calciumbinding protein expressed in granulocytes and monocytes, contains a potent enhancer element in the first intron. *J Biol Chem* **273**, 27026–27032.

Miyasaki, K. T., Bodeau, A. L., Murthy, A. R. *et al*. (1993) In vitro antimicrobial activity of the human neutrophil cytosolic S-100 protein complex, calprotectin, against Capnocytophaga sputigena. *J Dent Res* **72**, 517–523.

Miyasaki, K. T., Voganatsi, A., Huynh, T. *et al*. (1998) Calprotectin and lactoferrin levels in the gingival crevicular fluid of children. *J Periodontol* **69**, 879–883.

Nacken, W., Lekstrom-Himes, J. A., Sorg, C. *et al*. (2001) Molecular analysis of the mouse S100A9 gene and evidence that the myeloid specific transcription factor C/EBPepsilon is not required for the regulation of the S100A9/A8 gene expression in neutrophils. *J Cell Biochem* **80**, 606–616.

Nakamura, T., Kido, J., Kido, R., *et al*. (2000) The association of calprotectin level in gingival crevicular fluid with gingival index and the activities of collagenase and aspartate aminotransferase in adult periodontitis patients. *J Periodontol* **71**, 361–367.

Nisapakultorn, K., Ross, K. F. & Herzberg, M. C. (2001a) Calprotectin expression in vitro by oral epithelial cells confers resistance to infection by Porphyromonas gingivalis. *Infect Immun* **69**, 4242–4247.

Nisapakultorn, K., Ross, K. F. & Herzberg, M. C. (2001b) Calprotectin expression inhibits bacterial binding to mucosal epithelial cells. *Infect Immun* **69**, 3692–3696.

Nishimura, F., Terranova, V. P., Sawa, T. *et al*. (1999) Migration inhibitory factor related protein-8 (MRP-8) is an autocrine chemotactic factor for periodontal ligament cells. *J Dent Res* **78**, 1251–1255.

Nishimura, F. & Terranova, V. P. (1996) Comparative study of the chemotactic responses of periodontal ligament cells and

gingival fibroblasts to polypeptide growth factors. *J Dent Res* **75**, 986–992.

Passey, R. J., Xu, K., Hume, D. A., Geczy, C. L. (1999) S100A8: emerging functions and regulation. *J Leukoc Biol* **66**, 549–556

Rammes, A., Roth, J., Goebeler, M. *et al*. (1997) Myeloid-related protein (MRP) 8 and MRP14, calcium-binding proteins of the S100 family, are secreted by activated monocytes via a novel, tubulin-dependent pathway. *J Biol Chem* **272**, 9496–9502.

Reichert, S., Stein, J., Gautsch, A. *et al*. (2002) Gender differences in HLA phenotype frequencies found in German patients with generalized aggressive periodontitis and chronic periodontitis. *Oral Microbiol Immunol* **17**, 360–368.

Roth, J., Goebeler, M., Wrocklage, V. *et al*. (1994) Expression of the calcium-binding proteins MRP8 and MRP14 in monocytes is regulated by a calcium-induced suppressor mechanism. *Biochem J* **301**, 655–660.

Roth, J., Vogl, T., Sunderkotter, C. *et al*. (2003) Chemotactic activity of S100A8 and S100A9. *J Immunol* **171**, 5651.

Roulin, K., Hagens, G., Hotz, R. *et al*. (1999) The fatty acid-binding heterocomplex FA-p34 formed by S100A8 and S100A9 is the major fatty acid carrier in neutrophils and translocates from the cytosol to the membrane upon stimulation. *Exp Cell Res* **247**, 410–421.

Ryckman, C., Vandal, K., Rouleau, P. *et al*. (2003) Proinflammatory activities of S100: proteins S100A8, S100A9, and S100A8/A9 induce neutrophil chemotaxis and adhesion. *J Immunol* **170**, 3233–3242.

Schafer, B. W., Wicki, R., Engelkamp, D. *et al*. (1995) Isolation of a YAC clone covering a cluster of nine S100 genes on human chromosome 1q21: rationale for a new nomenclature of the S100 calcium-binding protein family. *Genomics* **25**, 638–643.

Sciacca, F. L., Ferri, C., Veglia, F. *et al*. (2002) IL-1 genes in myasthenia gravis: IL-1A -889 polymorphism associated with sex and age of disease onset. *J Neuroimmunol* **122**, 94–99.

Seeliger, S., Vogl, T., Engels, I. H. *et al*. (2003) Expression of calcium-binding proteins MRP8 and MRP14 in inflammatory muscle diseases. *Am J Pathol* **163**, 947–956.

Vandal, K., Rouleau, P., Boivin, A. *et al*. (2003) Blockade of S100A8 and S100A9 suppresses neutrophil migration in response to lipopolysaccharide. *J Immunol* **171**, 2602–2609.

Vendrell, J., Fernandez-Real, J. M., Gutierrez, C. *et al*. (2003) A polymorphism in the promoter of the tumor necrosis factor-alpha gene (-308) is associated with coronary heart disease in type 2 diabetic patients. *Atherosclerosis* **167**, 257–264.

Voganatsi, A., Panyutich, A., Miyasaki, K. T. *et al*. (2001) Mechanism of extracellular release of human neutrophil calprotectin complex. *J Leukoc Biol* **70**, 130–134.

Wang, R., Meng, H., Chen, Z. *et al*. (2002) Analysis of N-terminal amino acid sequence of 12 000-protein in gingival crevicular fluid and its clinical significance. *Zhonghua Kou Qiang Yi Xue Za Zhi* **37**, 297–299. (Chinese)

Yang, Z., Tao, T., Raftery, M. J. *et al*. (2001) Proinflammatory properties of the human S100 protein S100A12. *J Leukoc Biol* **69**, 986–994.

Yui, S., Mikami, M., Tsurumaki, K. *et al*. (1997) Growth-inhibitory and apoptosis-inducing activities of calprotectin derived from inflammatory exudate cells on normal fibroblasts: regulation by metal ions. *J Leukoc Biol* **61**, 50–57.

Yui, S., Mikami, M. & Yamazaki, M. (1995) Induction of apoptotic cell death in mouse lymphoma and human leukemia cell lines by a calcium-binding protein complex, calprotectin, derived from inflammatory peritoneal exudate cells. *J Leukoc Biol* **58**, 650–658.

Yui, S., Nakatani, Y. & Mikami, M. (2003) Calprotectin (S100A8/S100A9), an inflammatory protein complex from neutrophils with a broad apoptosis-inducing activity. *Biol Pharm Bull* **26**, 753–760.

Zhang, L., Meng, H., Zhao, H. *et al*. (2004) Estrogen receptor-alpha gene polymorphisms in patients with periodontitis. *J Periodontal Res* **39**, 362–366.

Zhao, Y., Meng, H. & Chen, Z. (2003) Calprotectin and total protein in gingival crevicular fluid during experimental gingivitis. *Beijing Da Xue Xue Bao* **35**, 668–670. (Chinese)

Zhou, H., Meng, H. & Chen, Z. (1998) Electrophoretic analysis of protein composition in gingival crevicular fluid. *Zhonghua Kou Qiang Yi Xue Za Zhi* **33**, 109–112. (Chinese)