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Analysis of plasma calprotectin and polymorphisms of S100A8 in patients with aggressive periodontitis

X. Sun, H. Meng, D. Shi, L. Xu, L. Zhang, Z. Chen, X. Feng, R. Lu
Department of Periodontology, Peking University School and Hospital of Stomatology, Beijing, China

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Background and Objective: Calprotectin is an important proinflammatory mediator in various inflammatory diseases and is composed of two subunits (S100A8 and S100A9). However, the level of calprotectin in plasma of patients with aggressive periodontitis and its relationship with gene polymorphisms of *S100A8* are unclear.

Material and Methods: The plasma concentrations of calprotectin were measured, using an enzyme immunoassay, in 139 patients with aggressive periodontitis and in 88 periodontally healthy control subjects. These patients were genotyped for the rs3795391 and rs3806232 polymorphisms of *S100A8*.

Results: The plasma concentration of calprotectin in patients with aggressive periodontitis was significantly higher than in controls (2.17 mg/L vs. 1.72 mg/L, respectively, $p = 0.001$). The percentage of the AA genotype of *S100A8* rs3795391 was significantly higher in patients than in controls (82% vs. 69.3%, respectively, $p = 0.027$), while the frequency of the allele G was decreased among patients compared with controls (9.6% vs. 16.1%, respectively, $p = 0.036$), which was especially apparent in men (rs3795391 genotype, $p = 0.005$; rs3795391 allele, $p = 0.015$). The mean probing depth in patients carrying the AA genotype was significantly higher than that of patients carrying the GA + GG genotype of two polymorphisms of *S100A8* (rs3795391, $p = 0.035$; rs3806232, $p = 0.040$), whereas the levels of calprotectin between different genotypes were not significantly different (rs3795391, $p = 0.11$; rs3806232, $p = 0.15$).

Conclusion: These findings indicate that aggressive periodontitis is associated with elevated levels of plasma calprotectin and that gene polymorphisms of *S100A8* may influence the susceptibility and severity of aggressive periodontitis.

Huanxin Meng, Department of Periodontology, Peking University School and Hospital of Stomatology, 22 Zhongguancun Nandajie, Haidian District, 100081 Beijing, China
Tel: 0086 10 62179977 ext. 5522 (O)
Fax: 0086 10 62173402
e-mail: kqhxmeng@bjmu.edu.cn.

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Calprotectin is a heterodimer of the calcium-binding proteins S100A8 and S100A9. It is constitutively expressed by neutrophils, activated monocytes, epithelial cells and keratinocytes (1–4). Calprotectin has been shown to be an important mediator in inflammatory reactions, and is involved in the transendothelial migration of myeloid cells upon neutrophil activation or endothelial adhesion of monocytes (5). Another important proinflammatory

property of calprotectin is its chemotactic effect (6,7). Elevated levels of calprotectin have been reported in numerous pathological conditions associated with inflammation (e.g. rheumatoid arthritis, systemic lupus erythematosus and chronic inflammatory bowel diseases) (8–10), More recently, numerous studies have emerged documenting that increased levels of calprotectin are present in patients with diabetes, cardiovascular diseases and various cancers (11–14).

Periodontitis is an infectious disease caused by periodontopathic bacteria. Inflamed gingiva were found to contain vast numbers of calprotectin-positive macrophages around the vessels in central connective tissue and adjacent to the epithelium (15). Calprotectin and its subunits have also been found in the gingival crevicular fluid from diseased gingival sites of periodontal patients, and the concentrations of calprotectin and its subunits were significantly higher in diseased sites than in healthy sites (16–20). The level of calprotectin in the gingival crevicular fluid of periodontitis patients was positively correlated with clinical parameters and biochemical markers, including probing depth, interleukin-1beta, prostaglandin E2, collagenase and aspartate aminotransferase (21,22), highlighting the potential importance of calprotectin in the inflammatory response mechanism of periodontal disease.

Accumulated evidence has shown that periodontitis is associated with raised systemic inflammation, measured as changes in plasma proteins, including acute-phase proteins, immunoglobulins and inflammatory mediators (23–26). However, the effect of periodontitis on the level of plasma calprotectin has not yet been reported.

Aggressive periodontitis is a severe type of periodontitis, characterized by rapid periodontal destruction and familial aggregation. Genetic predisposition to aggressive periodontitis has been a major focus of intense investigation for many years. Associations between polymorphisms of a number of genes, such as interleukin-1, Fc receptors and vitamin D receptor, and aggressive periodontitis have been reported, although no consistent pattern of inherited risk has emerged (27–29).

As discussed above, calprotectin is an important proinflammatory mediator with multiple regulatory functions in periodontitis and diverse inflammatory diseases. It is therefore reasonable to consider the calprotectin gene as an ideal candidate for conferring genetic susceptibility to periodontitis. S100A8 is the light subunit of calprotectin, and the single nucleotide polymorphisms (SNPs) rs3795391 A > G and rs3806232 A > G are found upstream of the ATG start codon of the *S100A8* gene. Our previous study demonstrated that a combined effect of these two SNPs and gender might be associated with susceptibility to aggressive periodontitis in Chinese people (30). Many gene polymorphisms may be significant in that they are likely to act via changes in the levels or activity of specific proteins. However, little is known about the relationship, if any, between *S100A8* gene polymorphisms and the level of plasma calprotectin in patients with inflammatory diseases.

The aim of the present study was to investigate the influence of aggressive periodontitis on the level of calprotectin in plasma, and to examine the relationship between *S100A8* gene polymorphisms and the level of calprotectin in patients with aggressive periodontitis.

Material and methods

Study population

One hundred and thirty-nine subjects with aggressive periodontitis were selected from the patients of the Department of Periodontology at Peking University Hospital of Stomatology. The diagnostic criteria were defined according to the classification proposed at the International Workshop for the Classification of Periodontal Diseases and Conditions in 1999. The details were as follows: periodontal disease onset before 35 years of age; and at least eight teeth (three or more of which were not first molars or incisors) with a probing depth of > 6 mm and radiographic evidence of alveolar bone loss.

Eighty-eight periodontally healthy control subjects were recruited from staff and students at the School of Stomatology, none of whom had any clinical evidence of periodontitis (probing depth ≤ 3 mm; and < 10% of sites with a bleeding index of ≥ 3).

None of the subjects had any systemic disease or were taking any medication known to affect periodontal status. All subjects were members of the Chinese Han race and nonsmokers. Exclusion criteria were treatment with antibiotics at least 3 mo before the start of the study and/or periodontal therapy at least 1 year before the start of the study. Pregnant women were also excluded. The present study was conducted after obtaining informed consent from all subjects and was approved by the Ethics Committee of Peking University Health Science Center.

Clinical parameters

Full-mouth periodontal examinations were conducted using a Williams periodontal probe. Probing depth was measured from the gingival margin to the base of the crevice/pocket at six sites (mesial, distal and the middle sites of both buccal and lingual sides) of all teeth except third molars. The attachment level was measured from the cemento–enamel junction to the base of the crevice/pocket at the respective site. Sites with both probing depth > 6 mm and attachment level > 5 mm were defined as severe. The percentage of severe sites was calculated. These examinations were performed by two skilled practitioners. For each patient, a set of full-mouth periapical radiographs was taken.

Blood collection and assessment

The blood samples were obtained from each fasting examinee by standard venipuncture using EDTA-containing tubes, between 08:00 and 10:00 h. The neutrophil count in the blood samples was measured using hematology analyzers. Then, the plasma was separated and immediately stored frozen at −70°C until required for further analyses. Plasma calprotectin levels were

measured using a commercially available ELISA (Phical test; Calpro AS, Oslo, Norway) according to the manufacturer's protocol.

S100A8 genotyping

The subjects' DNA was extracted from the EDTA-anticoagulated blood sample. The presence of *S100A8* rs3795391and rs3806232 polymorphisms were measured usingPCR–DNA amplification and agarose-gel electrophoresis. Primer sequences and PCR conditions used to determine the patients' genotypes for *S100A8* rs3795391 and *S100A8* rs3806232 were as described in our previous article (30).

Statistical methods

The results are presented as mean and standard deviation. The Student's *t*-test was used to compare the levels of calprotectin and neutrophil counts in different groups. Significance of differences of clinical parameters between two groups was determined using the Mann–Whitney *U*-test. Comparisons of genotype and allele distributions between groups were analyzed using the chi-square test. Correlations between the plasma calprotectin concentration and clinical and hematological parameters were calculated using Spearman correlation analyses.

Results

The characteristics and clinical parameters of the aggressive periodontitis and control groups are summarized in Table 1, which shows that these two groups were different with respect to probing depth, attachment level and percentage of severe sites. As expected, generally more severe clinical indices were observed in the group with aggressive periodontitis and less severe indices were observed in the control group. For body mass index, gender and age, no significant differences were observed between aggressive periodontitis and control groups. Table 1 shows that the neutrophil count (10^9 cells/L) of patients with aggressive periodontitis was significantly higher than that of the control group (4.17 ± 1.83 vs. 3.17 ± 0.88, respectively, $p < 0.01$).

The mean plasma concentration of calprotectin in patients with aggressive periodontitis was 2.17 mg/L, vs. 1.72 mg/L inthe control group (Fig. 1); the difference between these two groups was statistically significant ($p = 0.001$). Furthermore, the levels of calprotectin in aggressive periodontitis patients were significantly associated with probing depth, attachment level and neutrophil count, as seen in Table 2, while no significant associations were found between the levels of calprotectin and the percentage of severe sites in aggressive periodontitis patients.

The genotype and allele distributions of the two *S100A8* polymorphisms are presented in Table 3. They were in accordance with those expected under the Hardy–Weinberg equation. The percentage of the AA genotype of *S100A8* rs3795391 was significantly increased in aggressive periodontitis patients compared with controls (82% vs. 69.3%, respectively, $p = 0.027$), while the frequency of the allele G was decreased among aggressive periodontitis patients compared with controls (9.6% vs. 16.1%, respectively, $p = 0.036$). The frequency of the AA genotype of *S100A8* rs3806232 was also increased in aggressive periodontitis patients compared with controls (81.3% vs. 71.9%, respectively, $p = 0.089$), and the frequency of the allele G was decreased among aggressive periodontitis patients compared with controls (9.9% vs. 15.4%, respectively, $p = 0.061$). When the subjects were stratified by gender, the frequencies of the GA + GG genotype and of the G allele of these two polymorphisms in male patients were much lower than in controls (rs3795391: genotype: $p = 0.005$; allele: $p = 0.015$; rs3806232:

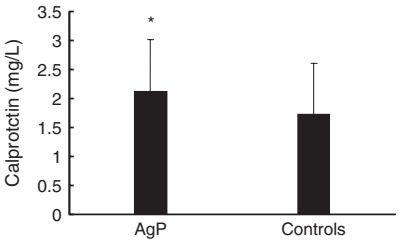

Fig. 1. Plasma levels of calprotectin in 139 aggressive periodontitis (AgP) patients and in 88 periodontally healthy controls. Values are shown as mean ± standard deviation. Significant differences between groups were obtained using the Student's *t*-test. The asterisk indicates a statistically significant difference ($p = 0.001$).

Table 2. Univariate analysis of associations of calprotectin concentrations with demographic and clinical variables in aggressive periodontitis (AgP) patients ($n = 139$)

	r	*p*-value
Age	0.050	0.553
Gender	−0.079	0.254
PD	0.267	0.002
AL	0.202	0.024
Percentage of severe sites	0.055	0.538
Neutrophil count	0.238	0.012

AL, attachment level; PD, probing depth.
p-values are based on Spearman correlation analysis.

Table 1. Characteristics, clinical parameters and neutrophil counts in aggressive periodontitis patients and control subjects

	Control ($n = 88$)	AgP ($n = 139$)
Age (years)	26.5 ± 4.7	26.9 ± 5.7
Gender (F/M)	55/33	91/48
BMI (kg/m^2)	21.3 ± 2.09	21.7 ± 3.7
Probing depth (mm)	1.92 ± 0.28	4.87 ± 1.06*
Attachment loss (mm)	0.03 ± 0.14	4.89 ± 1.83*
Severe sites (%)	0	34.7 ± 21.1*
Neutrophil count (10^9 cells/L)	3.17 ± 0.88	4.17 ± 1.83*

The data are given as mean ± SD, unless indicated otherwise.
BMI, body mass index; F, female; M, male.
Severe site: probing depth > 6 mm and attachment level > 5 mm.
*Compared with the control group, $p < 0.01$.

Table 3. The genotype distributions and allele frequencies of *S100A8* rs3795391 and rs3806232 in aggressive periodontitis (AgP) patients and control subjects

	AgP (%)	Control (%)	*p*-value
rs3795391	139	88	
Genotype			
AA	114 (82)	61 (69.3)	0.027
GA	23 (16.5)	25 (28.4)	
GG	2 (1.4)	2 (2.3)	
GA + GG	25 (17.9)	27 (30.7)	
Allele			
A	255 (90.4)	151 (83.9)	
G	27 (9.6)	29 (16.1)	0.036
rs3806232	139	88	
Genotype			
AA	113 (81.3)	63 (71.9)	0.089
GA	24 (17.3)	22 (24.7)	
GG	2 (1.4)	3 (3.4)	
GA + GG	26 (18.8)	25 (28.1)	
Allele			
A	254 (90.1)	154 (84.6)	
G	28 (9.9)	28 (15.4)	0.061

p-values were calculated using the chi-square test.

genotype: $p = 0.005$; allele: $p = 0.008$), as shown in Table 4. On the other hand, no differences were seen in female patients between aggressive periodontitis and control groups (rs3795391: genotype: $p = 0.468$, allele: $p = 0.379$; rs3806232: genotype: $p = 0.719$, allele: $p = 0.938$).

The levels of calprotectin and clinical parameters in aggressive periodontitis patients with different genotypes are shown in Table 5. The mean probing depth in patients carrying the AA genotype was significantly higher than that of the patients carrying the GA + GG genotype of *S100A8* rs3795391 and of *S100A8* rs3806232 (rs3795391: 4.96 ± 1.08 vs. 4.43 ± 0.84, $p = 0.035$; rs3806232: 4.96 ± 1.07 vs. 4.45 ± 0.82, $p = 0.040$). No significant differences were found in the level of calprotectin and attachment level between aggressive periodontitis patients with different genotypes of the two polymorphisms of *S100A8*. Table 6 shows the correlations between the plasma levels of calprotectin and the parameters evaluated (probing depth, attachment level and neutrophil count) in aggressive periodontitis patients with different genotypes. Significant associations were shown between the levels of plasma calprotectin and probing depth and neutrophil count in patients with the AA genotype of the two SNPs of *S100A8* (rs3795391, $p = 0.015$ and $p = 0.002$; rs3806232, $p = 0.012$ and $p = 0.001$), whereas no relationship was found between calprotectin levels and any of the evaluated variables (probing depth, attachment level and neutrophil count) in patients with the GA + GG genotype of the two SNPs of *S100A8*.

Table 4. Genotype distributions and allele frequencies of *S100A8* rs3795391 and rs3806232 in male and female subjects with and without aggressive periodontitis (AgP)

	Male			Female		
	AgP	Control	*p*-value	AgP	Control	*p*-value
rs3795391						
Genotype	48	33		91	55	
AA	43 (89.6)	21 (63.6)		71 (78)	40 (72.7)	
AG	4 (8.3)	12 (36.4)		19 (20.9)	13 (23.6)	
GG	1 (2.1)	0		1 (1.1)	2 (3.6)	
GA + GG	5 (10.4)	12 (36.4)	0.005	20 (22)	15 (27.3)	0.468
Allele						
A	92 (93.9)	54 (81.8)		163 (88.6)	97 (85.1)	
G	6 (6.1)	12 (18.2)	0.015	21 (11.4)	17 (14.9)	0.379
rs3806232						
Genotype						
AA	43 (89.6)	21 (63.6)		70 (76.9)	42 (76.4)	
AG	5 (10.4)	12 (36.4)		19 (20.9)	10 (18.2)	
GG	0	0		2 (2.2)	3 (5.5)	
GA + GG	5 (10.4)	12 (36.4)	0.005	21 (23.1)	13 (23.7)	0.719
Allele						
A	91 (94.8)	54 (81.8)		163 (87.6)	100 (86.2)	
G	5 (5.2)	12 (18.2)	0.008	23 (12.4)	16 (13.8)	0.938

Data are given as *n* (%), unless indicated otherwise.
p-values were calculated using the chi-square test.

Discussion

The present study showed that the plasma levels of calprotectin are elevated in aggressive periodontitis patients and are positively correlated with clinical parameters such as probing depth and attachment level. These results suggest that aggressive periodontitis may be associated with a raised systemic level of calprotectin.

There are several possible origins of the calprotectin found in the aggressive periodontitis patients in our study. Another study in our laboratory demonstrated that the concentration of calprotectin in gingival crevicular fluid obtained from the same group of patients as in the present study was 2131.39 mg/L (data unpublished), which was almost 1000 times higher than the plasma concentration (1.72 mg/L) found in this study. It has been reported that lipopolysaccharides

Table 5. The levels of calprotectin (mg/L) and clinical parameters in aggressive periodontitis patients with different genotypes of *S100A8*

	n	Calprotectin (mg/L)	PD	AL
rs3795391				
GA + GG	25	1.88 ± 1.08	4.43 ± 0.84	4.63 ± 1.52
AA	114	2.24 ± 1.04	4.96 ± 1.08*	4.94 ± 1.88
rs3806232				
GA + GG	26	1.91 ± 1.08	4.45 ± 0.82	4.60 ± 1.49
AA	113	2.22 ± 1.04	4.96 ± 1.07*	4.95 ± 1.88

*Compared with the GA + GG genotype ($p < 0.05$).
AL, attachment level; PD, probing depth.

Table 6. Correlations between plasma levels of calprotectin and the evaluated parameters (attachment level, probing depth and neutrophil count) in aggressive periodontitis patients with different genotypes of *S100A8*

	n		PD	AL	Neutrophil count
rs3795391					
AA	114	*r*	0.238	0.178	0.323
		p	0.015	0.075	0.002
GA + GG	25	*r*	0.413	0.318	0.175
		p	0.063	0.160	0.460
rs3806232					
AA	113	*r*	0.246	0.196	0.330
		p	0.012	0.05	0.001
GA + GG	26	*r*	0.387	0.237	0.254
		p	0.068	0.276	0.254

AL, attachment level; PD, probing depth.

of periodontal pathogens can induce the release of calprotectin from neutrophils, macrophages and gingival epithelial cells in periodontal tissue sites (31–33). These observations indicate that abundant calprotectin in local periodontal tissues could be a potential source of calprotectin in the plasma of aggressive periodontitis patients, through the 'dumping' of calprotectin from the local periodontal tissues into the blood circulation, thus increasing the level of calprotectin in plasma.

The present study revealed that the levels of calprotectin in plasma were positively correlated with the neutrophil count in aggressive periodontitis patients. Raised numbers of leukocytes and neutrophils have previously been reported in this cohort of patients (25). The increased amounts of peripheral neutrophils in aggressive periodontitis patients can be presumed to be associated with bacteremia, occurring as a result of the spread of periodontal pathogens and their virulence factors into the blood circulation (24). This relationship between the level of calprotectin in plasma and the neutrophil count implies that peripheral neutrophils may be another source of plasma calprotectin in aggressive periodontitis.

Calprotectin may enhance the binding activity of granulocyte CD11b/CD18 integrin receptors to the endothelium and modulate the transendothelial migration of leucocytes (5,34). Another possible proinflammatory property of calprotectin is the chemotactic effect, which further promotes leucocyte recruitment (6). The elevation of calprotectin observed in aggressive periodontitis patients in the present study might induce a positive feedback loop, in which primed phagocytes and stimulated endothelium facilitate the further recruitment of phagocytes to the site of periodontal inflammation. Additionally, increased plasma levels of calprotecin among healthy individuals may predict a risk of future cardiovascular events (35), and calprotectin could induce a thrombogenic, inflammatory response in human microvascular endothelial cells (36). Thereby, it appears that the elevated systemic levels of calprotectin in patients with aggressive periodontitis give further support to the association of periodontitis with cardiovascular diseases.

Genetic predisposition to aggressive periodontitis is a major subject of intense investigation (27–29). The present study demonstrates that the frequencies of the GA + GG genotype/G allele of *S100A8* rs3795391 are significantly decreased in patients with periodontitis. In male subjects, differences in the distributions of the genotype/allele of both *S100A8* rs3795391 and rs3806232 between aggressive periodontitis patients and controls are significant. These findings are consistent with, and extend the findings of, our early study (30), suggesting that these two SNPs of *S100A8* might be associated with susceptibility to aggressive periodontitis, and gender may play a role in an individual's predisposition to aggressive periodontitis. The combined effects of certain gene polymorphisms and gender on disease susceptibility have been demonstrated in coronary heart disease and lung cancer (37,38). Investigations of human leukocyte antigen (HLA) associations with periodontitis have revealed gender-dependent HLA deviations (39). In the Chinese population, the frequency of the *IL-1B* −511 A1/A2 heterozygote was significantly increased in male patients with aggressive periodontitis compared to periodontally healthy male controls (27), and the frequency of the XX genotype of the estrogen receptor-alpha gene was significantly higher in female patients with chronic periodontitis patients than in periodontally healthy female controls (40). The present study also indicated that the polymorphisms *S100A8* rs3795391 and rs3806232 may be associated with aggressive periodontitis in men. Men and women have significant differences in the strength of their immune and inflammatory responses to invading microbes, and likewise in the

prevalence of inflammatory and autoimmune diseases, and sex hormone levels may partly contribute to this difference. Therefore, in women, predisposition to aggressive periodontitis seems to be influenced by sex hormone levels and related factors, whereas genetic variations in the host response, caused by SNPs in *S100A8*, for example, are probably related to susceptibility to aggressive periodontitis in men.

The present study also showed that the probing depth in patients with the AA genotype was significantly higher than that of patients with the GA + GG genotype, suggesting that the two SNPs of the *S100A8* gene might be associated with the severity of aggressive periodontitis. This result confirms previous reports that certain polymorphisms in some genes, such as interleukin-1, CD14 and interleukin-6, may influence the severity of periodontitis (41,42).

Disease-associated gene polymorphisms may be functional by affecting the structure of gene products, causing quantitative differences in gene expression or influencing the innate, inflammatory and immunological response to microbial infection. In the present study, we did not find any difference in the plasma level of calprotecin between aggressive periodontitis patients with different genotypes of *S100A8*. However, significant relationships between the plasma levels of calprotectin and probing depth and neutrophil numbers were observed in patients with the AA genotype of *S100A8* rs3795391 and rs3806232. These findings indicate that bacterial challenge in patients with the AA genotype results in a vigorous immune-inflammatory response, leading to severe aggressive periodontitis. The hyper-inflammatory trait of these patients seems to be genetically determined, which may be associated with two SNPs of the *S100A8* gene.

In conclusion, the present study provides the first evidence that periodontal infection is associated with increased levels of plasma calprotectin, and this is the first functional study of association between polymorphisms of *S100A8* and plasma levels of calprotectin in inflammatory diseases. Owing to the limitations of the present study, further investigations are warranted to replicate and extend these findings in a larger population, and to explore the underlying mechanism of transcriptional regulation and the signaling pathway of calprotectin expression in aggressive periodontitis.

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