J Periodont Res 2009; 44: 184–192

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2008.01103.x

Family-based association analysis of *S100A8* genetic polymorphisms with aggressive periodontitis

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Ren XY, Xu L, Meng HX, Zhao HS, Lu RF, Chen ZB, Feng XH, Shi D, Zhang L, Tian Y. Family-based association analysis of S100A8 *genetic polymorphisms with aggressive periodontitis. J Periodont Res 2009; 44: 184–192. © 2009 The Authors. Journal compilation © 2009 Blackwell Munksgaard*

Background and Objective: It is known that S100A8, a member of the S100 calcium-binding protein family, is associated with inflammatory diseases, including periodontitis. Our previous population-based study found an association between two polymorphisms, rs3795391 (A > G) and rs3806232 (A > G), in the upstream region of the *S100A8* gene and aggressive periodontitis (AgP) in Chinese people. Based on those results, this investigation set out to analyze and corroborate whether the association also exists within families.

Material and Methods: Two hundred and four subjects from 73 nuclear families were recruited. All probands and their relatives were diagnosed according to the 1999 classification of periodontal diseases. Anticoagulated peripheral blood samples were collected from all the subjects, and DNA was extracted. The two single nucleotide polymorphisms (SNPs; rs3795391 and rs3806232) were detected and analyzed by standard polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) assay. Analysis of genotype/allele was performed by Family-Based Association Test (FBAT) software (http://www.biostat.harvard.edu/~fbat/default.html).

Results: There was a statistically significant association of the SNP rs3795391 with AgP in the additive genetic model ($\chi^2 = 3.9836$, d.f. = 1, $p = 0.0459$). Allele A showed significantly preferential transmission to the AgP affected individuals ($Z = 1.996$, $p = 0.0459$). The other SNP, rs3806232, showed no significant results in all models.

Conclusions: This family-based association study supports the previous findings that SNP rs3795391 (A > G) of the *S100A8* gene might contribute to AgP susceptibility. This is, to our knowledge, the first investigation about AgP using FBAT in genetic analysis.

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Key words: aggressive periodontitis; genetics; family; *S100A8* gene

Accepted for publication March 24, 2008

Aggressive periodontitis (AgP, formerly termed juvenile periodontitis, early onset periodontitis or rapidly progressive periodontitis) is a group of infrequent types of periodontal diseases with rapid attachment loss and bone destruction initiated at a young age. Though a variety of factors, such as microbial, environmental, behavioral and systemic disease, are suggested to influence the risk of AgP, an individual's genetic make-up is a crucial factor influencing their systemic or host response-related risk (1,2).

Many studies indicate that genetic differences among individuals may play an important role in risk of AgP. Genes may play a role in the predis-

position to and progression of AgP. Identification of susceptibility genes for AgP will shed light on the underlying genetic mechanisms. Such information is important for the design of new treatment to prevent or slow down AgP development. Several localized AgP loci on chromosomes 1, 4, 6 and 9 have been identified by parametric linkage analysis (3–5). Linkage analysis has been successful in identifying genes of large effect size in monogenic diseases showing typical Mendelian inheritance patterns, but has limited power in detecting small genetic effects in complex diseases (6–8).True linkage will also be missed if a wrong genetic model is assumed in parametric linkage analysis (9). A genetic association study provides an alternative that is more powerful in detecting small genetic effects in complex traits (6–8,10).

Kornman *et al*. (11) first reported an association between polymorphisms in the genes encoding for interleukin (IL)-1 and an increased severity of chronic periodontitis. After that, a lot of gene polymorphisms, such as IL-6, IL-4, IL-10, tumor necrosis factor-α (TNF-α), Fcγ receptors, human leukocyte antigen antigens, vitamin D receptors and *N*-formylpeptide receptors, have been investigated as possible markers of increased susceptibility to AgP (12–18). Conflicting results have been presented regarding the relationship between the genotype of these genes and susceptibility to AgP in different populations. These results may reflect the genetic heterogeneity of AgP.

The gene *S100A8* (NM-002964), which is located on chromosome 1q21, comprises three exons and encodes a structural protein of 93 amino acids called S100A8. The S100A8 protein belongs to the family of calcium-binding S100 proteins (synonyms CF-Ag, L1-Ag, CP-10, calgranulin A, MRP8; 19). It is massively expressed in developing as well as mature cells of the myeloid lineage, particularly in neutrophils, monocytes and activated macrophages (20–22). In the course of the inflammatory process, S100A8-positive cells appear very early at the site of inflammation (23,24). The S100A8/S100A9 surface-positive monocytes release large amounts of TNF-α and IL-1β, in contrast to their S100A8/S100A9 surface-negative counterparts (25). Therefore, it was proposed to use S100A8 as an inflammatory marker protein. Many studies showed that S100A8 was involved in many pathological conditions, including colorectal carcinoma, cystic fibrosis and rheumatoid arthritis (26–28). Studies (including results from our laboratory) demonstrated that the concentration of S100A8 and/or calprotectin in gingival crevicular fluid correlated with periodontal inflammation (29–33). The strong and highly specific expression pattern makes the *S100A8* gene an ideal candidate for disease genetic susceptibility. Therefore, three single nucleotide polymorphisms (SNPs; rs3795391, rs3806232 and rs3885688) in the upstream region of *S100A8* from the GenBank database were selected for our previous pilot study, and G^+ genotype/G allele in males was found to be associated with AgP compared with females with the AA genotype/A allele (12,34). Based on the previous case-control results, the present investigation was designed to utilize a novel method of genetic analysis known as Family-Based Association Test (FBAT) to confirm whether there is an association between two polymorphisms (rs3795391 and rs3806232) of *S100A8* and AgP within families.

Material and methods

Study population

The protocol for the study was reviewed and approved by the Ethics Committee of the Peking University Health Science Center. The probands and all the family members who agreed to attend the study provided written informed consent. Two hundred and four subjects from 73 nuclear families (including 73 probands, 42 fathers, 49 mothers, 35 siblings, three grandparents and two offspring) were recruited. All subjects were of Chinese Han descent. All the examinations were completed at the Department of Periodontology, School and hospital of Stomatology, Peking University. Aggressive periodontitis patients who encouraged their relatives to participate in the study are termed probands. All their available first-degree blood relatives attended for the examinations by appointment. All subjects were diagnosed according to the 1999 international classification of periodontal diseases (35).

Criteria of AgP probands. (1) Under 36 years old. (2) At least eight teeth, three of them not being first molars or incisors, had a probing depth ≥ 6 mm and clinical attachment loss ≥ 5 mm. (3) The clinical diagnosis was confirmed by evidence of interproximal bone loss on full-mouth periapical radiographs. The patients were generally healthy except for the presence of periodontitis. Female patients were not pregnant or lactating.

Diagnostic criteria. Diagnosis of AgP in older individuals is problematic owing to the difficulties of distinguishing between AgP and chronic periodontitis on the basis of clinical signs, even in older edentulous individuals. In this study, however, we use the Family-Based Association Test package (FBAT), in which the parental phenotypes have no contribution to the test statistic. Therefore, the same diagnostic criteria were used in all subjects. According to the 1999 AAP Classification, all subjects were diagnosed as healthy, gingivitis, chronic periodontitis or AgP based on full-mouth periodontal chartings (including assessments of probing depths, attachment loss, bleeding on probing at six sites per tooth) and full-mouth periapical radiographs (35). In the subsequent FBAT analysis, however, only probands and siblings with AgP were taken as affected individuals, while siblings who could not be definitely diagnosed with AgP were all taken as unaffected individuals (including healthy, gingivitis and chronic periodontitis), since the FBAT test statistic is based on the distribution of the offspring genotypes with any trait information and on the parental genotypes, not on the parental trait information. Thus, parental clinical diagnoses need not be classified as AgP or not-AgP in FBAT analysis. They were diagnosed

as healthy, gingivitis, chronic periodontitis or edentulous. Further classification of chronic periodontitis was based on the extent and severity of the clinically evident periodontal destruction and divided into mild, moderate and severe types.

Genetic analysis

Isolation of genomic DNA. Anticoagulated peripheral blood samples were collected from all of the 204 subjects. The DNA was extracted using a blood DNA mini kit (Watson Biotechnologies, Inc., Shanghai, China), following the procedure listed on the kit. The DNA integrity was checked and quantitated using agarose gel electrophoresis.

Genotyping of S100A8 and quality control. A standard polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) assay was used for *S100A8* genotyping. Both SNPs, rs3795391 and rs3806232, are single nucleotide substitutions (A > G); allele A completes a corresponding restriction endonuclease site, while allele G does not. To ensure the validity of the genotyping results, direct sequencing of PCR products was performed in 20% random samples using the forward or reverse primer. Details of the study design have been described fully in the previous report (34).

Statistical analysis

Tests of individual markers for association with AgP were performed with the Family-Based Association Test package (FBAT, version 2.0.2C; http://www.biostat.harvard.edu/~fbat/default.html; 36,37). The FBAT software provides numerous enhancements to standard association tests that are adjusted for admixture, as follows. (1) Dichotomous, measured, or time-to-onset traits maybe analyzed for association. Traits may be adjusted for covariate effects. With dichotomous traits, both affected and unaffected offspring can be used. (2) The program uses data from nuclear families, sibships, or a combination of the two, to test for association between traits and genotypes. If data are available on pedigrees, the program decomposes each pedigree into individual nuclear families or sibships. It can handle any number of offspring in a family. (3) The program computes both bi-allelic tests and multi-allelic tests of association. It uses standard genetic models (additive, dominant or recessive) to test association. The effect of an allele increases with its copy number in an additive model; it is the same for one or two copies in a dominant model; and it is demonstrated only with two copies of the allele in a recessive model. (4) The program constructs, by default, a test of the null hypothesis: no linkage and no association (38).

To assess linkage disequilibrium, Lewontin's D' and the correlation coefficient r^2 between the two SNPs were calculated (39). Calculation was done using the software online (http://analysis.bio-x.cn; 40).

Results

A total of 204 subjects were recruited in this study. The demographic and clinical characteristics of study participants are summarized in Table 1. Ten relatives had no periodontal chartings or X-rays because they were edentulous or had lost more than 22 teeth, and 31 relatives had either full-mouth periodontal chartings or full-mouth periapical radiographs. There was a

Table 1. General characteristics and genotype/allele distributions of AgP probands and their relatives

Variables	Probands (n = 73) N (%)	Relatives (n = 131) N (%)
Age (years)**		
Mean ± SD	25.8 ± 6.1	46.3 ± 14.2
Range	14.0–36.0	11.0–78.0
Gender**		
Male	23 (31.5)	61 (46.6)
Female	50 (68.5)	70 (53.4)
Smoker**		
Never	67 (91.8)	99 (75.6)
Former	1 (1.4)	1 (0.7)
Current	5 (6.8)	31 (23.7)
Probing depth (mm)†		
Mean ± SD	4.8 ± 1.2	3.6 ± 0.9
Attachment loss (mm)†		
Mean ± SD	4.3 ± 1.5	3.3 ± 1.6
Number of teeth lost		
Mean ± SD	1.6 ± 2.9	5.3 ± 7.9
Number of teeth with ≥ ½ bone lost†	11.6 ± 6.7	4.3 ± 5.6
rs3795391		
Genotype		
A/A	60 (82.2)	97 (74.0)
A/G	12 (16.4)	33 (25.2)
G/G	1 (1.4)	1 (0.8)
Allele		
A	132 (90.4)	227 (86.6)
G	14 (9.6)	35 (13.4)
rs3806232		
Genotype		
A/A	59 (80.8)	101 (77.1)
A/G	14 (19.2)	29 (22.1)
G/G	0 (0.0)	1 (0.8)
Allele		
A	132 (90.4)	231 (88.2)
G	14 (9.6)	31 (11.8)

**$p < 0.01$, probands vs. relatives.

†Data on relatives (n = 90). Ten relatives had no periodontal chartings or X-rays because they were edentulous or had lost more than 22 teeth, and 31 relatives had either full-mouth periodontal chartings or full-mouth periapical radiographs.

prominently increased female/male ratio in probands compared with relatives (68.5 and 53.4%, respectively, $p = 0.036$). The mean ages of the probands and their family members were 25.8 ± 6.1 and 46.3 ± 14.2 years, respectively. The frequency of current smokers in relatives was higher than that in probands (23.7 vs. 6.8%, respectively, $\chi^2 = 9.120$, $p = 0.003$). More severe clinical indices, including probing depth, attachment loss and bone loss, were observed in AgP probands than in relatives (probing depth, 4.8 ± 1.2 vs. 3.6 ± 0.9 mm; attachment loss, 4.3 ± 1.5 vs. 3.3 ± 1.6 mm; and bone loss, 11.6 ± 6.7 vs. 4.3 ± 5.6, respectively). However, the relatives had lost more teeth than probands (5.3 ± 7.9 vs. 1.6 ± 2.9). Periodontal clinical diagnoses of relatives are presented in Table 2.

Fifty-nine out of 73 probands provided fathers and/or mothers and 28 probands provided 35 siblings. The family structure included 32 case-parent trios (including two with grandparents), 10 case-fathers, 17 case-mothers, 35 case-siblings and 2 case-offspring. The family structure is shown in Table 3, and several typical family trees are presented in Fig. 1.

The Hardy–Weinberg equilibrium was satisfied for two SNPs (rs3795391, $\chi^2 = 0.43$, $p = 0.51$; and rs3806232, $\chi^2 = 1.18$, $p = 0.277$). Table 4 reports the results of SNP rs3795391 by FBAT analysis. Eighteen informative families (i.e. families in which at least there was one heterozygous parent or a sibling) were included in the additive model. Allele A (major allele) exhibited increased transmission ($Z = 1.996$, $p = 0.0459$), whereas allele G (minor allele) showed significantly reduced transmission to the AgP affected individuals ($Z = -1.996$, $p = 0.0459$), and the global statistic was also significant ($\chi^2 = 3.9836$, d.f. = 1, $p = 0.0459$). In the dominant genetic model, allele G also showed preferential reduced transmission to the AgP offspring at a marginal level ($Z = -1.946$, $p = 0.0517$), but only one informative family was included for allele A ($Z = 0.577$, $p = 0.5637$) in this model, and the global statistic was not significant ($\chi^2 = 3.9883$, d.f. = 2, $p = 0.1361$). Dominant and recessive models give reciprocal results when the marker is biallelic, and thus are equivalent to one test for the purpose of accounting for multiple testing. Therefore, the association was not apparent in dominant or recessive models.

Results of SNP rs3806232 are shown in Table 5. Fifteen informative families were included in the additive model. A similar trend of preferential transmission was also found for allele A, but was not statistically significant ($Z = 1.441$, $p = 0.1495$), and the global statistic was not significant ($\chi^2 = 2.0769$, d.f. = 1, $p = 0.1495$). The SNP rs3806232 showed no significant results in all the three models.

The general characteristics and genotype/allele distributions of probands and their siblings are shown in Table 6. There was no statistically significant difference in the distributions of the genotypes/alleles for rs3795391 and rs3806232 in AgP-affected and unaffected individuals. However, when classified by gender, there was a significant difference in the distributions of the genotype of rs3795391 in females. There was a prominent reduction in the frequency of the G^+ genotype at rs3795391 in female

Table 2. Periodontal clinical diagnosis of relatives

Diagnosis	Frequency, N	Percentage
Healthy	1	0.8
Gingivitis	5	3.8
Mild chronic periodontitis	31	23.7
Moderate chronic periodontitis	35	26.7
Severe chronic periodontitis	42	32.1
Edentulous	4	3.1
Nearly edentulous*	8	6.1
AgP	5	3.8
Total	131	100.0

*More than 22 teeth lost.

Table 3. Family structure of the study

	Number of sibships			
	0	1	≥ 2	Total
With mother	12	3	2	17
With father	8	2	0	10
With parents	24	4	2	30
Without parents	—	11	1	12
With parents and grandparent(s)	1	—	1	2
With offspring	—	2	0	2
Total	45	22	6	73

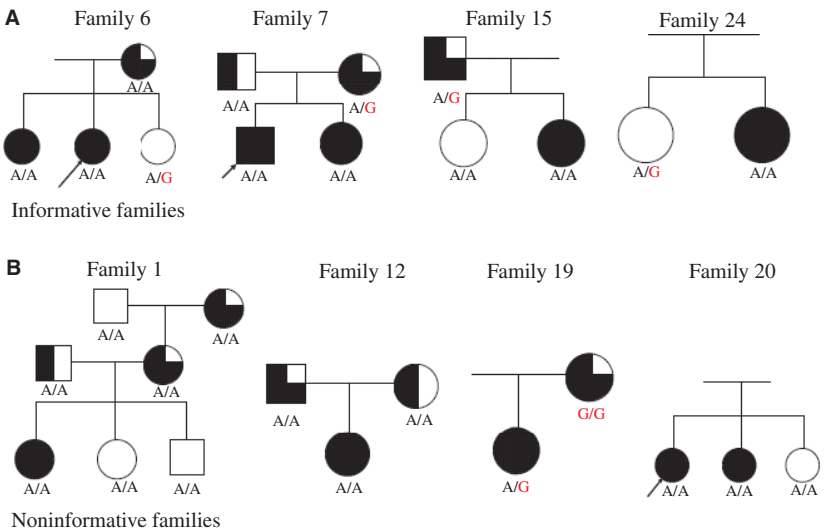

Fig. 1. Typical family trees of the study. The SNP rs3795391 is marked for each individual. Squares designate males and circles females. The proband is indicated with an arrow in families which have more than one AgP affected. Parents with gingivitis and mild chronic periodontitis were taken as healthy. (A) informative families and (B) non-informative families. Symbols: □○, healthy; ■●, AgP affected; ◣◔, adult affected by severe periodontitis; ◧◐, adult affected by moderate periodontitis.

Table 4. Association between AgP and SNP rs3795391 by FBAT analysis in 73 nuclear families

SNP rs3795391	Allele	N	Freq	Genotype	Observed	Expected		
	A	366	0.88	AA	160	161.0048		
	G	50	0.12	AG	46	43.9904		
				GG	2	3.0048		
Genetic model	Allele	Fam	*S*	*E* (*s*)	Var (*s*)	*Z*	*p*-value	Global statistic
Additive	A	18	34	29.5	5.1	1.996	0.0459*	$\chi^2(1) = 3.9836$
	G	18	6	10.5	5.1	−1.996	0.0459*	$p = 0.0459$*
Dominant	A	1	1	0.8	0.2	0.577	0.5637	$\chi^2(2) = 3.9883$
	G	18	6	10.3	4.8	−1.946	0.0517	$p = 0.1361$
Recessive	A	18	14	9.8	4.8	1.946	0.0517	$\chi^2(2) = 3.9883$
	G	1	0	0.3	0.2	−0.577	0.5637	$p = 0.1361$

Fam indicates number of informative families in which there is at least one heterozygous parent or a sibling; $Z = [S-E(s)]/\mathrm{SQRT}[\mathrm{Var}(s)]$; $S = \Sigma_{ij} T_{ij} X_{ij}$, X_{ij} denotes the genotype of the *j*-th offspring in family *i* at the locus being tested. The T_{ij} is the coded trait. $E(s)$ is the expected value. Var (s) means the variance; *p*, probability. The degree of freedom (*n*) is shown within parentheses after chi-square: $\chi^2(n)$, where $n = 1, 2$. *$p < 0.05$.

Table 5. Association between AgP and SNP rs3806232 by FBAT analysis in 73 nuclear families

SNP rs3806232	Allele	N	Freq	Genotype	Observed	Expected		
	A	370	0.889	AA	163	164.5432		
	G	46	0.111	AG	44	40.9135		
				GG	1	2.5433		
Genetic model	Allele	Fam	*S*	*E* (*s*)	Var (*s*)	*Z*	*p*-value	Global statistic
Additive	A	15	28	25	4.3	1.441	0.1495	$\chi^2(1) = 2.0769$
	G	15	6	9	4.3	−1.441	0.1495	$p = 0.1495$
Dominant	A	1	1	0.8	0.2	0.577	0.5637	$\chi^2(2) = 2.1111$
	G	15	6	8.8	4	−1.371	0.1702	$p = 0.3480$
Recessive	A	15	11	8.3	4	1.371	0.1702	$\chi^2(2) = 2.1111$
	G	1	0	0.3	0.2	−0.577	0.5637	$p = 0.3480$

Fam indicates number of informative families in which there is at least one heterozygous parent or a sibling; $Z = [S-E(s)]/\mathrm{SQRT}[\mathrm{Var}(s)]$; $S = \Sigma_{ij} T_{ij} X_{ij}$, X_{ij} denotes the genotype of the *j*-th offspring in family *i* at the locus being tested. The T_{ij} is the coded trait. $E(s)$ is the expected value. Var (s) means the variance; *p*, probability. The degree of freedom (*n*) is shown within parentheses after chi-square: $\chi^2(n)$, where $n = 1, 2$.

AgP patients compared with unaffected individuals (17.0 vs. 41.2%, respectively, $p = 0.039$, odds ratio = 0.292, 95% CI = 0.088–0.973; Table 7).

In all 204 subjects, the linkage disequilibrium between the two SNPs, rs3795391 and rs3806232, was strong. The D' value was 0.974 and correlation coefficient r^2 value was 0.860.

Discussion

This study recruited a total of 204 subjects (including 73 probands and their 131 relatives). Apart from three second degree blood relatives (grandparents), all relatives were first degree blood relatives, so this is a nuclear family study. There was a prominently increased female/male ratio in probands, which was probably a reflection of the greater number of females incorporated into the study, as expected from other similar studies (41,42).

The present family-based study showed a statistically significant association of the SNP rs3795391 of the *S100A8* gene with AgP in the additive genetic model. Allele A (major allele) exhibited increased transmission. The result supports our earlier population-based study (34). Although there were few published data on *S100A8* gene polymorphism in relation to diseases, the association is biologically plausible.

The *S100A8* gene is a member of the *S100* gene cluster on chromosome 1q21. The genes *S100A8*, *S100A9* and *S100A12* form a secondary cluster which is adjacent to the core cluster. The *S100A12* locus lies between *S100A8* and *S100A9* and the intron/exon structure more closely resembles that of *S100A8* (43).The SNPs rs3795391 (A > G) and rs3806232 (A > G) are both in the non-coding regions (intron and regulatory regions) of the *S100A8* gene and therefore do not affect the structure of gene products. However, these changes may have an effect on the regulatory aspects of *S100A8* expression such as transcription and mRNA processing.

The S100A8 protein is involved in the regulation of a number of cellular processes and extracellular regulatory activities (19). The S100A8 protein is constitutively expressed at high levels in neutrophils, monocytes and activated macrophages, and is associated with various inflammatory conditions, including periodontitis (44–46). Moreover, recent clinical and experimental data have suggested that changes in the expression and/or function of S100 proteins may represent a key step

Table 6. General characteristics and genotype/allele distributions of AgP probands and their siblings

Variables	Unaffected (n = 30) N (%)	AgP-affected (n = 78) N (%)	p	OR	95% CI
Age (years)	30 (27.8)	78 (72.2)			
Mean ± SD	28.27 ± 8.124	25.69 ± 6.027	0.075		
Gender					
Male	13 (43.3)	25 (32.1)			
Female	17 (56.7)	53 (67.9)	0.271		
Smoker					
Never	28 (93.3)	72 (92.3)	0.855		
Current	2 (6.7)	6 (7.7)			
rs3795391					
AA	20 (66.7)	65 (83.3)			
(AG + GG), G^+*	10 (33.3)	13 (16.7)	0.058	0.400	0.152–1.050
A	50 (83.3)	143 (91.7)			
G	10 (16.7)	13 (8.3)	0.075	0.455	0.188–1.101
rs3806232					
AA	20 (66.7)	64 (82.1)			
(AG + GG), G^+*	10 (33.3)	14 (17.9)	0.085	0.438	0.168–1.136
A	50 (83.3)	142 (91.0)			
G	10 (16.7)	14 (9.0)	0.107	0.493	0.206–1.180

*Owing to the low frequency of the homozygous polymorphic genotype, the GG genotype was combined with heterozygous genotype (AG) as the G-positive genotype (G^+).
OR, odds ratio.

Table 7. Combined associations of *S100A8* genotype and gender with AgP

	Gender		Unaffected N (%)	AgP-affected N (%)	p	OR	95% CI
rs3795391	Male	AA	10 (76.9)	21 (84.0)			
		G^+	3 (23.1)	4 (16.0)	0.593	0.635	0.119–3.392
		A	23 (88.5)	46 (92.0)			
		G	3 (11.5)	4 (8.0)	0.613	0.667	0.138–3.232
	Female	AA	10 (58.8)	44 (83.0)			
		G^+	7 (41.2)	9 (17.0)	**0.039***	**0.292**	**0.088–0.973**
		A	27 (79.4)	97 (91.5)			
		G	7 (20.6)	9 (8.5)	0.054	0.358	0.122–1.050
rs3806232	Male	AA	10 (76.9)	21 (84.0)			
		G^+	3 (23.1)	4 (16.0)	0.593	0.635	0.119–3.392
		A	23 (88.5)	46 (92.0)			
		G	3 (11.5)	4 (8.0)	0.613	0.667	0.138–3.232
	Female	AA	10 (58.8)	43 (81.1)			
		G^+	7 (41.2)	10 (18.9)	0.062	0.332	0.101–1.088
		A	27 (79.4)	96 (90.6)			
		G	7 (20.6)	10 (9.4)	0.083	0.402	0.140–1.155

*$p < 0.05$.
Bold values indicate that the odds ratio value is lower than 1, the G+ genotype might exert a protective effect in females against AgP.
OR, odds ratio.

during cancer development. Strong S100A8 and S100A9 upregulation was found in breast, lung, gastric, colorectal, pancreatic and prostate cancer (47–49).

Although the general consensus is that the S100A8 or S100A9 protein function is dependent on heterodimer formation, there is increasing evidence that S100A8, as well as S100A9, may exert specific functions as monomers (50,51). Studies have shown that monocytes and neutrophils express and secrete pro-inflammatory S100 proteins (S100A8, S100A9 and S100A12) specific for early recruited phagocytes (52,53), and S100A8 and S100A9 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock (54).

Calprotectin, a heterodimer of S100A8 and S100A9, is considered as the preferred form within cells. Calprotectin may exert different functions, e.g. inhibition of casein kinases, binding to cytoskeletal proteins and antimicrobial effects (24,55).The exact mechanism by which calprotectin crosses the cellular membrane remains to be elucidated, and its secretion by human monocytes and activated granulocytes depends on intact microtubules and involves protein kinase C activation (28,56). Calprotectin can interact with the cellular receptor for advanced glycation endproducts, triggering cellular activation and generation of key pro-inflammatory elements (57). Recently, the released calprotectin was proposed to enhance CD11b expression and/or affinity in human monocytes and to participate in the transendothelial migration mechanism, thus contributing substantially to the recruitment of monocytes to an inflammatory site (58).

How S100A8 and calprotectin contribute to the pathogenesis of periodontal diseases is unknown. Besides the high expressions in neutrophils, monocytes and activated macrophages, calprotectin is also expressed in oral epithelial keratinocytes. *In vitro*, the gingival epithelium cells expressing calprotectin confer resistance to invading bacteria including *Porphyromonas gingivalis*, *Listeria monocytogenes*, *Salmonella* and *Capnocytophaga* (59–61). In contrast, calprotectin has a broad apoptosis-inducing activity against various cell types, including tumor cells and normal fibroblasts, thus it might cause tissue destruction and influence the recovery of inflammatory tissue at a high concentration (62). In addition, S100A8 is an autocrine chemotactic factor for human periodontal ligament (PDL) cells (63) and specific for directing migration of PDL cells, but not for gingival fibroblasts (64). It was suggested that selective recruitment of PDL cells to a previously exposed root surface might enhance periodontal regeneration. In summary, S100A8 or calprotectin may

augment both the barrier protection and the innate immune functions of the gingival epithelium, exert a regulatory activity in inflammatory processes and may play an important role in human PDL cells. Many studies have demonstrated that the concentration of S100A8 and/or calprotectin in gingival crevicular fluid correlated with both gingivitis and periodontitis. Our recent work has shown that the plasma concentration of calprotectin was also correlated with the severity of periodontitis (X. J. Sun, H. X. Meng, D. Shi, L. Xu, L. Zhang, Z. B. Chen, X. H. Feng, X. Y. Ren, R. F. Lu and F. Q. Zhang, unpublished observations).

The SNP rs3795391 does not affect the structure of gene products, but it might cause quantitative differences in gene expression. Moreover, co-expression of several S100 proteins (e.g. S100A8, S100A9 and S100A12) might give rise to the supposition that these genes are not only structurally and functionally related but also, in terms of transcriptional control, probably use a common regulatory region in the cluster (65).

This study is a family-based association analysis; hence, the positive association results are robust to problems resulting from population admixture, stratification or model misspecification (36,37). The FBAT is an extension of the transmission disequilibrium test (TDT) and can deal with any kind of pedigree structure, including incomplete nuclear families. The Z and χ^2 tests produced by FBAT are based on the number of informative families in which there should be at least one heterozygous parent. The FBAT test statistic is based on the distribution of the offspring genotypes conditional on any trait information and on the parental genotypes, not on the parental trait information. Therefore, problems of diagnosis of AgP in old individuals can be better managed. It was known that genetic studies of AgP were hampered by a lot of factors, including a variable onset of the trait, lack of phenotypic information for edentulous family members or the problems of diagnosis in older individuals (1,66). Many genetic studies, especially segregation analysis and linkage studies of multigenerational pedigrees, have been flawed by having to use different inclusion criteria among probands and their family members (67–69).

Thus, the positive association of a family-based analysis can be due to either the direct effect of the marker tested or the marker tested being in linkage disequilibrium with an unknown causative sequence variation in the nearby region. The SNP rs3795391 is in a non-coding region of the *S100A8* gene. Not having a direct functional effect, it may be in linkage disequilibrium with the disease-predisposing locus.

Our previous study suggested (34) that the G^+ genotype/G allele may be considered to exert significant protective effects in males against AgP. In this study, in contrast, we found the protective effects of G^+ genotype in females. The discordance may result from the following factors. (1) The female/male ratio was reasonably well balanced in AgP patients and healthy control subjects in the previous study, but the ratio of female/male was not balanced in probands in this study (Table 1). (2) The control group of the previous study was composed of healthy subjects, but in the present study it was composed of unaffected individuals (including healthy subjects and those with gingivitis and chronic periodontitis).

In addition, we found a strong linkage disequilibrium between the two SNPs, with high D' and correlation coefficient values ($D' = 0.974$, $r^2 = 0.860$). Our recent work has also shown a correlation of plasma calprotectin with the two SNPs (X. J. Sun, H. X. Meng, D. Shi, L. Xu, L. Zhang, Z. B. Chen, X. H. Feng, X. Y. Ren, R. F. Lu and F. Q. Zhang, unpublished observations). Strictly, we should use the Bonferroni correction to maintain the desired overall significance level in statistical analyses. However, the two SNPs in the study exhibit a strong linkage disequilibrium ($D' = 0.974$), so it may be too strict for us to correct the p-values by Bonferroni correction. When background linkage disequilibrium exists between SNPs but they are assumed to be completely independent, the Bonferroni correction would markedly overcorrect for the inflated false-positive rate, resulting in a reduction in power (70). The additive model is the default model in FBAT. Though the program was performed in three genetic models in the study, a statistically significant association was only observed in the additive model. Moreover, the sample size of our study might not allow us to correct by Bonferroni. In China it is difficult to collect bigger families with more siblings.

In conclusion, this family-based association study supports the previous findings that SNP rs3795391 (A > G) of the *S100A8* gene might contribute to AgP susceptibility. To our knowledge, this is the first investigation on AgP and genetic analysis about the *S100A8* gene in families. Furthermore, this study proposes a novel genetic analysis (FBAT) in periodontitis. This study showed only a marginal significance and it should be tested in additional and larger family samples. Also, molecular and more functional studies are necessary to reveal the underlying mechanism of the associations we observed.

Acknowledgements

We thank Dr Dalong Ma (Laboratory of Medical Immunology and Peking University Center for Human Disease Genomics, Peking University Health Science Center, Peking University) for his critical views and help on this study. We are indebted to Dr Dafang Chen (Department of Epidemiology and Biostatistics, School of Public Health, Peking University Health Science Center, Peking University) for his excellent help with statistical analysis. This research was supported by the National Natural Science Foundations of China (30471882, 30271411, 30772420); the Special Fund for Promotion of Education, Ministry of Education PRC; the National High Tech Program ('863' Program; 2002AA217091); Clinical Research Fund, Ministry of Health PRC National Scientific and Technical Supporting Program in the 11th Five-Year Period (2007BAI18B02).

References

1. Kinane DF, Shiba H, Hart TC. The genetic basis of periodontitis. *Periodontol 2000* 2005;**39:**91–117.
2. Meng H, Xu L, Li Q, Han J, Zhao Y. Determinants of host susceptibility in aggressive periodontitis. *Periodontol 2000* 2007;**43:**133–159.
3. Boughman JA, Halloran SL, Roulston D *et al*. An autosomal-dominant form of juvenile periodontitis: its localization to chromosome 4 and linkage to dentinogenesis imperfecta and Gc. *J Craniofac Genet Dev Biol* 1986;**6:**341–350.
4. Hart TC, Marazita ML, McCanna KM, Schenkein HA, Diehl SR. Reevaluation of the chromosome 4q candidate region for early onset periodontitis. *Hum Genet* 1993;**91:**416–422.
5. Li Y, Xu L, Hasturk H, Kantarci A, DePalma SR, Van Dyke TE. Localized aggressive periodontitis is linked to human chromosome 1q25. *Hum Genet* 2004; **114:**291–297.
6. Lander ES, Schork NJ. Genetic dissection of complex traits. *Science* 1994;**265:**2037–2048.
7. Johnson GC, Todd JA. Strategies in complex disease mapping. *Curr Opin Genet Dev* 2000;**10:**330–334.
8. Tabor HK, Risch NJ, Myers RM. Candidate-gene approaches for studying complex genetic traits: practical considerations. *Nat Rev Genet* 2002;**3:**391–397.
9. Ott J. *Analysis of Human Genetic Linkage*, 3rd edition. Baltimore/London: Johns Hopkins University Press, 1999.
10. Hodge SE. What association analysis can and cannot tell us about the genetics of complex disease. *Am J Med Genet* 1994;**54:**318–323.
11. Kornman KS, Crane A, Wang HY *et al*. The interleukin-1 genotype as a severity factor in adult periodontal disease. *J Clin Periodontol* 1997;**24:**72–77.
12. Li QY, Zhao HS, Meng HX, Zhang L, Xu L, Chen ZB. The relationship between a nucleotide substitution of S100A8 gene and aggressive periodontitis. *Zhonghua Kou Qiang Yi Xue Za Zhi* 2004;**39:**181–184 (Chinese).
13. Kinane DF, Hodge P, Eskdale J, Ellis R, Gallagher G. Analysis of genetic polymorphisms at the interleukin-10 and tumour necrosis factor loci in early-onset periodontitis. *J Periodont Res* 1999; **34:**379–386.
14. Sun JL, Meng HX, Cao CF *et al*. Relationship between vitamin D receptor gene polymorphism and periodontitis. *J Periodont Res* 2002;**37:**263–267.
15. Fu Y, Korostoff JM, Fine DH, Wilson ME. Fcγ receptor genes as risk markers for localized aggressive periodontitis in African-Americans. *J Periodontol* 2002;**73:** 517–523.
16. Hodge PJ, Riggio MP, Kinane DF. No association with HLA-DQB1 in European Caucasians with early-onset periodontitis. *Tissue Antigens* 1999;**54:**205–207.
17. Michel J, Gonzales JR, Wunderlich D, Diete A, Herrmann JM, Meyle J. Interleukin-4 polymorphisms in early onset periodontitis. *J Clin Periodontol* 2001;**28:** 483–488.
18. Shapira L, Stabholz A, Rieckmann P, Kruse N. Genetic polymorphism of the tumor necrosis factor (TNF)-α promoter region in families with localized early-onset periodontitis. *J Periodont Res* 2001;**36:**183–186.
19. Schafer BW, Wicki R, Engelkamp D, Mattei MG, Heizmann CW. 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* 1995;**25:**638–643.
20. Kligman D, Hilt DC. The S100 protein family. *Trends Biochem Sci* 1988;**13:**437–443.
21. Lagasse E, Clerc RG. Cloning and expression of two human genes encoding calcium-binding proteins that are regulated during myeloid differentiation. *Mol Cell Biol* 1988;**8:**2402–2410.
22. Zwadlo G, Bruggen J, Gerhards G, Schlegel R, Sorg C. Two calcium-binding proteins associated with specific stages of myeloid cell differentiation are expressed by subsets of macrophages in inflammatory tissues. *Clin Exp Immunol* 1988; **72:**510–515.
23. Hessian PA, Edgeworth J, Hogg N. MRP-8 and MRP-14, two abundant Ca^{2+}-binding proteins of neutrophils and monocytes. *J Leukoc Biol* 1993;**53:**197–204.
24. Sorg C. The calcium binding proteins MRP8 and MRP14 in acute and chronic inflammation. *Behring Inst Mitt* 1992; **91:**126–137.
25. Bhardwaj RS, Zotz C, Zwadlo-Klarwasser G *et al*. The calcium-binding 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* 1992;**22:**1891–1897.
26. Kristinsson J, Roseth A, Fagerhol MK *et al*. Fecal calprotectin concentration in patients with colorectal carcinoma. *Dis Colon Rectum* 1998;**41:**316–321.
27. Golden BE, Clohessy PA, Russell G, Fagerhol MK. Calprotectin as a marker of inflammation in cystic fibrosis. *Arch Dis Child* 1996;**74:**136–139.
28. Frosch M, Strey A, Vogl T *et al*. Myeloid-related proteins 8 and 14 are specifically secreted during interaction of phagocytes and activated endothelium and are useful markers for monitoring disease activity in pauciarticular-onset juvenile rheumatoid arthritis. *Arthritis Rheum* 2000;**43:**628–637.
29. Zhou H, Meng H, Chen Z. Electrophoretic analysis of protein composition in gingival crevicular fluid. *Zhonghua Kou Qiang Yi Xue Za Zhi* 1998;**33:**109–112 (Chinese).
30. Li J, Meng H, Chen Z. Electrophoresis of proteins in gingival crevicular fluid of patients with adult periodontitis before and after treatment. *Zhonghua Kou Qiang Yi Xue Za Zhi* 1998;**33:**276–278 (Chinese).
31. Wang R, Meng H, Chen Z, Cao C. 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* 2002;**37:**297–299 (Chinese).
32. Nakamura T, Kido J, Kido R *et al*. 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* 2000;**71:**361–367.
33. Zhao Y, Meng H, Chen Z. Calprotectin and total protein in gingival crevicular fluid during experimental gingivitis. *Beijing Da Xue Xue Bao* 2003;**35:**668–670 (Chinese).
34. Li Q, Meng H, Zhang L *et al*. Correlation between single nucleotide polymorphisms in a calprotectin subunit gene and risk of periodontitis in a Chinese population. *Ann Hum Genet* 2007;**71:**312–324.
35. Armitage GC. Development of a classification system for periodontal diseases and conditions. *Ann Periodontol* 1999;**4:**1–6.
36. Rabinowitz D, Laird N. A unified approach to adjusting association tests for population admixture with arbitrary pedigree structure and arbitrary missing marker information. *Hum Hered* 2000;**50:**211–223.
37. Laird NM, Horvath S, Xu X. Implementing a unified approach to family-based tests of association. *Genet Epidemiol* 2000;**19** (Suppl 1)**:**S36–S42.
38. Horvath S, Xu X, Laird NM. The family based association test method: strategies for studying general genotype–phenotype associations. *Eur J Hum Genet* 2001; **9:**301–306.
39. Devlin B, Risch N. A comparison of linkage disequilibrium measures for fine-scale mapping. *Genomics* 1995;**29:**311–322.
40. Shi YY, He L. SHEsis, a powerful software platform for analyses of linkage disequilibrium, haplotype construction, and genetic association at polymorphism loci. *Cell Res* 2005;**15:**97–98.
41. Hart TC, Marazita ML, Schenkein HA, Diehl SR. Re-interpretation of the evi-

dence for X-linked dominant inheritance of juvenile periodontitis. *J Periodontol* 1992;**63:**169–173.
42. Hart TC, Marazita ML, Schenkein HA, Brooks CN, Gunsolley JG, Diehl SR. No female preponderance in juvenile periodontitis after correction for ascertainment bias. *J Periodontol* 1991;**62:**745–749.
43. Ravasi T, Hsu K, Goyette J *et al*. Probing the S100 protein family through genomic and functional analysis. *Genomics* 2004;**84:**10–22.
44. Roth J, Goebeler M, Sorg C. S100A8 and S100A9 in inflammatory diseases. *Lancet* 2001;**357:**1041.
45. Semprini S, Capon F, Tacconelli A *et al*. Evidence for differential S100 gene overexpression in psoriatic patients from genetically heterogeneous pedigrees. *Hum Genet* 2002;**111:**310–313.
46. Kido J, Nakamura T, Kido R *et al*. Calprotectin in gingival crevicular fluid correlates with clinical and biochemical markers of periodontal disease. *J Clin Periodontol* 1999;**26:**653–657.
47. Stulik J, Osterreicher J, Koupilova K *et al*. The analysis of S100A9 and S100A8 expression in matched sets of macroscopically normal colon mucosa and colorectal carcinoma: the S100A9 and S100A8 positive cells underlie and invade tumor mass. *Electrophoresis* 1999;**20:**1047–1054.
48. Cross SS, Hamdy FC, Deloulme JC, Rehman I. Expression of S100 proteins in normal human tissues and common cancers using tissue microarrays: S100A6, S100A8, S100A9 and S100A11 are all overexpressed in common cancers. *Histopathology* 2005;**46:**256–269.
49. Hermani A, Hess J, De Servi B *et al*. Calcium-binding proteins S100A8 and S100A9 as novel diagnostic markers in human prostate cancer. *Clin Cancer Res* 2005;**11:**5146–5152.
50. Nacken W, Roth J, Sorg C, Kerkhoff C. S100A9/S100A8: Myeloid representatives of the S100 protein family as prominent players in innate immunity. *Microsc Res Tech* 2003;**60:**569–580.
51. Sampson B, Fagerhol MK, Sunderkotter C *et al*. Hyperzincaemia and hypercalprotectinaemia: a new disorder of zinc metabolism. *Lancet* 2002;**360:**1742–1745.
52. Roth J, Vogl T, Sorg C, Sunderkotter C. Phagocyte-specific S100 proteins: a novel group of proinflammatory molecules. *Trends Immunol* 2003;**24:**155–158.
53. Foell D, Hernandez-Rodriguez J, Sanchez M, Vogl T, Cid MC, Roth J. Early recruitment of phagocytes contributes to the vascular inflammation of giant cell arteritis. *J Pathol* 2004;**204:**311–316.
54. Vogl T, Tenbrock K, Ludwig S *et al*. Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. *Nat Med* 2007;**13:**1042–1049.
55. Murao S, Collart FR, Huberman E. A protein containing the cystic fibrosis antigen is an inhibitor of protein kinases. *J Biol Chem* 1989;**264:**8356–8360.
56. Rammes A, Roth J, Goebeler M, Klempt M, Hartmann M, Sorg C. 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* 1997;**272:**9496–9502.
57. Hofmann MA, Drury S, Fu C *et al*. RAGE mediates a novel proinflammatory axis: a central cell surface receptor for S100/calgranulin polypeptides. *Cell* 1999;**97:**889–901.
58. Eue I, Pietz B, Storck J, Klempt M, Sorg C. Transendothelial migration of 27E10+ human monocytes. *Int Immunol* 2000;**12:**1593–1604.
59. Nisapakultorn K, Ross KF, Herzberg MC. Calprotectin expression inhibits bacterial binding to mucosal epithelial cells. *Infect Immun* 2001;**69:**3692–3696.
60. Nisapakultorn K, Ross KF, Herzberg MC. Calprotectin expression in vitro by oral epithelial cells confers resistance to infection by Porphyromonas gingivalis. *Infect Immun* 2001;**69:**4242–4247.
61. Miyasaki KT, Bodeau AL, Murthy AR, Lehrer RI. In vitro antimicrobial activity of the human neutrophil cytosolic S-100 protein complex, calprotectin, against *Capnocytophaga sputigena*. *J Dent Res* 1993;**72:**517–523.
62. Yui S, Nakatani Y, Mikami M. Calprotectin (S100A8/S100A9), an inflammatory protein complex from neutrophils with a broad apoptosis-inducing activity. *Biol Pharm Bull* 2003;**26:**753–760.
63. Nishimura F, Terranova VP, Sawa T, Murayama Y. Migration inhibitory factor related protein-8 (MRP-8) is an autocrine chemotactic factor for periodontal ligament cells. *J Dent Res* 1999;**78:**1251–1255.
64. Nishimura F, Terranova VP. Comparative study of the chemotactic responses of periodontal ligament cells and gingival fibroblasts to polypeptide growth factors. *J Dent Res* 1996;**75:**986–992.
65. Wicki R, Marenholz I, Mischke D, Schafer BW, Heizmann CW. Characterization of the human S100A12 (calgranulin C, p6, CAAF1, CGRP) gene, a new member of the S100 gene cluster on chromosome 1q21. *Cell Calcium* 1996;**20:**459–464.
66. Boughman JA, Beaty TH, Yang P, Goodman SB, Wooten RK, Suzuki JB. Problems of genetic model testing in early onset periodontitis. *J Periodontol* 1988; **59:**332–337.
67. Marazita ML, Burmeister JA, Gunsolley JC, Koertge TE, Lake K, Schenkein HA. Evidence for autosomal dominant inheritance and race-specific heterogeneity in early-onset periodontitis. *J Periodontol* 1994;**65:**623–630.
68. Diehl SR, Wang Y, Brooks CN *et al*. Linkage disequilibrium of interleukin-1 genetic polymorphisms with early-onset periodontitis. *J Periodontol* 1999;**70:**418–430.
69. Hodge PJ, Teague PW, Wright AF, Kinane DF. Clinical and genetic analysis of a large North European Caucasian family affected by early-onset periodontitis. *J Dent Res* 2000;**79:**857–863.
70. Nyholt DR. A simple correction for multiple testing for single-nucleotide polymorphisms in linkage disequilibrium with each other. *Am J Hum Genet* 2004;**74:**765–769.