

ORIGINAL ARTICLE

Mitochondrial polymorphisms and dysfunction related to aggressive periodontitis: a pilot study

X Wang¹, Q Luan¹, Q Chen², L Zhao², Y Guo¹

¹Department of Periodontology, Peking University School and Hospital of Stomatology, Beijing, China; ²State Key Laboratory of Biomembrane and Membrane Biotechnology, Institute of Zoology, Chinese Academy of Sciences, Chaoyang District, Beijing, China

AIM: To investigate whether aggressive periodontitis (AgP) is associated with specific mtDNA polymorphisms or point mutations and furthermore whether mitochondrial dysfunction occurs in gingival fibroblasts of AgP patients.

MATERIALS AND METHODS: The mitochondrial DNA coding regions were amplified and sequenced in 22 overlapped fragments in 34 patients with AgP and 28 healthy controls for initial screening. We selected eleven SNPs for detailed investigation in the rest 30 AgP patients and 26 healthy controls, because all other variants occurred at relatively low frequencies or had no difference between two groups. Logistic regression models were used to analyze the association between mtDNA variants and AgP. Gingival fibroblasts were cultured from four patients with AgP and four healthy controls, and then mitochondrial membrane potential, reactive oxygen species production and cell proliferation were analyzed.

RESULTS: Significant association was observed between aggressive periodontitis and eight mitochondrial polymorphisms: '8701A-9540T-10400C-10873T-14783T-15043G-15301G' (OR = 3.471 (1.610–7.483), $P = 0.001$) and 10398A (OR = 3.238 (1.481–7.078), $P = 0.003$). Compared with the controls, patients with aggressive periodontitis had a decrease in mitochondrial membrane potential and an increase in reactive oxygen species production.

CONCLUSION: In conclusion, we propose that mitochondrial dysfunction and '8701A-9540T-10400C-10873T-14783T-15043G-15301G, 10398A' are associated with and may increase the susceptibility to AgP in the Han Chinese population.

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Keywords: aggressive periodontitis; mitochondrial polymorphism; susceptibility; reactive oxygen species; mitochondrial membrane potential

Introduction

Periodontal diseases are inflammatory disorders caused by complex interactions between the immune response of the host and pathogenic bacteria. Periodontal diseases are commonly referred to as gingivitis and periodontitis. Gingivitis, the mildest form of periodontal disease, is highly prevalent and readily reversible by effective oral hygiene producers (Albandar and Rams, 2002). Periodontitis is a severe form of periodontal disease in which the inflammation extends deep into the tissues and causes the loss of supporting connective tissue and alveolar bone, resulting in the formation of soft tissue pockets between the gingiva and roots of the teeth. Periodontitis commonly includes chronic periodontitis and aggressive periodontitis. Chronic periodontitis occurs in localized and generalized forms, and people with significant disease tend to be around 35 years of age. Aggressive periodontitis (AgP) also includes localized aggressive periodontitis and generalized aggressive periodontitis. AgP is a type of rare and severe periodontal disease, which affects systemically healthy individuals. It is characterized by the early age of onset, rapid rate of disease progression and familial aggregation (Lang *et al*, 1999). Imbalance between dental microbial plaque and host immune responses is generally considered to be responsible for periodontal tissue destruction in AgP-affected individuals, resulting in attachment loss and alveolar bone loss. The response of individuals to microbial plaque is unique in each case, leading to differences in the quality and quantity of local inflammatory and immune reactions (Page *et al*, 1997). <>Both familial and twin studies indicate that host heredity is an important susceptibility factor in the development of periodontal disease (Marazita *et al*, 1994; Hassell and Harris, 1995; Michalowicz *et al*, 2000). Gene variations contribute partially to the host response to microbial plaque, and thus, numerous candidate genes related to inflammatory and

Correspondence: Qingxian Luan, Department of Periodontology, Peking University School and Hospital of Stomatology, 22 Zhongguancun Nandajie, Haidian District, Beijing 100081, China. Tel: +8610 82195497, Fax: +8610 62173402, E-mail: kqluanqx@126.com

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immune reactions have been investigated with the aim of identifying the genetic basis of susceptibility to AgP (Kinane and Hart, 2003; Loos *et al*, 2005). So far, research on the genetic background of AgP has focused mainly on nuclear DNA. For example, the role of cathepsin C (CTSC) gene variants has been studied in AgP associated with and without Papillon-Lefèvre syndrome (PLS), which is a rare autosomal recessive condition characterized by palmoplantar hyperkeratosis and severe early-onset periodontitis. The possible role of CTSC variants differs in PLS-associated and non-PLS-associated AgP. CTSC mutations are not associated with all forms of early-onset periodontal disease, and currently there is no evidence for the existence of a class of patients who do not have the full PLS disease phenotype, but suffer isolated aggressive periodontitis because they have a low-activity CTSC variant (Hewitt *et al*, 2004). The role of genetic factors in aggressive periodontitis is clear; however, there is as yet no clear evidence for this in the general population (Genco and Borgnakke, 2013).

The mitochondrial genome, which was discovered in 1963, is an extra-nuclear genome unique to humans. Human mitochondrial DNA (mtDNA) is a circular double-stranded molecule of 16 569 bp localized near the mitochondrial inner membrane. The genome contains 37 genes: two rRNA genes, 22 tRNA genes and 13 genes essential for oxidative phosphorylation. Each cell contains several hundred or thousands of mitochondria, and each mitochondrion has 2–10 copies of mtDNA molecules. The mitochondrial genome is susceptible to damage due to the absence of histones, continued exposure to abundant oxidative stress and limited DNA repair capacity (Yakes and van Houten, 1997). The deleterious effects of increased oxidative stress are termed oxidative damage, which generally occurs after exposure to a relatively high concentration of ROS and/or a decrease in the antioxidant (AO) defense system. Oxidative attack by free radicals of mitochondrial origin is likely to be the primary cause of mtDNA damage due to the close proximity of a reactive oxygen species (ROS) rich environment within the mitochondria (Barja and Herrero, 2000). Increasing evidence implicates ROS in the pathogenesis of a variety of inflammatory disorders, and, more recently, periodontitis (Chapple, 1997; Canakci *et al*, 2005; Canakçi *et al*, 2009).

In the past few years, numerous mtDNA polymorphisms and pathogenic mutations including large-scale deletions, point mutations have been found to be responsible for or related to several human diseases (Singh and Kulawiec, 2009). In the periodontal field, studies on mtDNA are few and limited, although previous reports have concentrated mainly on the association between large-scale deletions of mtDNA and chronic periodontitis (CP). Investigations of the relationship between CP and a '4977-bp deletion' which is termed as '5-kbp common deletion' have revealed that this mutation is found more frequently in the gingival tissues of CP patients than in unaffected control patients (Canakçi *et al*, 2006). Several point mutations have also been found to be associated with CP (Govindaraj *et al*, 2011). Mitochondrial dysfunction has also been suggested in gingival fibroblasts of patients with CP including increased ROS production and

decreased mitochondrial membrane potential (Canakçi *et al*, 2006). AgP, such as CP, is considered to be caused by specific oral pathogens that induce host defense reactions. Therefore, this study was conducted to investigate the hypothesis that AgP is associated with specific mtDNA polymorphisms or point mutations and, furthermore, that mitochondrial dysfunction occurs in gingival fibroblasts of AgP patients.

To address these issues, we analyzed the mitochondrial genetics and mitochondrial dysfunction in AgP patients. Consistent with our hypothesis, mitochondrial single-nucleotide polymorphisms (mtSNPs) were found to be associated with AgP, and mitochondrial dysfunction was found in patients with AgP.

Materials and methods

Subjects and medical records review

The study was approved by the Institutional Review Board and Ethics Committee of Peking University Health Science Center. Each patient provided written informed consent to participation in this study. A total of 118 unrelated Han Chinese were enrolled in the study. All the patients were recruited at Department of Periodontology, Peking University School and Hospital of Stomatology, China. We selected 64 patients (mean age, 27.63; female: male, 36:28) with AgP according to the recommendations of the 1999 International Classification Workshop (Lang *et al*, 1999). All 54 controls (mean age, 27.02; female: male, 32:22) showed only minimal signs of periodontal disease based on clinical examinations. The detailed information of subjects, including clinical, radiographic, historical and laboratory characteristics, were collected. Subjects were excluded due to pregnancy, systemic disease and obvious malocclusion or orthodontic treatment history.

Mutational screening of mtDNA coding region

Blood analysis, DNA isolation and mtDNA amplification. Peripheral blood samples were collected into two tubes from each fasting participant by venipuncture. Blood in the tube containing EDTA was divided into two parts. Total DNA of the blood sample was extracted using a genomic DNA isolation kit (DP1801; Biotek, Beijing, China) and then stored at -20°C for later use. The other part was used for blood cell analysis by hematology analyzers (SYSMEX KX-21, Sysmex, Kobe, Japan). The other tube did not contain EDTA and was used for serum protein analysis using a biochemical analyzer (HITACHI 7060, Hitachi, Tokyo, Japan).

To amplify the mtDNA coding region, 22 separate PCRs were performed in a 25 μl reaction volume containing 5 μl 5 \times PrimeSTAR Buffer (Mg²⁺ Plus), 35 ng total DNA, 200 μM per each dNTP (TaKaRa, Japan), 0.2 μM per each primer, 0.625 units PrimeSTAR[®] HS DNA Polymerase (TaKaRa) and 15.75 μl sterile distilled water. Twenty-two pairs of corresponding primers were used to amplify 22 overlapping fragments (Rieder *et al*, 1998). The thermal cycling condition for all amplification reactions were as follows: initial denaturation at 98 $^{\circ}\text{C}$ for 5 min followed by 30 cycles at 98 $^{\circ}\text{C}$ for 30 s, 61 $^{\circ}\text{C}$ for

10 s and 72°C for 1 min, with a final extension incubation at 72°C for 10 min [DNA Engine PTC-200 (Bio-Rad, California, USA)]. After amplification, PCR products (892 bp on average) were detected by 1.5% agarose gel electrophoresis followed by staining with Goldview. Fragments were then purified using a PCR purification kit (TIANGEN, Beijing, China) according to manufacturer's instructions.

DNA sequencing and sequence analysis. Purified PCR products were sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems). The reaction product was purified by ethanol precipitation and then analyzed using an ABI 3730 automated sequencer according to the manufacturer's protocols. To identify possible mtSNP loci and mutations, we compared these sequences with the revised Cambridge reference (NC_012920) sequences using BLASTn in NCBI (Anderson *et al*, 1981; Andrews *et al*, 1999; <http://blast.ncbi.nlm.nih.gov/>). In addition, mitomap (<http://www.mitomap.org>) and MitoTool (Fan and Yao, 2010; <http://www.mitotool.org/>) were used to investigate the gene variations. Variations selected for further investigation were amplified again and sequenced in the reverse direction to confirm the accuracy.

Cell culture and mutational screening of mtDNA coding region

Cell culture. Gingival tissue samples (50–100 mg) were collected from the AgP patients and controls during flap and crown lengthening surgery. Gingival tissues from four patients with AgP and four controls were paired on the basis of age- and sex-matching. A portion of the tissues was used to culture gingival fibroblasts, while the residual tissue was used for mtDNA analysis. Tissues were placed in a 1.5 ml sterile Eppendorf tubes containing the Dulbecco's modified Eagle medium (DMEM) with 2% antibiotics (penicillin, streptomycin, gentamycin). The tissue sample was cut into small pieces (approximately 1 mm³) using ophthalmic scissors in a 60-mm tissue culture dish and rinsed three times with D-Hanks' Balanced Salt Solution containing 4%, 2% and 1% antibiotics, respectively. The minced explant tissues were plated in a 25-mm² cell culture flask and cultures in a CO₂ incubator at 37°C. DMEM supplemented with 10% fetal bovine serum (FBS) was added after 4 h. The medium was replaced three times each week when the cells reached confluence. Fibroblasts were trypsinized, resuspended in DMEM supplemented with 50% FBS and 10% dimethyl sulfoxide (DMSO) and frozen over liquid nitrogen for later use. When required, cells were thawed, and passages 4 to 7 were used in experiments. Four paired cell lines were used for cell function analysis.

Mutational screening of mtDNA coding region. DNA was extracted from gingival tissue and gingival fibroblasts using a tissue genomic DNA isolation kit (DP1802, Biotek). The mtDNA coding region was amplified and sequenced. These sequences were then compared with the revised Cambridge reference sequences. It should be noted that gingival tissue used to culture gingival fibroblasts and

that used to extract DNA derived from the same gingival region.

Cell function analysis

Mitochondrial membrane potential, ROS production and cell proliferation were analyzed, and each experiment was repeated at least twice.

Measurement of mitochondrial membrane potential. Gingival fibroblast cells were plated in a six-well dish (1.8 × 10⁵ per well) and allowed to attach for 24 h in a CO₂ incubator at 37°C. As a positive control, cells were incubated with 40 mM carbonyl cyanide 3-chlorophenylhydrazone (CCCP) at 37°C for 20–30 min. Cells were washed with PBS and then treated with the fluorescent probe 3, 3'-dihexyloxacarbocyanine iodide [DIOC6 (3)] (40 nM) at 37°C for 20–30 min. Cells were washed twice with PBS and collected by trypsinization with the platelet dissolved in 500 µl of PBS. Fluorescence intensity of DIOC6 (3) was measured with excitation at 488 nm and emission at 538 nm using a multimode microplate reader (PerkinElmer, Waltham, MA, USA).

Measurement of ROS. Gingival fibroblast cells were trypsinized and washed with cold PBS. 1.8 × 10⁵ cells were resuspended with 1 ml DMEM media containing 1 µl of 10 mM carboxy-2',7'-dichloro-fluorescein diacetate (carboxy-H₂DCFHDA) and incubated in the dark in CO₂ incubator for 15 min. Cells were centrifuged at 1000 g for 5 min at room temperature, washed three times with PBS and dissolved in 500 µl of PBS. The ROS content of cells was analyzed based on measurement of the fluorescence intensity of carboxy-H₂DCFHDA with excitation at 488 nm and emission at 525 nm using a multimode microplate reader (PerkinElmer).

Cell proliferation assay. Gingival fibroblasts (7000 cells per well) were plated in 96-well plates. Basal cell proliferation was measured after approximately 12 h in culture, and proliferation was measured again 24 h later. Samples were washed once in D-Hanks' Balanced Salt Solution to remove non-viable cells before adding 100 µl DMEM with 10% FBS containing 10 µl of CCK-8 solution to each well. The plates were incubated in the dark in a CO₂ incubator for a further 2 h. Optical density was measured at 450 nm using a microplate reader (BioTek, Winooski, VT, USA).

Statistical analysis

Age, sex ratio and blood examination results were compared between AgP patients and controls by using the unpaired Student's *t*-test. Eleven SNPs were selected for focused investigation after comparison with revised Cambridge reference sequences. The difference in the allele frequency of each SNP was assessed using the Chi-square test, and Holm's method was used to correct the multiple comparisons. In detail, mitochondrial DNA coding region was amplified and sequenced in 34 patients with AgP and 28 healthy controls for initial screening. After Chi-square test, frequencies of nine particular SNPs were found to be different in the AgP group and control group. Then these

nine SNPs were further investigated in the rest 30 AgP patients and 26 controls. To avoid omissions of SNPs, which may distribute differently between two groups after expanding the sample size, we randomly choose two SNPs further investigated in the rest 30 AgP patients and 26 controls. Therefore, these 11 SNPs have been studied in the whole 64 AgP patients and 54 controls. Multivariate logistic regression models were further used to analyze the association between mtDNA variants and AgP after adjustment for age and sex and smoking status. Paired Student's *t*-tests were used to compare MMP, ROS production and cell proliferation between the two groups. All statistical analyses were performed using the Statistical Package for Social Science program (International Business Machines Corporation, Armonk, NY, USA).

Results

Patient characteristics

Background characteristics. A total of 118 subjects were involved in the study. The background characteristics such as age, sex, smoking status and fasting blood glucose levels were compared. The quantitative data are presented as mean ± s.d. There were no statistically significant differences in the background characteristics between the AgP and control groups (Table 1).

Clinical periodontal characteristics. Severe alveolar bone loss was observed in subjects of the AgP group. For example, the full-mouth X-ray film of a 23-year-old woman with AgP showed radiolucent projection from the crest into the interdental septum indicating severe, generalized alveolar bone loss in all erupted teeth. Wedge-shaped alveolar bone loss was observed in the first right mandibular molar, while in other three first molars, Arc-shaped loss of alveolar bone was observed extending from the distal surface of the second premolar to the mesial surface of the second molar. The bone loss of incisors extended to the root tip, and alveolar bone loss of the other tooth was observed (Figure 1a).

In contrast, the full-mouth X-ray film of a 25-year-old woman with gingivitis showed the normal appearance of interdental septa (Figure 1b).

Mutational screening of mtDNA coding regions and association of variants with AgP

Gene alleles of blood samples. After initial screening in 34 patients with AgP and 28 controls, a total of 421

variants were found in the study (Table S1). We selected eleven SNPs for detailed investigation (T6392C, A8701G, T9540C, G10310A, A10398G, C10400T, T10873C, C12705T, T14783C, G15043A and G15301A), because all other variants occurred at relatively low frequencies or had no difference between two groups. The eleven SNPs were amplified from blood samples and sequenced at least twice for all 118 subjects. Statistically significant differences in allele distribution were observed between the AgP and control groups at eight positions analyzed by the Chi-square test: A8701G, T9540C, A10398G, C10400T, T10873C, T14783C, G15043A and G15301A. Frequencies of 8701A, 9540T, 10398A, 10400C, 10873T, 14783T, 15043G and 15301G were higher in AgP group than in the control group ($P = 0.001, 0.001, 0.003, 0.001, 0.001, 0.001, 0.001, 0.001$, respectively; Table 2). The association between AgP and the eight alleles was further evaluated by independent logistic regression analysis. The distribution frequencies of 8701A, 9540T, 10400C, 10873T, 14783T, 15043G and 15301G were the same in both groups; therefore, these seven SNPs were included in the logistic regression analysis as a single entity '8701A-9540T-10400C-10873T-14783T-15043G-15301G'. Significant association was observed between AgP, and the two factors adjusted for the age, sex and smoking status: '8701A-9540T-10400C-10873T-14783T-15043G-15301G' (OR = 3.471, $P = 0.001$), 10398A (OR = 3.238, $P = 0.003$) as shown in Table 3.

Gene alleles of gingival tissue and gingival fibroblasts

The mitochondrial DNA of cultured gingival fibroblasts and residual tissue was extracted, amplified and sequenced. Identical variants of these eight mtSNPs were detected in gingival fibroblasts, gingival tissue and blood from the same subject. The allele distribution in the four sets of paired gingival fibroblasts was as follows: 8701A-9540T-10400C-10873T-14783T-15043G-15301G and 10398A, two AgP cell lines; '8701G-9540C-10400T-10873C-14783C-15043A-15301A', 10398G, two control cell lines; '8701A-9540T-10400C-10873T-14783T-15043G-15301G', 10398A, one AgP and one control cell line; '8701G-9540C-10400T-10873C-14783C-15043A-15301A', 10398G, one AgP cell line; '8701A-9540T-10400C-10873T-14783T-15043G-15301G', 10398A, one control cell line.

Cell function analysis

Mitochondrial membrane potential. DIOC6 (3) was used to evaluate mitochondrial membrane potential using a

Table 1 Background characteristics of total study subjects

Characteristics	Control group (n = 54)	AgP group (n = 64)	P value
Age, mean ± s.d. (year)	27.02 ± 5.261	27.63 ± 4.593	0.505
Sex ratio (Female:Male)	32:22	36:28	0.742
Smoking status (Non-smoking:Smoking)	51:3	59:5	0.627
WBC, mean ± s.d. ($\times 10^9 l^{-1}$)	6.0522 ± 1.64502	6.4426 ± 1.90285	0.268
Glucose, mean±s.d. (mmol l^{-1})	5.2212 ± 0.37361	5.2479 ± 0.35166	0.728
TP, mean ± s.d. (g l^{-1})	77.1333 ± 4.61027	78.5690 ± 4.45686	0.113

WBC, white blood cell count; Glucose, fasting blood glucose levees; TP, total protein concentration; *P* value, values observed after '*t*-test' for: age, sex ratio, smoking status, WBC, Glucose, TP.

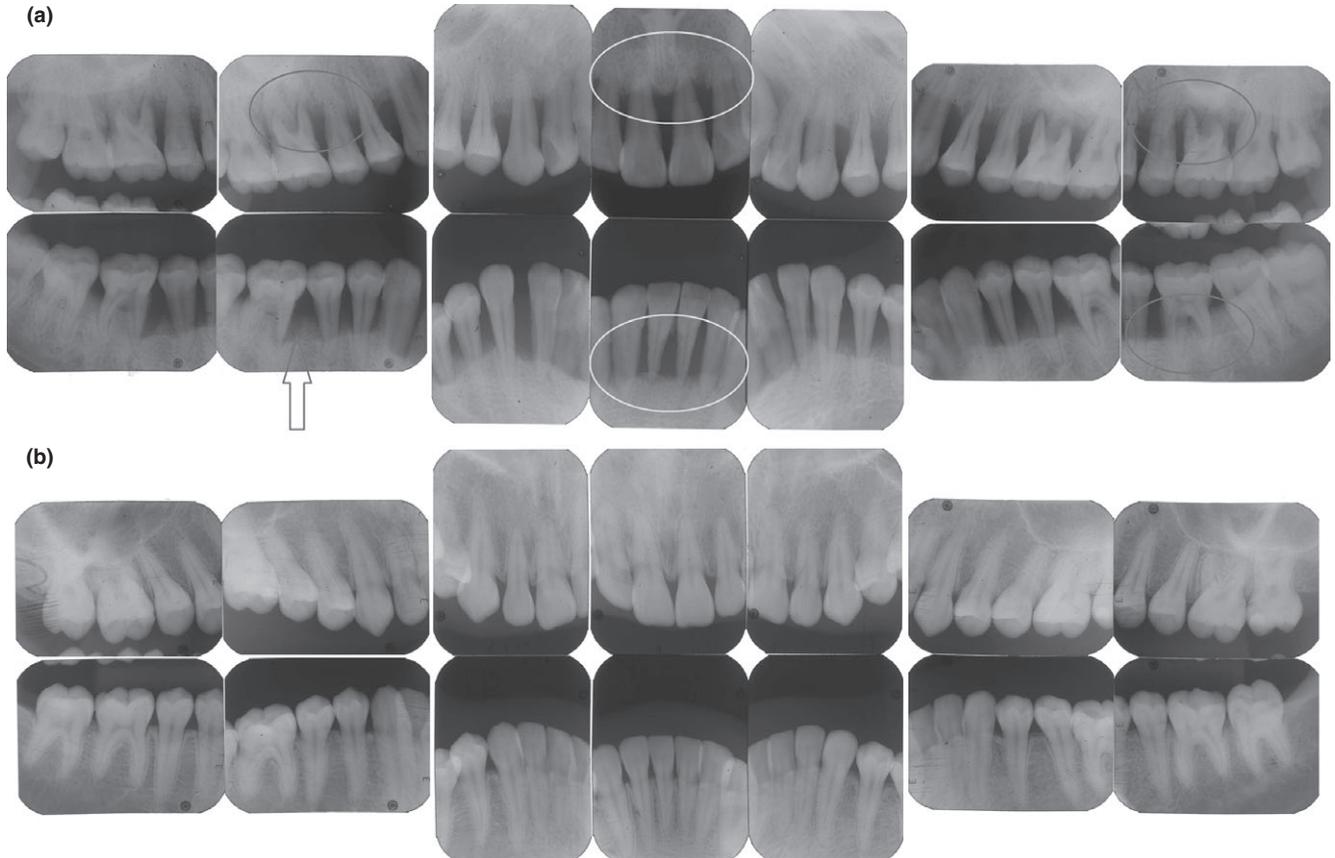


Figure 1 Full-mouth X-ray films of a severe generalized AgP patient and a healthy control individual. (a) Severe generalized aggressive periodontitis in a 23-year-old female patient. Radiolucent projection from the crest into the interdental septum indicates alveolar bone loss. Arc-shaped loss of alveolar bone in the first molar extends from the distal surface of the second premolar to the mesial surface of the second molar (oval box in posterior teeth area). The arrow shows the wedge-shaped alveolar bone loss. The bone resorption of incisors extended to the root tip (oval box in anterior teeth area), and alveolar bone loss of the other tooth can also be observed. (b) Normal radiopaque lamina dura around the roots and interdental septum in a 25-year-old female control individual

Table 2 Univariate data analysis of eleven SNPs

Gene	Position	Control group	AgP group	P value ^a
COX1	6392T	49	52	0.144
	6392C	5	12	
ATP6	8701G	38	26	0.001
	8701A	16	38	
COX3	9540C	38	26	0.001
	9540T	16	38	
ND3	10311G	49	52	0.144
	10311A	5	12	
ND3	10398G	40	30	0.003
	10398A	14	34	
ND3	10400T	38	26	0.001
	10400C	16	38	
ND4	10873C	38	26	0.001
	10873T	16	38	
ND5	12705T	41	38	0.057
	12705C	13	26	
Cytb	14783C	38	26	0.001
	14783T	16	38	
Cytb	15043A	38	26	0.001
	15043G	16	38	
Cytb	15301A	38	26	0.001
	15301G	16	38	

^aP value - a χ^2 test was used.

multimode microplate reader. Patients with AgP had a decrease in mitochondrial membrane potential compared with the controls (3102.85 ± 707.391 vs 1870.38 ± 396.264 , $P < 0.01$)(Figure 2). Each AgP gingival fibroblast line showed decreased mitochondrial membrane potential ('8701A-9540T-10400C-10873T-14783T-15043G-15301G, 10398A', $n = 3$ and '8701G-9540C-10400T-10873C-14783C-15043A-15301A, 10398G', $n = 1$) compared with the matched control in each pair.

ROS production. ROS production was analyzed by carboxy- $H_2DCFHDA$ using a multimode microplate reader. ROS production was higher in patients with AgP compared with the controls (2995.96 ± 445.767 vs 4498.32 ± 590.095 , $P < 0.01$; Figure 2). Each AgP gingival fibroblast showed an increase in ROS production ('8701A-9540T-10400C-10873T-14783T-15043G-15301G, 10398A', $n = 3$ and '8701G-9540C-10400T-10873C-14783C-15043A-15301A, 10398G', $n = 1$) compared with the control.

Cell proliferation. Cell proliferation was measured by CCK-8 assay. Basal cell proliferation (0 h) appeared similar in the two groups. After 24 h, the cell proliferation rate of patients with AgP was lower than that in controls

Table 3 Logistic analysis adjusting for age, sex and smoking status

SNP	AA variant	Control group (n = 54)		AgP group (n = 64)		OR(95%CI)	P value
		N	%	N	%		
G8701A- C9540T- T10400C- C10873T- C14783T- A15043G- A15301G- G10398A	A59T Synonymous Synonymous Synonymous Synonymous Synonymous T114A	16	29.63	38	59.4	3.471 (1.610–7.483)	0.001
		14	25.93	34	53.13	3.238 (1.481–7.078)	0.003

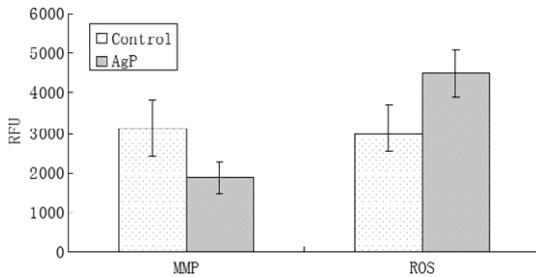


Figure 2 Measurement of mitochondrial membrane potential (MMP) and reactive oxygen species (ROS). Bar graph represents a decrease in MMP and an increase in ROS production in the AgP group compared with the control group (*represents $P < 0.01$)

($P > 0.05$), although there was no statistical significance when considering the total four pair of AgP cells and controls. However, proliferation of AgP gingival fibroblasts with ‘8701A-9540T-10400C-10873T-14783T-15043G-15301G, 10398A’ was significantly lower than the paired controls (0.938 ± 0.115 vs 1.149 ± 0.114 , $P < 0.01$). In contrast, AgP gingival fibroblasts with ‘8701G-9540C-10400T-10873C-14783C-15043A-15301A, 10398G’ proliferated significantly faster than the control with ‘8701A-9540T-10400C-10873T-14783T-15043G-15301G, 10398A’ (0.868 ± 0.049 vs 0.506 ± 0.023 , $P < 0.01$; Figure 3).

Discussion

Periodontal tissue destruction in AgP individuals is generally considered to be the result of an imbalance between dental microbial plaque and host immune responses. The periodontopathogen *Aggregatibacter* (previously *Actinobacillus*) *actinomycetemcomitans* (Aa) is a gram-negative, capnophilic fermentative coccobacillus. Aa has been identified as the major pathogen responsible for localized AgP and has been implicated in generalized AgP and septicemia during pregnancy (Shalini *et al*, 1995; Xynogala *et al*, 2009; Casarin *et al*, 2010). Patients with AgP are a group of people who are highly susceptible to periodontitis. Susceptibility to periodontal disease is influenced by gene polymorphisms, and a variety of nuclear genetic factors are known to be associated with AgP. Recently, an association between the T(-) genotype of the CD28 polymorphism and AgP has been reported (E Silva *et al*, 2013)

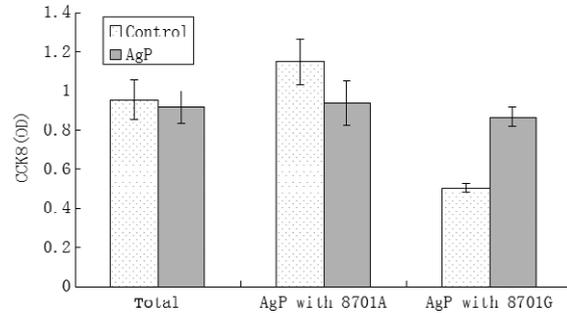


Figure 3 Measurement of cell proliferation. The first group of bars represent the total four paired AgP gingival fibroblasts and controls. The second group of bars include three AgP gingival fibroblasts with alleles of ‘8701A-9540T-10400C-10873T-14783T-15043G-15301G, 10398A’, two controls with ‘8701G-9540C-10400T-10873C-14783C-15043A-15301A, 10398G’ and one control with ‘8701A- 9540T-10400C-10873T-14783T-15043G-15301G, 10398A’. AgP gingival fibroblasts with alleles of ‘8701A-9540T-10400C-10873T-14783T-15043G-15301G, 10398A’ show proliferation impairment compared with the controls.(*represents $P < 0.01$). The third group of bars represent one cell strain of AgP with ‘8701G-9540C-10400T-10873C-14783C-15043A-15301A, 10398G’ and one cell strain of control with ‘8701A-9540T-10400C-10873T-14783T-15043G-15301G, 10398A’ and the AgP gingival fibroblasts proliferated faster than the control (*represents $P < 0.01$)

and others, such as the cathepsin C gene p.I453V variant, the osteoclastogenesis inhibitory factor gene G1181C polymorphism and the vitamin D receptor fok I polymorphism have also been reported to be responsible for increased susceptibility in generalized AgP (Park *et al*, 2006, 2008; Noack *et al*, 2008). CTSC mutations have also been studied in AgP; however, weak CTSC mutations are not a cause of aggressive periodontitis (Hewitt *et al*, 2004). Nonetheless, the list of genetic factors involved in this disease is still incomplete, and more effort is needed to identify biomarkers and genetic alterations that would allow the selection of individuals at high risk for early diagnosis, prevention and treatment programs.

The mitochondrial genome is an extranuclear genome unique to humans, which is susceptible to damage, and mtDNA has been described as a ‘real Pandora’s box’ of pathogenic mutations associated with a wide range of clinical syndromes (Dimauro and Davidzon, 2005). However, it does not mean that the mitochondrial background (mtSNP) is not important (Ruiz-Pesini *et al*, 2007). Specific mtSNPs have been shown to contribute to the

mitochondrial dysfunction in patients with diabetes. Recently, oxidative damage and mitochondrial dysfunction have been found in CP patients. Increased oxidative stress may lead to premature oxidative DNA damage in the gingival tissue of periodontitis patients and the salivary 8-hydroxy-2'-deoxyguanosine level may signify premature oxidative mtDNA damage in diseased gingival tissue (Canakçi *et al*, 2009). All conditions that affect mitochondria efficiency can alter the production of ROS, having a direct and critical effect on oxidative stress (Kowaltowski *et al*, 2009). ROS levels are increased in CP patients compared with controls, indicating high production of ROS during the inflammation of the gingival tissues. Studies of the mtDNA sequences among the CP patients suggest that the affected tissue is prone mitochondrial dysfunction, which renders the cells even more vulnerable (Govindaraj *et al*, 2011). A decrease in MMP has also been found in CP patients. Discharge of the MMP has a number of consequences for the cell, including apoptosis (Abramov *et al*, 2010; Govindaraj *et al*, 2011). Aa-LPS induces apoptosis in human trophoblasts via the mitochondria-dependent pathway, and this effect may contribute to the pathogenesis of periodontitis (Li *et al*, 2011). Thus, we hypothesized that mtDNA alterations are candidate markers of AgP susceptibility and that patients with AgP have mitochondrial dysfunction. In accordance with our hypothesis, a significant association was observed between AgP and mtSNPs including '8701A-9540T-10400C-10873T-14783T-15043G-15301G' and 10398A. These data indicate that alleles of '8701A-9540T-10400C-10873T-14783T-15043G-15301G' and 10398A significantly increase the risk of AgP compared with '8701G-9540C-10400T-10873C-14783C-15043A-15301A' and 10398G. Furthermore, our results provide evidence of mitochondrial dysfunction among AgP patients.

Although mtDNA variations and mitochondrial dysfunction have been studied in CP patients, no mtSNPs have yet been found to be associated with CP (Govindaraj *et al*, 2011). In contrast, we found eight mtSNPs related to AgP. This discrepancy may be due to the types of periodontitis investigated and ethnic differences in the patients involved in the studies. Mitochondrial dysfunction has also been studied in several types of cells in the periodontal field. H₂S increased ROS and caused a significant reduction in the mitochondrial inner transmembrane potential in human gingival epithelial cells (Calenic *et al*, 2010), while CoCl₂ induced disruption of the MMP and caused mitochondrial dysfunction in periodontal ligament cells (Song *et al*, 2012). Furthermore, high levels of mitochondrial ROS production were observed in PBMCs of periodontitis patients (Bullon *et al*, 2011, 2012), and a reduction in the MMP was observed in skin fibroblasts from CP patients compared with controls following LPS treatment (Bullon *et al*, 2011). Similar to their study, decreased MMP and increased ROS production were observed in each gingival fibroblasts from AgP patients compared with cells from controls. Additionally, we also explored proliferation of gingival fibroblast from aggressive periodontitis; however, no difference was observed between two groups.

All the genes in which the eight mtSNPs related to AgP were located are important for oxidative phosphorylation

complexes involved in ATP production. G8701A and G10398A are non-synonymous polymorphisms, while C9540T, T10400C, C10873T, C14783T, A15043G and A15301G are synonymous. G8701A (Ala59Thr) is in the ATP synthetase6 (ATP6) gene of complex V and G10398A (Ala114Thr) is in the NADH dehydrogenase subunit3 (ND3) gene of complex I (Ruiz-Pesini *et al*, 2007). G10398A is one of the most extensively studied mitochondrial polymorphisms. For example, it has been reported that 10398A is associated with Parkinson's disease (Gui *et al*, 2012), type 2 diabetes (Liao *et al*, 2008) and metabolic syndrome (Juo *et al*, 2010). Functions of G8701A and G10398A have been studied in other diseases. G8701A and G10398A were found to be closely linked and shown to alter the mitochondrial matrix pH and intracellular calcium dynamics, which are suggested to regulate the efficiency of ATP synthesis (Kazuno *et al*, 2006). Even a small decrease in ATP production may influence the motility of mitochondria and result in an imbalanced distribution. Consequently, decreased ATP production leads to higher ROS generation (Yu Wai Man *et al*, 2005). It was found that production of ROS increased due to the function alteration of complex I caused by G10398A (Ross *et al*, 2001; van der Walt *et al*, 2003; Canter *et al*, 2005; Mims *et al*, 2006; Kulawiec *et al*, 2009). Maintenance of a membrane potential is another essential property of mitochondrial function, and decreased MMP has been found in variety of aging cell types from several mammalian species (Sugrue and Tutton, 2001). In addition, low mitochondrial membrane potential was observed in the G10398A cybrid (Kazuno *et al*, 2008; Kulawiec *et al*, 2009).

In the present study, decreased MMP and increased ROS production were observed in AgP gingival fibroblasts with '8701A-9540T-10400C-10873T-14783T-15043G-15301G, 10398A' compared with cells in the control group, thus indicating their possible role in altering ROS production and MMP. Interestingly, an AgP gingival fibroblast line with '8701G-9540C-10400T-10873C-14783C-15043A-15301A, 10398G' also showed decreased MMP and increased ROS production compared with the control with '8701A-9540T-10400C-10873T-14783T-15043G-15301G, 10398A', thus indicating that other factors may affect these parameters. Previous studies have demonstrated that cells with defects in the mitochondrial respiratory chain exhibit growth impairments. It was shown that increased concentrations of CH3SH have an inhibitory effect on both cell growth and proliferation in human oral epithelial cell lines (Setoguchi *et al*, 2002). Furthermore, a lower rate of proliferation in the case of a 10398A cybrid has been observed in African-American women with breast cancer compared with the 10398G cybrid (Kulawiec *et al*, 2009). Indeed, cells from AgP patients with '8701A-9540T-10400C-10873T-14783T-15043G-15301G, 10398A' showed growth impairments when compared with controls. Interestingly, one gingival fibroblast line with '8701G-9540C-10400T-10873C-14783C-15043A-15301A, 10398G' alleles proliferated faster than paired control cells with '8701A-9540T-10400C-10873T-14783T-15043G-15301G, 10398A' alleles. This suggested that '8701A-9540T-10400C-10873T-14783T-

15043G-15301G, 10398A' contributes to the defects in the mitochondrial respiratory chain in patients with AgP, thus providing further evidence for the association between '8701A-9540T-10400C-10873T-14783T-15043G-15301G, 10398A' and AgP.

To our knowledge, this work represents the first effort to research mtDNA coding regions in AgP, and link mtSNPs to AgP. Furthermore, it is the first study of mitochondrial dysfunction in AgP.

In conclusion, we propose that mitochondrial dysfunction and '8701A-9540T-10400C-10873T-14783T-15043G-15301G, 10398A' are associated with and may increase the susceptibility to AgP in the Han Chinese population. We propose that 8701A-9540T-10400C-10873T-14783T-15043G-15301G, and 10398A may be suitable markers for identifying high-risk subjects in the Han Chinese population who require early periodontal prevention, diagnosis and treatment.

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Author contributions

Xiaoxuan Wang and Qingxian Luan designed the research. Xiaoxuan Wang and Yuan Guo participated in acquisition of data. Xiaoxuan Wang analysed data and interpreted data with Qingxian Luan, Quan Chen and Lidong Zhao. Xiaoxuan Wang drafted the paper and the other four authors revised the paper critically. All authors have approved the submitted version.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 A total of 421 variants found in the study after initial screening.