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Variants of the human *PPARG* locus and the susceptibility to chronic periodontitis

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Abstract

Apart from its regulatory function in lipid and glucose metabolism, peroxisome proliferator-activated receptor (PPAR)γ has impact on the regulation of inflammation and bone metabolism. The aim of the study was to investigate the association of five polymorphisms (rs10865710, rs2067819, rs3892175, rs1801282, rs3856806) within the *PPARG* gene with chronic periodontitis. The study population comprised 402 periodontitis patients and 793 healthy individuals. Genotyping of the *PPARG* gene polymorphisms was performed by PCR and melting curve analysis. Comparison of frequency distribution of genotypes between individuals with periodontal disease and healthy controls for the polymorphism rs3856806 showed a *P*-value of 0.04 but failed to reach significance after correction for multiple testing ($P < 0.01$). Two single nucleotide polymorphisms (SNPs) (rs10865710 and rs3892175) were found to be in strong linkage disequilibrium to rs2067819 ($D' > 0.90$). A 3-site analysis (rs2067819-rs1801282-rs3856860) revealed five haplotypes with a frequency of ≥1% among cases and controls. Following adjustment for age, gender and smoking, none of the haplotypes was significantly different between periodontitis and healthy controls after Bonferroni correction. This study could not show a significant association between *PPARG* gene variants and chronic periodontitis.

Keywords

Heritability, innate immunity, oral, attachment loss, metabolism, diabetes

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Introduction

Periodontal disease is defined as a bacterial-derived chronic infection of the gingival crevicular area.[1,2] In order to explain the considerable discrepancies in individual host susceptibility, a strong genetic background for the pathogenesis of chronic periodontitis has been suggested. A previous study on twins estimated that the heritability of this entity reaches even 50%.[3] According to a common pathogenetic model, the genetic impact on individual susceptibility and/or severity of periodontitis are mediated by either an inappropriate or exaggerated immune response against a given bacterial stimulus.[4,5] Hence, polymorphisms of genes that are involved in the stimulation and regulation of inflammatory processes are excellent candidates for the elucidation of the genetic background of the periodontal pathogenesis.[6]

The peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily and are ligand activated transcription factors (for review, see Rizzo et al.[7]). Three subtypes have been identified so far: PPARα (NR1C1), PPARβ/δ (NR1C2) and PPARγ (NR1C3). The PPARs are activated by different natural ligands, *i.e.* fatty acids, eicosanoids, and oxidized fatty acids, as well as by lipid-lowering fibrates and the anti-diabetic glitazones.[8,9]

The PPARs are primarily involved in lipid and lipoprotein metabolism, glucose homeostasis, cell proliferation and differentiation.[10] Moreover, a growing body of evidence suggests that at least PPARγ plays a significant role in regulation and mediation of

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inflammatory reactions.[11] Particularly, PPARγ influences the differentiation of monocytes and attenuates the expression of various pro-inflammatory mediators (*e.g.* TNF-α, IL-1β and IL-6).[12–14] Also matrix metalloproteinase (MMP) 9, which is involved in periodontal inflammatory process, is down-regulated by PPARγ.[14]

Various polymorphisms at the human *PPARG* locus have yet been described. It has been suggested that some of these variants (rs10865710, rs2067819, rs3892175, rs1801282, rs3856806) might be functionally effective, particularly leading to impaired anti-inflammatory effects of PPARγ.[15–17] For instance, the variants rs1801282 and rs10865710 were shown to be associated with the risk of myocardial infarction[18] and with systemic levels of inflammatory markers in patients with end-stage renal disease.[19]

The present study aimed to assess whether these polymorphisms of the *PPARG* gene contribute to the susceptibility for periodontal disease.

Subjects and methods

Patient population

Individuals with severe medical disorders including diabetes mellitus, immunological disorders, increased risk for bacterial endocarditis and pregnant females were excluded from the study. The study conformed to the ethical guidelines of the Helsinki Declaration and was approved by the local ethics committee. Participants provided written, informed consent prior to their enrolment into the study. Study subjects in both groups were adult, unrelated Caucasians from the south of Germany (region of Upper Bavaria). In order to improve genetic homogenicity of the study sample, all subjects must have parents and grandparents of the German ethnic group.

Subjects in both study groups received a standardized periodontal examination protocol including the evaluation of: (i) the probing pocket depth measured at six locations on each tooth (mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual, disto-lingual) using a Michigan type 'O' probe; (ii) furcation involvement using a Naber type probe; (iii) bleeding on probing registered as present or absent; and (iv) bone loss as assessed by orthopantomographs. The probing pocket depth was defined as the distance from the free gingival margin to the base of the periodontal pocket keeping the probe in line with the long axis of the tooth. For the examination of furcation defects, horizontal probing from the furcation entrance to the base of the defect was used. The furcation involvement was classified according to the protocol of Nyman and Lindhe.[20] Due to ethical reasons, radiographic examination using orthopantomographs was done for individuals having periodontitis only.

A total of 402 patients from the Department of Periodontology, Ludwig-Maximilians University (Munich, Germany) were enrolled. The median age in the periodontitis group was 54.1 years (SD ± 10.9) and the age ranged from 18–85 years (Table 1).

The patients fulfilled the following clinical criteria: (i) a total of at least 15 teeth *in situ*; (ii) ≥8 teeth with a probing pocket depth of ≥5 mm at least at one location and/or a furcation involvement ≥class II; and (iii) evidence of bone loss manifested as the distance between the alveolar crest and the cemento-enamel junction of ≥3 mm around the affected teeth.

A total of 793 unrelated, ethnically matched, Caucasian individuals without periodontitis comprised the healthy control group. The absence of periodontal disease was determined according to the following criteria: (i) a minimum of 22 teeth *in situ*; (ii) ≤1 site with probing pocket depth ≥3 mm; and (iii) lack of any kind of furcation involvement at any tooth. Within the control group, the median age was 47.3 years (SD ± 9.2) and the age ranged from 18– 73 years.

Information regarding smoking habit was available for 354 of the patients with periodontitis and 538 of the control persons. Individuals have been classified as smokers if they had a self-reported history of smoking of at least 10 cigarettes/day for at least one year.

Blood samples and DNA isolation

Peripheral venous blood samples of 9 ml were drawn from each individual by standard venepuncture. Each blood sample was collected in sterile tubes containing K_3EDTA solution. DNA was isolated using partly the QIAamp® DNA Blood Midi Kit (Qiagen, Hilden, Germany), partly the salting out procedure.[21]

Table 1. Demographic data of the two study groups with periodontitis patients and healthy individuals

	Control ($n = 793$)	Periodontitis ($n = 402$)	*P*-value
Age at diagnosis years (mean)	8–73 (47.3)	21–85 (54.1)	0.001
Sex			
Male (%)	531 (67.4)	178 (47.5)	
Female (%)	257 (32.6)	197 (52.5)	0.001
Smoking			
Smokers (%)	67 (12.5)	113 (31.9)	
Non-smokers (%)	471 (87.5)	241 (68.1)	0.001
Number of teeth	ND	25.3 (±4.9)	
Cases per severity			
Mild	ND	86 (37.4)	
Moderate	ND	81 (35.2)	
Severe	ND	63 (27.4)	

Genotyping of the polymorphisms within the PPARG gene

Five *PPARG* single nucleotide polymorphisms (SNPs) (rs10865710, rs2067819, rs3892175, rs1801282, rs3856806) were genotyped partly by PCR and restriction fragment length polymorphism (RFLP) analysis as described elsewhere[22] and partly by PCR and melting curve analysis using a pair of fluorescence resonance energy transfer (FRET) probes in a LightCycler® 480 Instrument (Roche Diagnostics, Mannheim, Germany). The donor fluorescent molecule (fluorescein) at the 3'-end of the sensor probe is excited at its specific fluorescence excitation wavelength (533 nm) and the energy is transferred to the acceptor fluorescent molecule at the 5'-end (LightCycler Red 610, 640 or 670) of the anchor probe. The specific fluorescence signal emitted by the acceptor molecule is detected by the optical unit of the LightCycler 480 Instrument. The sensor probe is exactly matching to one allele of each SNP, preferentially to the rarer allele, whereas in the case of the other allele there is a mismatch resulting in a lower melting temperature. The total volume of the PCR was 5 µl containing 25 ng of genomic DNA, 1 × Light Cycler 480 Genotyping Master (Roche Diagnostics), 2.5 pmol of each primer and 0.75 pmol of each FRET probe (TIB MOLBIOL, Berlin, Germany). In the case of rs10865710, the concentration of the forward primer was reduced to 1.25 pmol. The PCR comprised an initial denaturation step (95°C for 10 min) and 45 cycles (95°C for 10°C s, primer annealing temperature as given in supplementary material (Table S1) for 10 s, 72°C for 15 s). The melting curve analysis comprised an initial denaturation step (95°C for 1 min), a step rapidly lowering the temperature to 40°C and holding for 2 min, and a heating step slowly (1 acquisition/°C) increasing the temperature up to 95°C and continuously measuring the fluorescence intensity. The results of melting curve analysis have been confirmed by analyzing two patient samples for each possible genotype using sequence analysis. For sequencing, the total volume of the PCR was 100 µl containing 250 ng of genomic DNA, 1 × PCR-buffer (Qiagen, Hilden, Germany), the final $MgCl_2$ concentration as given in Table 2B, 0.2 mM of a dNTP-Mix (Sigma, Steinheim, Germany), 2.5 units of HotStar Plus Taq™ DNA polymerase (Qiagen) and 10 pmol of each primer (TIB MOLBIOL). The PCR comprised an initial denaturation step (95°C for 5 min), 35 cycles (denaturation at 94°C for 30 s, primer annealing temperature as given in Table S1 for 30 s, extension at 72°C for 30 s) and a final extension step (72°C for 10 min). The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) and sequenced by a commercial sequencing company (Sequiserve, Vaterstetten, Germany). All sequences of primers and FRET probes and primer annealing temperatures used for genotyping and for sequence analysis are given in supplementary material (Tables S1 and S2).

Statistical analysis

Deviations from the Hardy–Weinberg equilibrium were tested in the control group separately for each genetic marker using an Exact test and a type 1 error level of 0.05.[23] Statistical power analysis was performed under the assumption that the minor allele frequency equals at least 0.1 roughly corresponding to the lower limit for the marker allele frequencies among control individuals (<http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/power2.pl>). Significance of associations with or between single locus genotypes were assessed with Fisher's exact test or the chi-squared test with Yates correction where appropriate. Using Bonferroni correction for five non-independent tests (5 SNPs), the nominal probability value was set at $P = 0.01$. Subgroup analysis was done according to gender, smoking and severity of disease. For comparison of quantitative variables (*i.e.* age), the Student's *t*-test was used. Analysis was performed using PASW software, v18.0 (SPSS Inc., Chicago, IL, USA). For the determination of the mode of inheritance that could best explain associations, Akaike's Information Criterion was used.[24] Each SNP was considered under a co-dominant, dominant, recessive and over-dominant effect model using SNPStats (<http://bioinfo.iconcologia.net/index.php?module=Snpstats>). For haplotype analysis, linkage disequilibrium

Table 2. Frequency distribution of genotypes for the five polymorphisms at the *PPARG* locus

		Genotypes periodontitis (%)				Genotypes control (%)				
SNP (alleles)		N 11	N 12	N 22	MAF	N 11	N 12	N 22	MAF	HWE
rs10865710	(C/G)	221 (55.7)	157 (39.5)	19 (4.8)	24.6	414 (53.8)	296 (38.5)	59 (7.7)	26.9	0.58
rs2067819	(G/A)	248 (62.3)	136 (34.2)	14 (3.5)	20.6	461 (59.3)	268 (34.5)	48 (6.2)	23.4	0.27
rs3892175	(G/A)	345 (86.7)	52 (13.1)	1 (0.2)	6.8	621 (80.1)	145 (18.7)	9 (1.2)	10.5	0.85
rs1801282	(C/G)	293 (73.8)	96 (24.2)	8 (2.0)	14.1	581 (75.5)	173 (22.5)	16 (2.0)	13.3	0.44
rs3856806	(C/T)	295 (74.5)	97 (24.5)	4 (1.0)	13.3	561 (72.3)	193 (24.9)	22 (2.8)	15.3	0.27

N, number of individuals; 1, major allele; 2, minor allele; MAF, minor allele frequency; HWE, analysis of Hardy–Weinberg equilibrium.

measures were estimated from inferred haplotypes using the Haploview v4.2 software.[25] Haplotype frequencies were estimated with the expectation maximation algorithm.[26] Analysis of associations between haplotypes and phenotypes was performed with SNPStats after adjustment for age, gender, and smoking as confounding variables using logistic regression. Since 3-site haplotypes have been used for this analysis, the level of significance was set at $P < 0.017$ after Bonferroni correction.

Results

Study sample

A total of 402 patients with chronic periodontitis and 793 healthy control individuals have been genotyped for the five polymorphisms at the human *PPARG* locus. The power calculation revealed that the minimum sample size for patients with periodontitis required 383 individuals and 778 for the healthy controls.

Frequency distribution of alleles and genotypes

Minor allele and genotype frequencies of the five polymorphisms of the peroxisome proliferator-activated receptor *PPARG* gene within both study groups are shown in Table 2. For each SNP, the genotype frequencies were in agreement with the Hardy–Weinberg equilibrium.

The minor allele frequencies for the five SNPs varied between 6.8% and 24.6% in the periodontitis group and between 10.5% and 26.9% in the control population. Considering different effect models with Akaike's Information Criterion showed that a recessive model does best fit to the data as observed herein. Doing association analysis under a recessive effect model after adjustment for age, gender, and smoking revealed an obviously significant association for the polymorphism rs3856806 ($P = 0.04$). The mutant allele was more prevalent among healthy individuals (rs3856806 T allele: 15.3%) than in periodontitis patients (rs3856806 T allele: 13.3%; Table 3). However, after performing Bonferroni correction for multiple testing, this association remained non-significant. Also, subclass analysis according to gender and smoking revealed no nominal significant association between any of the tested polymorphisms and periodontal disease (supplementary Table S3).

Linkage disequilibrium and haplotype analysis

Linkage disequilibrium between the five tested loci is given as Lewontin's D' in Table 4. Two SNPs (rs10865710 and rs3892175) were in strong LD with rs2067819 ($D' > 0.9$). A 3-site haplotype analysis excluding rs10865710 and rs3892175 revealed a frequency of $\geq 1\%$ for a total of five haplotypes in both study groups (Table 5). Three of these haplotypes composed of rs2067819, rs1801282 and rs3856806 seemed to be significantly associated with periodontitis (A-G-C: $P = 0.019$; OR 0.44, 95% CI 0.22–0.87;

Table 3. Analysis of associations between single locus genotypes for the five polymorphisms at the *PPARG* locus with periodontitis using five different effect models, *i.e.* co-dominant, dominant, recessive, and over-dominant

	rs10865710	rs2067819	rs3892175	rs1801282	rs3856806
Co-dominant model					
P-value	0.32	0.63	0.07	0.75	0.07
OR_{het} (95% CI)	0.96 (0.69–1.32)	0.99 (0.71–1.36)	1.31 (0.86–1.99)	0.87 (0.60–1.25)	1.19 (0.84–1.69)
OR_{hom} (95% CI)	1.59 (0.82–3.09)	0.96 (0.66–3.08)	NA	0.99 (0.32–3.05)	4.15 (0.99–17.47)
AIC	980.4	991	984.6	982.5	981.6
Dominant model					
P-value	0.82	0.86	0.13	0.47	0.17
OR (95% CI)	1.03 (0.75–1.40)	1.03 (0.75–1.40)	1.37 (0.91–2.08)	0.88 (0.62–1.25)	1.28 (0.90–1.80)
AIC	980.6	989.9	985.6	980.5	982.9
Recessive model					
P-value	0.14	0.34	0.06	0.98	0.04
OR (95% CI)	1.62 (0.84–3.10)	1.44 (0.67–3.07)	NA	1.02 (0.33–3.14)	3.96 (0.94–16.61)
AIC	978.4	989	984.2	981	980.5
Over-dominant model					
P-value	0.56	0.81	0.22	0.45	0.42
OR (95% CI)	0.91 (0.67–1.25)	0.96 (0.70–1.32)	1.29 (0.85–1.96)	0.87 (0.60–1.25)	1.15 (0.81–1.64)
AIC	980.3	989.9	986.4	980.5	984.2

Results obtained following adjustment for smoking, gender, and age. OR, odds ratio; OR_{het}, odds ratio for the heterozygote carriership of the rare allele; OR_{hom}, odds ratio for the homozygote carriership of the rare allele, 95% CI, confidence interval at 95%; AIC, Akaike's Information Criterion.

Table 4. Linkage analysis for the five polymorphisms at the *PPARG* locus given as Lewontins D' value for multiple comparisons

	rs10865710	rs2067819	rs3892175	rs1801282	rs3856806
rs10865710	–	0.9009	0.8863	0.8505	0.6823
rs2067819	–	–	0.9314	0.8139	0.5325
rs3892175	–	–	–	0.6759	0.8363
rs1801282	–	–	–	–	0.5877
rs3856806	–	–	–	–	–

Table 5. Frequency estimates for the 3-site haplotypes composed of the genotypes for rs2067819, rs1801282 and rs3856806

	Estimated frequencies				
	Periodontitis	Controls	Cumulative	OR (95% CI)	*P*-value
G-C-C	0.7475	0.6877	0.7088	1.00	NA
A-C-C	0.0712	0.1115	0.8059	1.53 (1.01–2.32)	0.04
A-G-T	0.0886	0.0796	0.8886	1.05 (0.70–1.59)	0.80
G-C-T	0.0397	0.0511	0.9353	1.91 (1.07–3.41)	0.03
A-G-C	0.0461	0.0277	0.9696	0.44 (0.22–0.87)	0.02

Association analysis was performed for the haplotypes with an estimated frequency of $\geq$1%. OR, odds ratio; 95% CI, confidence interval at 95%.

G-C-T: $P = 0.028$, OR 1.91, 95% CI 1.07–3.41; A-C-C: $P = 0.046$; OR: 1.53, 95% CI 1.01–2.32). However, none of these associations reached the level of nominal significance after Bonferroni correction ($P < 0.017$).

Discussion

For the delineation of the genetic background of chronic periodontitis directly, population-based association studies on genetic variants affecting the regulation of innate immunity seem to be particularly attractive.[27] Peroxisome proliferator-activated receptor γ was suggested to have regulatory functions in innate immune response. Recent data suggest that activators of PPARγ, *e.g.* thiazolidinediones, can down-modulate inflammatory processes. So far, the expression of PPARγ has been confirmed in human osteoclasts[28] and keratinocytes[29] of the oral mucosa both of which are involved in the manifestation and perpetuation of periodontitis. Thus, it seems reasonable to assume that PPARγ might contribute to the susceptibility for periodontitis. The potential role of PPARγ in the pathogenesis of periodontitis is further supported by findings in animal studies. In rodents, exogenous PPARγ ligands, *i.e.* glitazones, were able to reduce the manifestation of experimental periodontitis effectively.[30] The results of *in vitro* experiments suggest that, upon activation, PPARγ inhibits RANKL-mediated osteoclastogenesis ultimately leading to the attenuation of periodontal tissue destruction.[31]

Based on recent data, the *PPARG* variant rs1801282 could be of particular interest since it seemed to be associated with the risk for periodontitis in pregnant women.[32] Pregnant carriers of the rare rs1801282-G allele have a significantly higher susceptibility for periodontal attachment loss as compared to carriers of the wild-type allele. Although a slightly higher prevalence of the mutant allele was found among periodontitis patients than in healthy control subjects in the present study, this difference did not reach significance. Also the variant rs3856806 justifies specific interest in the context of our study, since it was shown to be associated with Crohn's disease.[17] It was hypothesized that periodontitis and Crohn's disease share a common immunopathogenetic pathway.[33] The rare allelic variant seems to be linked to an attenuated inflammatory response in consequence lowering the individual susceptibility for Crohn's disease. Also, among periodontitis patients, the rare variant was more common as compared with healthy controls, but statistical analysis did not confirm a significant difference between cases and controls.

As in other complex diseases, genetic association studies in periodontitis are difficult to design since multiple genetic factors and, in addition, various phenotypic factors are thought to have impact on the pathogenesis. A particular challenge is to take into account all established risk factors for chronic periodontitis. Herein, we could include at least smoking and gender into multivariate statistical analysis. Moreover, certain phenotypic variables might have caused stratification of the study sample. In this respect the influence of vascular diseases might be of particular importance. It has been shown that the *PPARG* polymorphisms tested in this study are associated with coronary heart disease and arterial hypertension.[18,34] Since no data were available on the disease status of the study

subjects considering both entities, the present findings could not be adjusted for these potentially confounding variables.

Although power analysis revealed that the study sample has enough power to detect associations between periodontitis and the genetic variants of the PPARG locus, it might drop considerably if the relative risk for heterozygous or homozygous carriers of a functional variant that leads to periodontitis is smaller than assumed in power calculation. For example, the C to T transition of the polymorphism rs3856806 is a silent mutation. One can assume that this polymorphism showing differences in allele frequencies between both study groups is in linkage disequilibrium to a further, yet unknown, functional variant that ultimately leads to an impaired immunological reaction in periodontitis. Moreover, it was hypothesized that probably more than 20 genetic loci contribute to the heritability of chronic periodontitis, all of which might show only very low penetrance.[35] As previously done in other complex genetic disorders, a genome-wide association study probably comprises a more suitable approach to delineate those genetic regions that are in fact involved in the pathogenesis of periodontal disease.[36]

Conclusions

The findings of the present study reject the hypothesis that variants of the human *PPARG* gene have impact on the pathogenesis of chronic periodontitis.

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