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CARD15 gene variants in aggressive periodontitis

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Abstract

Objective: The *CARD15* gene encodes a protein that acts as an intracellular receptor of bacterial products, thus playing an important role in the innate immune response. Recently, *CARD15* gene variants have been identified as a cause of increased susceptibility to Crohn's disease. The present study aimed to examine a potential association of *CARD15* gene variants with aggressive periodontitis susceptibility.

Material and Methods: The three main known *CARD15* gene variants (p.R702W, p.G908R, and p.L1007fsX1008) were analysed by direct sequencing of exon 4, 8, and 11 of the gene in a total of 86 generalized aggressive periodontitis patients in comparison with 67 healthy controls.

Results: The mutant allele frequencies of the *CARD15* variants were low in the generalized aggressive periodontitis group as well as in the control group and not significantly different (R702W: 3.5% versus 5.2%; G908R: 1.7% versus 1.5%; L1007fsX1008: 5.2% versus 4.5%). Two rare variants (A755V and R791Q), previously described only in patients with other inflammatory diseases, were observed in three patients having aggressive periodontitis but not in controls.

Conclusions: Unlike in Crohn's disease, our results did not show an association between the three main *CARD15* mutations and aggressive periodontitis. The role of rare variants remains unclear.

Key words: aggressive periodontitis; *CARD15* gene; genetic risk; innate immune response; mutation

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Aggressive periodontitis (AgP) comprises rare, but severe and progressive forms of periodontal diseases in otherwise healthy patients. It is characterized by a rapid destruction of periodontal tissue, and familial aggregation is possible (Lang et al. 1999). AgP, like chronic periodontitis (CP), is considered to be caused by specific oral pathogens that induce host defence reactions. Tissue destruction results from an imbalance in host protective and destructive mechanisms. The host response to the bacterial challenge, i.e. the quality and quantity of the local inflammatory and immune reaction, is at least in part genetically determined. Thus, numerous candidate genes involved in host responses have been considered for an increased AgP susceptibility (Kinane & Hart 2003, Loos et al. 2005, Shapira et al. 2005).

Recently, *CARD15* (former named *NOD2*) gene mutations have been identified to increase the susceptibility to inflammatory diseases. *CARD15* encodes an intracellular protein that has been implicated in the innate immune response by recognition of invading bacteria and induction of inflammatory response. It is composed of two caspase recruitment domains (CARD), a central nucleotide binding domain (NOD) and 10 leucin-rich repeats (LRR; Ogura et al. 2001b). The LRRs are involved in intracellular recognition of Gram-positive and Gram-negative bacterial cell wall components. Muramyl dipeptide (MDP) derived from peptidoglycan (PGN) was identified as the bacterial structure recognized by CARD15 (Girardin et al. 2003, Inohara & Nunez 2003). In response to bacterial MDP exposure, CARD15 induces apoptosis and activates the nuclear factor κB (NF-κB). The latter is a protein that plays an important role in pro-inflammatory responses by regulating the transcription of cytokine genes (Inohara et al. 2001, Ogura et al. 2001b).

Three major variants, namely R702W, G908R, and L1007fsX1008, as well as multiple rare variants in the *CARD15* gene have been associated with susceptibility to different inflammatory diseases. A strong association with the *CARD15* variants has been shown especially in Crohn's disease (CD), with carrier frequencies of one or more variant *CARD15* alleles between 22% and 60% (Hampe et al. 2001, 2002, Hugot et al. 2001, Abreu et al. 2002, Lesage et al. 2002, Ogura et al. 2001a, Sun et al. 2003, Tukel et al. 2004). Other inflammatory diseases

with reported *CARD15* variants are spondylarthropathy (SpA; Miceli-Richard et al. 2002), or psoriatic arthritis (PsA; Rahman et al. 2003).

The disease-associated CARD15 protein variants are functionally relevant. They are impaired in their ability to sense PGN or MDP, and also in their NF-κB response to MPD (Girardin et al. 2003, Inohara et al. 2003). A recent study showed that cultured macrophages derived from primary peripheral blood mononuclear cells of CD patients harbouring the R702W, G908R, or L1007fsX1008 mutation exhibit a significant defect in the MDP-dependent, NF-κB-mediated activation of several pro-inflammatory cytokines (Li et al. 2004). The authors concluded from their data that defects in the initial innate response to bacterial exposure result in increased susceptibility to inflammation. Cytokines play an important role in the regulation of host defence mechanisms against oral pathogens as well as in host-mediated periodontal tissue destruction if inappropriately expressed. This pathogenetic role of cytokines in periodontitis, as well as the function of CARD15 in cytokine activation, may imply that *CARD15* gene variants could also be involved in cytokine-mediated inflammatory responses in periodontitis, at least in a subgroup of diseased patients like in CD. One previous study failed to show an association of the L1007fsX1008 variant and CP (Folwaczny et al. 2004). A second study analysed both the R702W as well as the L1007fsX1008 variants in periodontitis with similar negative results (Laine et al. 2004). However, this study did not consider a stratification for AgP or CP. AgP and CP are different types of periodontitis with clearly identifiable clinical and laboratory findings (Lang et al. 1999). Thus, the aim of our study was to analyse the frequencies of the three most common variations of the *CARD15* gene (R702W, G908R, and L1007fsX1008) in generalized AgP (G-AgP) compared with healthy controls, assessing the association of *CARD15* variants with an increased susceptibility to this subgroup of periodontitis.

Material and Methods

Study population

A total of 153 unrelated Caucasian German subjects volunteered to participate in this case–control association study. One hundred and thirty-eight patients were recruited at the Department of Conservative Dentistry and Periodontology, Medical Faculty, Dresden University of Technology, Germany, and 15 patients were recruited from the School of Dental Medicine, Department of Periodontology at the University Greifswald, Germany.

Each patients received an appropriate description of the study protocol and signed a consent form, approved by the local ethics committee (reference no. EK 151102000 and EK 26022005, respectively). A thorough history of systemic and dental diseases and smoking history was obtained by interviews. Subjects having severe systemic disorders (including diabetes mellitus, immunological disorders, chronic inflammatory diseases, or increased risk for bacterial endocarditis) were not enroled in the study. The categories of smoking history were recorded as: never smoker, smoker (regardless of the number of cigarettes smoked per day), or former smokers (who have quit smoking for at least 5 years).

Eighty-six patients with G-AgP were selected based on clinical and radiographic findings, according to the recommendations of the 1999 international classification workshop (Lang et al. 1999). The criteria for G-AgP were: generalized inter-proximal attachment loss (AL) at least on three teeth other than first molars and incisors. This loss of clinical attachment had to be $\geqslant 5$ mm, and inter-proximal bone loss had to be $\geqslant 50\%$ of the root length as assessed by full-mouth radiographs or orthopantomographs. Proximal AL of $\geqslant 5$ mm in $\geqslant 30\%$ of teeth was used for threshold level in the case definition to include only cases with substantial periodontitis extent and severity as proposed to identify risk factors at the 5th European Workshop on Periodontology (Tonetti & Claffey 2005).

Although the current classification system of periodontal diseases is no longer based principally on the age of the patient, in most cases, AgP is characterized by an early age of clinical manifestation. Thus, all G-AgP patients were less than 40 years old at the time of diagnosis (mean age 34.1 ± 6.0 years).

The control group comprised a total of 67 unrelated individuals. They were periodontally healthy or showed only minimal signs of periodontal AL. In accordance with the recommendations of Morton & Collins 1998 for genetic studies regarding diseases with early onset (''Sample-Based Case–Control Design with hypernormal controls''), we included only controls older than 40 years of age (mean age 53.7 ± 9.7 years) to minimize the likelihood of a late onset of AgP. Controls were selected based on the following criteria: at least 22 teeth in situ (no history of tooth loss because of increased tooth mobility due to periodontitis), at least 90% of measured tooth sites with probing pocket depth (PPD) < 4 mm and AL $\leqslant 1$ mm, no PPD $\geqslant 5$ mm and no AL > 2 mm, as well as no vertical bone defects present in radiographs.

Blood samples and DNA isolation

Peripheral blood was obtained from all subjects and genomic DNA was purified from whole blood using the QiaAmp blood DNA purification kit (Qiagen, Hilden, Germany).

Sequence analysis of the *CARD15* gene

The three major CD-associated *CARD15* gene variants were genotyped by amplifying the last part of exon 4 (530 bp) and complete exons 8 and 11 using primer pairs and protocols described previously (Sun et al. 2003). Purified PCR products were directly sequenced using the ABI PRISM® BigDye® Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase v3·0 and v3·1 (Applied Biosystems, Forster City, CA, USA) and capillary sequencing devices (ABI 3730 DNA Analyzer, Applied Biosystems). The sequences were generated by the Sequencher 4.2 software (Gene Codes Corp., AnnArbor, MI, USA) and aligned with the published *CARD15* gene sequence (GenBank accession no. AF178930). Identified DNA sequence variants were confirmed by sequencing of at least two independent PCR products.

Data analysis

For association analysis, sample size calculation was performed as proposed (Fleiss 1981) using a calculator available online (http://statpages.org/proppowr.html). As no data are available for all analysed *CARD15* genotype frequencies and allele frequencies in European periodontitis populations, we used published allele frequencies for a

European study on CD (Lesage et al. 2002). In that study, the total frequency of variant alleles in the CD and the control group was approximately 25% and 7%, respectively. The sample size for each group was calculated on the basis of a α significance level of 0.05 and a power level of 80%. The sample size calculation showed that 65 control subjects and 84 AgP patients were required in case of a control-to-case ratio of 1 to 1.3, which reflects the ratio in our sample.

Statistical analysis was performed using SPSS software version 12.0 for windows (SPSS Inc., Chicago, IL, USA). Allele and carrier frequencies of the three main *CARD15* variants in the three study groups were calculated. Fisher's exact test was used to compare frequencies among the groups. A potential association between aggressive periodontitis and *CARD15* mutations was assessed by using multiple logistic regression models after adjusting for other confounding factors (gender and smoking). Adjusted odds ratios and 95% confidence intervals (95% CI) were calculated. To determine whether the study population is in Hardy–Weinberg equilibrium for the particular determined genotypes, χ^2 goodness-of-fit tests were performed.

Thresholds for significant *p*-value for all tests was considered to be 0.05, if needed, corrected for the number of hypotheses tested (Bonferroni approach).

Table 1. Characteristics of the study population

	G-AgP* group ($n = 86$)	Control group ($n = 67$)	*p*-value
No. teeth present (mean ± SD)	25.3 ± 3.2	25.4 ± 2.7	0.959
Mean AL ± SD (mm)	4.4 ± 0.9	1.3 ± 0.5	<0.0001†
No. of teeth with AL ⩾5 mm	18.3 ± 5.0	None	
No. of proximal sites with bone loss ⩾50%	31.1 ± 7.5	None	
Mean age (years ± SD)	34.1 ± 6.0	53.7 ± 9.7	<0.0001†
No. (%) never smoker	57 (66.3)	56 (83.6)	
No. (%) former smoker	7 (8.1)	4 (6.0)	
No. (%) smoker	22 (25.6)	7 (10.4)	0.042‡
No. (%) male	37 (43.0)	23 (34.3)	0.318§

*Generalized aggressive periodontitis.
†Unpaired *t*-test.
‡Overall *p*-value from χ^2 test, post hoc testing: Fisher's exact test, comparing smoker and non-smoker (never and former) between the groups, $p = 0.02$.
§Fisher's exact test.

Table 2. *CARD15* variants (carrier and allele frequencies)

Nucleotide position and change	Peptide change	No (%) of variant alleles in study groups		No (%) of variant carriers in study groups		Adjusted OR A for G-AgP	
		G-AgP ($n = 172$)	Control ($n = 134$)	G-AgP ($n = 86$)	Control ($n = 67$)	OR*	95% CI†
c.2104 C>T	p.R702W	6 (3.5) $p = 0.571$	7 (5.2)	6 (7.0)	6 (9.0) $p = 0.765$	0.67	0.23;1.99
c.2722 G>C	p.G908R	3 (1.7) $p = 1.000$	2 (1.5)	3 (3.5)	2 (3.0) $p = 1.000$	1.16	0.18;7.48
c.3020insC	p.1007fs	9 (5.2) $p = 0.797$	6 (4.5)	9 (10.5)	6 (9.0) $p = 0.792$	1.24	0.40;3.80

p-Values were calculated by Fisher's exact test.
*Odds ratio (OR) adjusted for gender and smoking (carrier status was included in the model).
†[95% CI], 95% confidence interval.

Results

The periodontal and demographic characteristics of the study population are given in Table 1. The groups differ in their periodontal findings according to the case and control definition. As expected, the percentage of smokers was also significantly higher in the test group than in the control group, as smoking is an established risk factor for periodontitis. The G-AgP patients were also significantly younger than the controls according to the inclusion criteria for the groups.

The genotype frequencies of the whole study population as well as the frequencies of the three study groups were in Hardy–Weinberg equilibrium. The carrier and allele frequencies of the three variants between the groups were very similar (Table 2), and the differences in these frequencies between the groups were not statistically significant. All carriers of any mutation were heterozygous, except one control subject, who harboured the homozygous 2104C>T mutation (R702W). Although this control person had abdominal symptoms reminiscent of CD several years ago, clinical and histological examination did not confirm the diagnosis. No subject was a compound heterozygous carrier of two mutations. All study patients were further classified into carriers and non-carriers of at least one of the three mutations. No significant differences in the carrier frequencies was found between the G-AgP group (20.93%) and the control group (20.90%). The logistic regression analysis adjusted for gender and smoking showed no association between the carrier status of any of the studied *CARD15* variants and periodontal status (Table 2).

Lastly, two rare sequence variants (A755V and R791Q) were observed in three patients with G-AgP but were not found in the control population.

Discussion

AgP, like chronic periodontitis, is a multifactorial inflammatory disease, that results from complex interactions between the microbial challenge and specific host reactions. A genetic predisposition for the disease is of particular significance. Numerous studies carried out in the last 10 years supported the hypothesis that the host immune reactions and the quality and quantity of the local inflammatory response are at least in part genetically determined (Kinane & Hart 2003, Loos et al. 2005, Shapira et al. 2005, Nibali et al. 2006).

Cytokines play an important role in inflammatory host reactions and thus in the pathogenesis of periodontitis (Seymour & Gemmell 2001). For this reason, polymorphisms in genes coding for pro-inflammatory cytokines such as IL-1, TNF-α, or IL-10 have attracted much attention as potentially crucial variants influencing the host response in perio-

dontitis (Kinane & Hart 2003). Furthermore, periodontal pathogens induce cytokine production via a pathway involving bacterial LPS-recognizing receptors-like Toll-like receptors (TLR) (Madianos et al. 2005). A recent study established an association of single-nucleotide polymorphisms (SNP) in the *TLR-4* gene with periodontitis (Schröder et al. 2005). We have analysed the *CARD15* (*NOD2*) gene coding for a protein that acts as a sensor for intracellular pathogens. Three frequent *CARD15* mutations, R702W, G908R, and L1007fsX1008, as well as multiple rare variants have been found to impair NF-κB-mediated expression of pro-inflammatory cytokines in response to bacterial challenge (Ogura et al. 2001a, Girardin et al. 2003, Inohara et al. 2003, Li et al. 2004). Patients harbouring these mutations are more susceptible to inflammatory diseases such as CD (Hampe et al. 2001, 2002, Hugot et al. 2001, Ogura et al. 2001a, , Lesage et al. 2002, Sun et al. 2003, Tukel et al. 2004), SpA (Miceli-Richard et al. 2002), or PsA (Rahman et al. 2003). However, in our study, we could not detect an association between G-AgP and any of the three major mutations in the *CARD15* gene. The allele frequencies in the G-AgP and control groups for the 2104T allele (3.5% and 5.2%), the 2722C allele (1.7% and 1.5%), as well as for the 3020insC allele (5.2% and 4.5%), respectively, were similar to the allele frequencies in healthy non-CD controls of other European studies ranging from 3.5% to 5.3%, from 0.3% to 1.4%, and from 0.8% to 4.4%, respectively (Hampe et al. 2001, Hugot et al. 2001, Ahmad et al. 2002, Cuthbert et al. 2002, Lesage et al. 2002, Radlmayr et al. 2002, Arnott et al. 2004).

The data presented here are in line with two recent studies that failed to show an association between the R702W or the L1007fsX1008 mutation and chronic and severe periodontitis, respectively (Folwaczny et al. 2004, Laine et al. 2004). The allele frequency especially for the L1007fsX1008 mutation in our AgP group (5.2%) was slightly increased compared with 1.9% in CP (Folwaczny et al. 2004) and 2.5% in severe periodontitis (Laine et al. 2004). However, the number of subjects harbouring a *CARD15* variant still remained very low. This could be an explanation for the absence of significant differences between our study groups. A further reason could be that different bacteria are implicated in the development of CD and periodontitis. Thus, one can speculate that in periodontitis, receptors other than CARD15 are involved in bacterial recognition and initiating antibacterial host reactions, e.g. Toll-like receptors. Impaired NF-κB activation by the PGNs via *CARD15* mutations would therefore have only minor overall relevance in the pathogenesis of periodontitis. However, it cannot be excluded that rare variants discovered in three AgP subjects and previously described in CD subjects (Lesage et al. 2002) as well as in SpA patients (Miceli-Richard et al. 2002), or variants in other regions of the *CARD15* gene may have an impact on periodontitis susceptibility.

Our findings, verifying no common genetic background of CD and G-AgP susceptibility, in turn support two previous studies (Flemmig et al. 1991, Grössner-Schreiber et al. 2006), who found that CD patients are not more susceptible to severe forms of periodontitis than the general population or healthy controls, respectively.

In conclusion, our results reject the hypothesis that the major functionally relevant *CARD15* gene mutations are involved in G-AgP susceptibility. Carrier and allele frequencies among G-AgP patients and controls were not different for any of the three studied SNPs. The implication of the NF-κB pathway in periodontitis suggests that other genes involved in this pathway may be candidates for periodontitis susceptibility. Assuming that periodontitis is a multifactorial disease, it would be of interest to study the impact of a complex genetic background of bacterial recognition and host inflammation in periodontitis.

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Clinical Relevance

Scientific rationale for the study: CARD15 is a protein that acts as an intracellular receptor of bacterial products. It plays an important role in pro-inflammatory responses by inducing regulation of transcription of cytokine genes and apoptosis. *CARD15* gene variants are known to increase the susceptibility to inflammatory diseases, especially to Crohn's disease.

Principal findings: In this study, no association was found between aggressive periodontitis susceptibility and *CARD15* gene variants.

Practical implications: Currently, there is no evidence for *CARD15* gene variants to act as genetic risk markers for aggressive periodontitis, resulting in no practical implications for disease prevention and treatment.