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CARD15 gene mutations in periodontitis

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Abstract

Objectives: The *CARD15* gene encodes the Nod2 protein, which is involved in intracellular recognition of bacterial products like peptidoglycan, activates inflammation and regulates apoptosis through nuclear factor-kappa B, a transcription factor that plays a central role in the innate immunity. Two functional mutations, an insertion mutation at nucleotide 3020 (3020insC) and a missense mutation C2104T in the *CARD15* gene (originally *NOD2* gene) have been reported to be associated with Crohn's disease. Our aim was to investigate the occurrence of *CARD15* gene polymorphisms in adult patients with periodontitis taking into account smoking and presence of putative periodontal pathogens as additional variables.

Material and methods: A case–control study was performed in 104 Dutch Caucasian patients with severe adult periodontitis (54 non-smokers and 50 smokers, mean age 46 years) and in 97 ethnically matched, periodontal healthy controls (73 non-smokers and 24 smokers, mean age 40 years). DNA isolated from a mouthwash was typed with PCR technology. Presence of putative periodontal pathogens was established by culture technique.

Results: Frequencies of the *CARD15* 3020insC and 2104T mutations were similar in the periodontitis group and in the control group (5.1% and 13.3%; 5.2% and 10.3%, respectively). The highest carrier frequency of *CARD15* mutations was found in non-smoking patients without *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* (29.4% versus 17.4% in controls); however it did not reach statistical significance.

Conclusion: Our results suggest no role for *CARD15* 3020insC and C2104T mutations in adult periodontitis.

Keywords: *Actinobacillus actinomycetemcomitans*; adult periodontitis; *CARD15*; mutation; *Porphyromonas gingivalis*; smoking

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Adult periodontitis is a chronic infectious and inflammatory disorder that affects the periodontal tissues and alveolar bone, which may result in tooth loss. Twin studies suggest that up to 82% of the susceptibility to periodontitis in adults can be attributed to genetic factors (Michalowicz et al. 1991). Genetic factors that determine and modify host responses to the microbial challenge are major determinants of susceptibility to periodontitis and influence the rate and extent of disease progression and severity (Page et al. 1997). Previous studies on genetic factors have shown that *IL-1* gene polymorphisms may determine the severity of periodontitis in adults (Kornman et al. 1997, Gore et al. 1998, Laine et al. 2001).

Bacteria are essential but insufficient to cause periodontal disease. *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* have been implicated as major pathogens in periodontitis (Haffajee & Socransky 1994). A number of additional bacteria have been implicated in the progression of periodontitis and, include among others, *Bacteroides forsythus, Prevotella intermedia, Peptostreptococcus micros, Fusobacterium nucleatum* and *Campylobacter rectus* (Haffajee & Socransky 1994).

Cigarette smoking (Bergström 1989) and diabetes mellitus are two other well-documented risk factors for periodontitis (Thorstensson & Hugoson 1993, Collin et al. 1998).

Nod2 belongs to the recently described family of nod proteins that have been implicated in intracellular recognition of bacterial components (Inohara et al. 2001). At first it was thought that Nod2 recognizes bacterial lipopolysaccharide but recent studies suggest specific recognition of peptidoglycan (Ogura et al. 2001b, Girardin et al. 2003, Inohara et al. 2003). Biological and functional analyses identified muramyl dipeptide derived from peptido-

glycan as the essential structure in bacteria recognized by Nod2 (Girardin et al. 2003, Inohara et al. 2003). Nod2 also regulates apoptosis and induces activation of nuclear factor-kappa B (NF-κB), a protein that plays an important role in transcription of cytokine genes (Ogura et al. 2001b, Sun & Andersson 2002).

The Nod2 protein encoded by the *CARD15* gene (originally *NOD2* gene) is located on chromosome 16, and is predominantly expressed in peripheral monocytes (Ogura et al. 2001a, Gutierrez et al. 2002). The gene is characterized by two amino-terminal caspase recruitment domains (CARD), a nucleotide (ATP or GTP) binding domain and a distal leucine-rich repeat (LRR) region.

A functional frameshift mutation caused by a cytosine insertion at nucleotide 3020 (3020insC) in the *CARD15* gene has recently been described as a susceptibility factor for Crohn's disease (Hampe et al. 2001, Hugot et al. 2001, Ogura et al. 2001a, Murillo et al. 2002, Heliö et al. 2003). This mutation in the LRR results in a 33 amino acid shorter version of a 1040-amino-acid protein. Another mutation is the C2104T missense mutation, which is also located in the LRR domain of *CARD15* and is associated with Crohn's disease (Hugot et al. 2001).

Our aim was to investigate the occurrence of the two most common mutations of the *CARD15* gene in adult population with and without periodontitis taking into account smoking and presence of periodontal pathogens as additional variables.

Material and methods

Patients

After informed consent, 104 patients with periodontitis and 97 periodontally healthy controls were selected consecutively for the present study, according to previously described clinical criteria (Laine et al. 2001). Shortly, the periodontal status was based on radiographic and clinical examination. Subjects were evaluated clinically for probing pocket depths (PPD) and clinical periodontal attachment level. Bitewing radiographs were evaluated for interproximal bone loss measurements from the cemento–enamel junction of the tooth to the bone crest. We selected patients with severe periodontitis, i.e., $\geqslant 7$ interproximal sites with $\geqslant 50\%$ bone loss. None of the controls had PPD > 4 mm, and there were no sites with radiographic evidence of alveolar bone loss. The study protocol was approved by the ethical committee of the Vrije Universiteit Medical Center Amsterdam. Subjects, who were self-reported current smokers or had stopped smoking $\leqslant 1$ year ago, were considered smokers and subjects, who had never smoked or had stopped smoking > 1 year ago were considered non-smokers. All subjects were unrelated, over 25 years of age, and had both parents and grandparents of Dutch Caucasian heritage.

Microbiology

Subgingival plaque samples were obtained from all individuals. Paper-point samples were taken from the deepest subgingival site in each quadrant of the dentition (Mombelli et al. 1991, 1994) of the patients and controls. The samples were analyzed for the presence of *A. actinomycetemcomitans,* using TSBV agar plates (Slots 1982), and for *P. gingivalis* using the anaerobic non-selective blood agar plates as previously described (van Winkelhoff et al. 1986).

Analysis of the gene polymorphisms

The *CARD15* 3020insC was genotyped by allele specific multiplex PCR (Hampe et al. 2001), and the *CARD15* C2104T polymorphism (NCBI SNP Id rs2066844) by PCR–RFLP with genomic DNA isolated with mouthwash method (Laine et al. 2000).

Statistical methods

The *CARD15* 3020insC and C2104T allele and carrier frequencies, their combinations and the carriers of at least one *CARD15* mutation were compared between the control and periodontitis group by Fisher's two-tailed exact test, and odds ratios (ORs) with 95% confidence intervals (CIs) were determined. Subsequently, differences in distribution of genotypes in *P. gingivalis* and *A. actinomycetemcomitans* negative patients and controls (as binary variables) were analyzed with logistic regression models. Age, gender and smoking were entered in the models, the significant determinants were determined and adjusted and ORs and CIs were calculated. Differences in genotype frequencies between patient and control subgroups were explored. A p-value < 0.05 was considered statistically significant. Statistical analysis was performed using SPSS version 10.0 for windows (SPSS Inc., Chicago, IL, USA).

Results

Demographic data of the cohorts is summarized in Table 1. The carrier frequencies of the *CARD15* 3020insC and 2104T mutations in patients and controls are presented in Table 2. The genotype frequencies in patients and controls were in Hardy–Weinberg equilibrium. The prevalence of the insertion 3020insC mutation for the total periodontitis group and the total control group was 5.1% and 5.2%, respectively (Table 2). Similar prevalence of 3020insC mutation in patients and controls was also found after subdivision to non-smokers and smokers (in patients 7.8% and 2.1%; in controls 5.5% and 4.2%, respectively). Frequencies for *CARD15* 2104T allele were similar in patients and controls (13.1% versus 10.0%, respectively) (Table 2). In non-smokers no differences were found between the patients and controls (11.1% versus 11.0%, respectively). 2104T allele tend to be more frequent in smoking patients (15.6% versus 8.3% in controls) but did not reach statistical significance. None of the patients or controls were homozygous for the insertion or missense mutation and none were carrier of both mutations.

Patients and controls were further classified in carriers and non-carriers of at least one of the *CARD15* mutations (Table 3). No significant differences were found between patients and controls. Subgroups of the *CARD15* mutation carriers were further explored and interestingly, the highest prevalence of *CARD15* mutation carriers was found in

Table 1. Demographic characteristics of adult patients with severe periodontitis and periodontal healthy controls

	All patients	Controls
no. of subjects	104	97
mean age (years)	46.4	40.4
age range (years)	28–66	25–77
sex (F/M)	52/52	55/42
smokers/non-smokers	50/54	24/73
Pg- and Aa-	43 (41.3%)	86 (88.7%)

Pg-, *Porphyromonas gingivalis*, not detectable; Aa-, *Actinobacillus actinomycetemcomitans*, not detectable.

Table 2. Carrier frequencies of the *CARD15* 3020insC and 2104T mutations in the adult patients with severe periodontitis and controls

Carriers of *CARD15*	Patients	Controls
3020insC		
total	5/99 (5.1%)	5/97 (5.2%)
non-smokers	4/51 (7.8%)	4/73 (5.5%)
Pg- and Aa-	3/41 (7.3%)	5/86 (5.8%)
non-smokers Pg- and Aa-	3/21 (14.3%)	4/69 (5.8%)
2104T		
total	12/90 (13.3%)	10/97 (10.3%)
non-smokers	5/45 (11.1%)	8/73 (11.0%)
Pg- and Aa-	6/36 (16.7%)	9/86 (10.5%)
non-smokers Pg- and Aa-	2/17 (11.8%)	8/69 (11.6%)

No homozygous individuals were found for the 3020insC and 2104T mutations.
Pg-, *Porphyromonas gingivalis*, not detectable; Aa-, *Actinobacillus actinomycetemcomitans*, not detectable.

Table 3. The *CARD15* mutations carrier status in patients with severe periodontitis and controls

Variable	Carriers of *CARD15* mutations	
	Patients	Controls
total	17 (19.3%)	15 (15.5%)
non-smokers	9 (20.5%)	12 (16.4%)
smokers	8 (18.2%)	3 (12.5%)
Pg- and Aa-	9 (25.7%)	14 (16.3%)
non-smokers Pg- and Aa-	5 (29.4%)	12 (17.4%)

Pg-, *Porphyromonas gingivalis*, not detectable; Aa-, *Actinobacillus actinomycetemcomitans*, not detectable.

a subgroup of non-smoking patients without *P. gingivalis* and *A. actinomycetemcomitans,* however, no statistical significant differences were found (29.4% versus 17.4% in controls).

The logistic regression analysis adjusted for smoking, gender and age showed no significant association for the *CARD15* 3020insC and 2104T mutations or carriers of one of the *CARD15* mutations in patients and controls without *P. gingivalis* and *A. actinomycetemcomitans*.

Discussion

Previously, we have reported on an association between severe adult periodontitis and carriage of the less common allele in *IL-1A-889, IL-1B+593* and *IL-1RN* VNTR genes (Laine et al. 2001). The highest prevalence was found in a group of patients without two major risk factors for adult periodontitis i.e. smoking, and presence of *P. gingivalis* and *A. actinomycetemcomitans.* These individuals seem to be, because of host's genetic background, susceptible to otherwise weakly pathogenic bacteria.

Using a similar approach, the present study shows the highest frequency of the *CARD15* 3020insC and 2104T mutation carriers in non-smoking patients without cultivable *P. gingivalis* and *A. actinomycetemcomitans.* No significant association was found but a trend in a subgroup with the risk exposure conditions. *CARD15* mutation carriage is therefore an uncommon risk condition.

The age limit of 25 years was also a selection criterion for the patients, as for the periodontal healthy controls in the present study. There is a risk for including young subjects in the control group who may be susceptible to the disease but not yet developed detectable symptoms.

Prevalence of the *CARD15* 3020insC and 2104T mutants in the periodontal healthy individuals of the present study (5.2% and 10.3%, respectively) is similar to previous reports on these mutations in healthy European populations (2.0% and 4.0%, respectively) (Hugot et al. 2001, Murillo et al. 2002).

Previous studies have reported on diminished ability of the *CARD15* 3020insC mutant cells to detect bacterial peptidoglycan when compared with the wild-type cells (Girardin et al. 2003, Inohara et al. 2003). The NF-κB activation is completely abolished in the cells with the *CARD15* 3020insC mutation whereas the cells with the *CARD15* C2104T mutation showed reduced activity when compared with wild-type *CARD15* (Inohara et al. 2003). A deficiency in sensing bacteria by monocytes/macrophages might result in an unbalanced inflammatory response.

Our results suggest no role for *CARD15* 3020insC and C2104T mutations in adult periodontitis. However, a larger study is needed to confirm if the trend in *P. gingivalis* and *A. actinomycetemcomitans* negative subjects is significant. In future studies, it would be of interest to assess whether the *CARD15* mutations are associated with susceptibility to adult periodontitis in combination with polymorphisms in other genes such as *IL-1* cluster, *TLR4* and *TLR2*.

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