

Polymorphisms of pro-inflammatory cytokine genes and the risk for acute suppurative or chronic nonsuppurative apical periodontitis in a Colombian population

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Abstract

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Aim To determine the association of functional single nucleotide polymorphisms in genes of the pro-inflammatory cytokines tumour necrosis factor- α , interleukin-1 β , interleukin-8 and interleukin-12B with the development of two clinical forms of apical periodontitis (AP): acute suppurative and chronic nonsuppurative.

Methodology The study included 120 patients from Bucaramanga City, Colombia, 63 diagnosed with acute suppurative AP (ASAP) and 57 diagnosed with chronic nonsuppurative AP (CNAP). Genotyping for *IL1B* +3954 (rs1143634), *IL8/CXCL8* –251 (rs4073), *IL12B* +1188 (rs3212227) and *TNFA* –308 (rs1800629) was performed by the PCR–restriction fragment length polymorphisms method. The statistical analysis was performed using STATA 10.0 and PLINK V1.07 software.

Results Significant differences in the distribution of *IL8/CXCL8* –251 A allele (P adjusted = 0.041; OR adjusted = 0.41, CI adjusted = 0.31–0.97) and *IL8/CXCL8* –251 TT genotype (P adjusted = 0.04; OR adjusted = 2.24, CI adjusted = 1.04–4.84) were observed comparing patients diagnosed with ASAP and CNAP. No association was observed in genotype and allele distribution for other genetic polymorphisms analysed.

Conclusion This study provides molecular epidemiological evidence that suggests in the present cohort that *IL8/CXCL8* –251 T allele, which is associated with higher production of IL8/CXCL8, is also associated with a higher risk of developing acute suppurative form of AP, whereas *IL8/CXCL8* –251 A allele, which is associated with lower production of IL8/CXCL8, is associated with chronic nonsuppurative form of AP. This suggests a pivotal role for IL-8/CXCL8 in periapical disease because of its ability to induce chemotaxis and modulating the directed migration of neutrophils to the site of inflammation in response to microbial infection of pulp.

Keywords: apical periodontitis, Colombia, cytokines, inflammation, single nucleotide polymorphisms.

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Introduction

Apical periodontitis (AP) of endodontic origin is a group of inflammatory disorders of the periradicular tissues caused mainly by a mixed microbial infection in the root canal system of the affected tooth

(Ebersole and Taubman 1994, Nair 1997, Abbott 2004). Clinically, AP includes an acute phase and a chronic phase, which, in most cases, are suppurative and nonsuppurative inflammatory lesions, respectively (Nair 1997). In addition, these clinical forms not only are progressive in nature, which means that an initial acute inflammation gradually shifts to a chronic inflammatory reaction, but they are also dynamic in nature, which means that a secondary acute AP is an acute exacerbation of an existing chronic AP lesion (Nair 1997).

Even though microorganisms play an important role in the aetiology of AP, the presence of inflammatory cells such as T and B lymphocytes, macrophages and neutrophils in human periapical lesions suggests that immune response is also involved in the pathogenesis of the disease (Metzger 2000, Nair 2004). It is well known, for instance, that the immune-inflammatory process in AP is double-faced as a result of the rate of recruitment, activation and death of neutrophils in the site of infection. Thus, on the one hand, it tries to remove the microorganisms from the root canal to avoid microbial invasion into the periapical tissues, and, on the other hand, it promotes the destruction of soft and mineralized periodontal tissues (Bendre *et al.* 2003, Pixley and Stanley 2004). Therefore, and as a consequence of this immune-inflammatory process in response to microbial infection of the pulp, the clinical presentation of AP, acute or chronic, may be explained, at least in part, by differences in the immune response and its effect on influx of immune cells to the site of infection (Nair 1997).

Cytokines play an important role in the influx of leucocytes displaying pro-inflammatory and/or anti-inflammatory properties. The type of immune response, pro- or anti-inflammatory, elicited by cytokines is determined, amongst other factors, by type of cells present in the tissue (Ataoglu *et al.* 2002, Nair 2004). Numerous cell types, including neutrophils, T and B lymphocytes, macrophages and plasma cells, have been identified in periapical lesions. However, because macrophages and Th1 cells have been considered principal immunological constituents of periapical lesions (Metzger 2000, Lukic *et al.* 2006), cytokines such as tumour necrosis factor- α (TNF- α), interferon- γ (INF- γ), interleukin-1 α (IL-1 α), interleukin-1 β (IL-1 β), interleukin-6 (IL-6), interleukin-8 (IL-8/CXCL8) and interleukin-12p40 (IL-12 β) can have a potential role in the development of AP (Artese *et al.* 1991, Ataoglu *et al.* 2002, Nair 2004, Reichert *et al.* 2008). For example, the IL-8/CXCL8, the most potent

known chemokine, mediates the activation and migration of neutrophils into the periradicular tissue from peripheral blood; moreover, in contrast to most inflammatory cytokines, IL-8/CXCL8 is virtually unique because it may be produced early in the inflammatory response but will persist for a prolonged period of time, for days or even weeks (Remick 2005). Therefore, this cytokine could play an important role in the development of AP.

In spite of the fact that a large number of immune cells and cytokines involved in AP lesions have been described, the molecular mechanisms underlying the pathogenesis because of these constituents are not fully understood. Over the past several years, genetic variation has been studied extensively in humans because it may affect the development and clinical presentation of several diseases (Pacheco and Moraes 2009). The single nucleotide polymorphisms (SNPs) are the most common type of genetic variation in the human DNA sequence. A SNP occurs when a single nucleotide, such as an adenine (A), replaces one of the other three nucleotides, which are cytosine (C), guanine (G) or thymine (T). Besides, because of the fact that the human genome is diploid, two possible alleles (e.g. A and T) made up the genotype (e.g. AA, AT or TT) of an individual at one or more loci for any given SNP. The position of SNPs within the reference genome is known, nevertheless, the relative location of SNPs on the gene could be defined respectively by negative or positive numbers counting back or forward from the transcription start site. Finally, even though the vast majority of SNPs are neutral variants, only a small fraction of them influence the gene expression or the overall activity of the protein; these effects are mainly attributed to SNPs that occur upstream (promoter region) or downstream (coding region) within genes, respectively. In this way, genetic association studies are performed to determine and compare the allele and genotype frequency of SNPs in populations of individuals with and without a particular characteristic to assess the risk of disease (Manolio 2010).

Many SNPs at cytokine genes have been characterized and associated with the development of several inflammatory diseases mainly by modification of the balance between pro- and anti-inflammatory responses. Previous reports have demonstrated that the SNPs *TNFA* -308 G/A and *IL8/CXCL8* -251 T/A in the promoter region, as well as the *IL1B* +3954 C/T in the coding region and *IL12B* +1188 in the 3'-untranslated region, have been correlated with the

level of cytokine production (Pociot *et al.* 1992, Wilson *et al.* 1997, Hull *et al.* 2000, Seegers *et al.* 2002). In addition, these SNPs have been reported to be associated with inflammatory processes of the oral cavity where the immune system plays a pivotal role (Yoshie *et al.* 2007, Morsani *et al.* 2011).

Considering that the evidence strongly suggests an association between SNPs in cytokine genes and the development of several inflammatory diseases, as well as by the fact that the infection in the root canal system and the immune response against this infection cannot explain by themselves why people develop different clinical forms of the same disease, the aim of this study was to investigate whether genetics polymorphisms in the inflammatory response genes *IL1B*, *IL8/CXCL8*, *IL12B* and *TNFA* would be associated with acute suppurative and chronic nonsuppurative AP. The null hypothesis is that there will be no association between any of the SNPs.

Materials and methods

Population

The study was designed as an analytical observational case-control study, which included 120 patients from Bucaramanga City, Colombia, who were diagnosed according to radiographic findings and clinical manifestations. The diagnosis of AP was made in one tooth per patient. The radiological examination of all patients revealed periapical radiolucency compatible with AP of pulp origin. The classification of the patients was carried out on their signs and symptoms (Gutmann *et al.* 2009). On the one hand, the group of patients with acute suppurative AP (ASAP), the case group, consisted of individuals with moderate-to-severe signs and symptoms, which included intraoral oedema or exuding from the root canal, or both; swelling; heat; redness; occasionally fever and/or lymphadenopathy; marked tenderness to percussion and pain when pressure is applied to the tooth which, besides, exhibits varying degrees of mobility; no pulp reaction to cold, heat or electrical stimulation; and radiographically from a slight thickening of the periodontal ligament space to a radiolucent lesion. On the other hand, the group of patients with chronic nonsuppurative AP (CNAP), the control group, consisted of individuals with no or only mild signs and symptoms and without previous exacerbation, which included radiographically a thickening of the periodontal ligament space compatible with periapical

Table 1 Demographic characteristics of study population

Parameter	ASAP	CNAP
Number of subjects	63	57
Mean age (years $\pm$ SD)	32.8 $\pm$ 10.4	35.4 $\pm$ 11.6
Age range	From 18 to 55	From 19 to 60
Sex		
Female	37	40
Male	26	17

ASAP, acute suppurative apical periodontitis; CNAP, chronic nonsuppurative AP; SD, standard deviation.

lesion; without sensitivity or with a slight sensitivity to percussion when pressure is applied to the tooth; and the tooth did not respond to thermal or electrical stimulus because of necrotic pulp. Patients with chronic AP with fistulas were excluded, as well as people with a weakened immune system induced by HIV infection or metabolic diseases, blood dyscrasias, chemotherapy or radiation treatment, and transplant. The gender distribution was similar in both groups (Table 1). The population from this region does not have concentration of ethnical groups, and it is not structured (Hincapié *et al.* 2009), meaning that it has one subpopulation that is the population itself. This study complies with the current laws of Colombia and fulfilled all criteria required by the Medical Code of Ethics and the Helsinki statement. The Ethical Committee of the Universidad Santo Tomas approved this study, and a written informed consent was obtained from all patients.

Genotyping

Genomic DNA was isolated from 7 mL of EDTA non-coagulated blood samples using the standard salting-out technique (Miller *et al.* 1988). Genotyping for *IL1B* +3954 (rs1143634), *IL8/CXCL8* -251 (rs4073), *IL12B* +1188 (rs3212227) and *TNFA* -308 (rs1800629) was performed by the PCR-restriction fragment length polymorphisms method as previously described (Sakellari *et al.* 2003, Sanchez *et al.* 2005, Hong *et al.* 2007, Liu *et al.* 2011). The PCR was carried out in a total volume of 20 μ L using GoTaq DNA polymerase (Promega Corporation, Madison, WI, USA). The PCR products were digested using appropriate restriction enzymes according to the manufacturer's instructions (Fermentas International Inc, Glen Burnie, MD, USA). The visualization of the products was performed in an agarose gel electrophoresis, and each allele was recognized according to its size. Individuals with known genotypes and exclusion of

the restriction enzyme were used as controls. The SNPs in candidate genes were selected principally according to functional considerations (gene expression levels or amino acid change) in models of infection diseases, as well as on the basis of minor allele frequency >10% published in HapMap2 Release 24 using the European-American (CEU) population as reference population. In addition, these SNPs were previously genotyped in a sample of 100 healthy people from Santander, Colombia, showing the following frequencies: *IL1B* +3954 T at 14.7%, *IL8/CXCL8* -251 A at 35.4%, *IL12B* C +1188 at 29.1% and *TNFA* -308 A at 7.8% (no published data).

Statistical analysis

Allele and genotype frequencies were obtained by direct counting. Differences between allele and genotype frequencies were determined by chi-square (χ^2) test of independence and Fisher's exact test when was appropriate. A *P*-value < 0.05 was considered statistically significant according to the fifth-class association in an independent population sample using the Better Associations for Disease and GENes (BADGE) system (Manly 2005). Odds ratios (OR) and 95% confidence intervals (CI) were calculated according to the Woolf's method (Woolf 1955). The software used was STATA 10.0 (College Station, TX, USA). Hardy-Weinberg equilibrium (HWE) was tested for each SNP with Arlequin 3.11 software (Excoffier et al. 2005). A *P*-value < 0.01 was considered such evidence of deviation from HWE. Correction for multiple testing was performed using the false discovery rate method (FDR) and by permuting the association results 10 000 times to define the smallest empirical significance level. The correlation of each genetic polymorphism with periodontitis status was assessed using a logistic regression model with cases or control as the dependent variables, and age and gender as covariates in the model, these analyses were carried out using PLINK V1.07 software (Purcell et al. 2007).

Results

The *IL1B*, *IL8/CXCL8*, *IL12B* and *TNFA* genotypic and allelic frequencies analysed in patients with ASAP (the group of cases) and CNAP (the group of controls) are listed in Table 2. The genotype frequencies for every group, cases and controls, were distributed in accordance with HWE when the expected and observed genotype frequencies were compared.

Table 2 Genotype frequencies of *IL1B*, *IL8*, *IL12B* and *TNFA* polymorphisms in patients with acute suppurative and chronic nonsuppurative AP

	Genotype frequencies				
SNPs	ASAP N = 63 (%)	CNAP N = 57 (%)	P	OR (95% CI)	
<i>IL1B</i>					
+3954					
CC	48 (76.2)	48 (84.2)	NS		
CT	14 (22.2)	9 (15.8)	NS		
TT	1 (1.6)	0	NS		
C	110 (87.3)	105 (92.1)	NS		
T	16 (12.7)	9 (7.9)	NS		
<i>IL8/CXCL8</i>					
-251					
TT	28 (44.4)	15 (26.3)	0.039 ^a	2.22 (1.04–4.84)	
TA	29 (46)	32 (56.1)	NS		
AA	6 (9.5)	10 (17.5)	NS		
T	85 (67.5)	62 (54.4)	NS		
A	41 (32.5)	52 (45.6)	0.038 ^b	0.57 (0.34–0.97)	
<i>IL12B</i>					
+1188					
AA	20 (31.7)	23 (40.4)	NS		
AC	31 (49.2)	26 (45.6)	NS		
CC	12 (19)	8 (14)	NS		
A	71 (56.3)	72 (63.2)	NS		
C	55 (43.7)	42 (36.8)	NS		
<i>TNFA</i>					
-308					
GG	50 (79.4)	52 (91.2)	NS		
GA	12 (19)	4 (7)	NS		
AA	1 (1.6)	1 (1.8)	NS		
G	112 (88.9)	108 (94.7)	NS		
A	14 (11.1)	6 (5.3)	NS		

AP, apical periodontitis; ASAP, acute suppurative AP; CNAP, chronic nonsuppurative AP; NS, no significance; OR, odds ratio; SNPs, single nucleotide polymorphisms; 95% CI, 95% confidence interval.

The result of *IL8/CXCL8* -251 means what the risk of developing acute suppurative form of AP is higher if people are homozygous for the T allele (TT), whereas people homozygous for the A allele (AA) or heterozygous (AT) could develop chronic nonsuppurative form of AP.

^a*P* adjusted = 0.040; OR = 2.24, 95% CI = 1.04–4.84.

^b*P* adjusted = 0.041; OR = 0.55, 95% CI = 0.31–0.97.

Statistically significant differences in the distribution of *IL8/CXCL8* -251 A allele (*P* = 0.038, OR = 0.57, CI = 0.34–0.97 and *P* adjusted = 0.041; OR adjusted = 0.41, CI adjusted = 0.31–0.97) and *IL8/CXCL8* -251 TT genotype (*P* = 0.039, OR = 2.22, CI = 1.04–4.84 and *P* adjusted = 0.04; OR adjusted = 2.24, CI adjusted = 1.04–4.84) were observed comparing cases with controls. These associations were respectively fitted into a model of dominant and recessive inheritance by logistic regression and adjusted for age and gender (Table 2). They

remained significant after 10 000 permutation tests (empirical P -value = 0.045), but they were not significant by correction for multiple testing (FDR > 0.1). No significant differences were observed between genotype and allele frequencies of the *IL1B* +3954, *IL12B* +1188 and *TNFA* -308 SNPs with cases and controls (Table 2).

To find the possible influence of genetic polymorphisms together, an analysis of the occurrence of alleles was carried out in both groups of patients; only the group of alleles *IL1B* +3954 C, *IL12B* +1188 A, *TNFA* -308 G and *IL-8/CXCL8* -251 A showed a significant difference (P = 0.029, OR = 0.48, CI = 0.23–0.98), which occurred at a frequency of 17.6% in cases and 28.6% in controls.

Discussion

Inflammatory mediators play an important role in mediating the extravasation and accumulation of immune cells in acute and chronic inflammatory processes of the oral cavity and other inflammatory diseases. In patients with AP, it has been shown that dental-pulp cells and periodontal tissues are able to respond to cytokines and microorganisms through chemokine and chemokine receptor synthesis, which regulate the kinetics and leucocyte influx involved in the pathogenesis of the AP (Silva et al. 2007).

The results provide molecular epidemiological evidence that *IL8/CXCL8* -251 SNP is associated with two clinical features of AP; on the one hand, the A allele is associated with the development of CNAP and, on the other hand, the TT genotype, and hence, the T allele is associated with the development of ASAP (Table 2). The *IL8/CXCL8* -251 SNP has been associated with variations in gene expression; therefore, a higher up-regulation of this chemokine in patients with ASAP than in patient with CNAP would display increased chemotaxis and induce activation of monocytes/macrophages and neutrophils in infected tissue to avoid the infection and, as a consequence, favoured the inflammatory aggression against to the dental pulp (Silva et al. 2007). In the same way, both pulps and periapical tissues exhibit variable concentration of IL-8/CXCL8 according to clinical status. Elevated levels of IL-8/CXCL8 have been detected in gingival fibroblasts isolated from patients with periodontitis and in pulps diagnosed with irreversible pulpitis, whereas only negligible amounts were present in healthy controls and normal pulps, respectively (Dongari-Bagtzoglou & Ebersole 1998, Huang et al.

1999); moreover, in approximately 90% of periapical exudates collected from root canals during routine endodontic treatment show detectable levels of IL-8/CXCL8, which were significantly higher in periapical exudates from lesions that had painful symptoms (Shimauchi et al. 2001). These results suggest that the modulation of *IL8/CXCL8* gene expression, at least in part, may have a critical role in the development of the acute and chronic forms of AP.

However, even though *IL8/CXCL8* -251 A/T polymorphism has been associated with variations of expression of *IL8/CXCL8* gene, the functional effect of this polymorphism has been controversial. Contradictory experimental evidences using reporter genes in different cell models have showed that the *IL8/CXCL8* -251 A or T alleles could increase the promoter activity of *IL-8/CXCL8* *in vitro*. These experiments were carried out using constructs with the *IL8/CXCL8* -251 T/A polymorphism into different promoter lengths. Thus, a region from nucleotide -317 to +7 with the A allele shows a higher transcriptional activity than with the T allele (Ohyauchi et al. 2005), whereas a region from nucleotide -1479 to +45 with the T allele shows a higher transcriptional activity than with the A allele (Lee et al. 2005). In contrast, no significant differences were showed in the expression pattern of *IL8/CXCL8* -251A and -251T plasmids transfected in human cells following TNF- α stimulation (Hacking et al. 2004). These experimental findings suggest that the *IL8/CXCL8* -251 T/A polymorphism is necessary, but not sufficient to modulate the *IL-8/CXCL8* gene expression and, therefore, other SNPs could be involved in the transcriptional and translational activities. In fact, it has been reported that the expression of *IL-8/CXCL8* influenced by *IL8/CXCL8* -251 T/A polymorphism may also be regulated by *IL8/CXCL8* +781 C/T polymorphism, which are in linkage disequilibrium within the same haplotype and are being part of a transcription factor binding complex (Hacking et al. 2004, Kim et al. 2010). Moreover, higher levels of the *IL-8/CXCL8* mRNA were found in patients with periodontitis, mainly in individuals who presented the TA genotype (Andia et al. 2011). Therefore, more experimental evidence will be necessary to understand the functional role of the *IL8/CXCL8* -251 T/A polymorphism in a haplotype context with AP; thus, in addition to the blood sample used for genotyping, a sample of pulp tissue will be required to demonstrate the effect of these polymorphisms in the gene expression and, consequently, on the clinical presentation of AP.

Finally, previous studies have established the association of SNPs *IL1B*, *IL12B* and *TNFA* genetic polymorphisms with periodontal diseases (de Sa et al. 2007, Nikolopoulos et al. 2008, Reichert et al. 2008, Abu-Saleh 2010, Trevilatto et al. 2011). In addition, the effect of the allelic variants of *IL1B* +3954 T, *IL12B* +1188 C and *TNFA* –308 A has been correlated with increased level of each cytokine production (Pociot et al. 1992, Wilson et al. 1997, Seegers et al. 2002). However, on the one hand, although these alleles showed a higher frequency in cases than in controls, no significant differences were identified between these groups (Table 2), whereas, on the other hand, the association analysis by combining the four polymorphisms suggests that *IL1B* +3954 C, *IL12B* +1188 A, *TNFA* –308 G and *IL-8/CXCL8* –251 A alleles may act together as a protective factor in the development of ASAP, but not of CNAP. It is possible that sample size and differences in allele frequency amongst the studied populations may explain some failures to find association with individual alleles.

Nevertheless, these results should be validated in other populations or in this population with a larger sample size. This is because the statistically significant results could occur by chance due to the fact that the associations were not significant after adjustment for multiple testing despite remaining significant after 10 000 permutation test. In addition, it is well known that SNPs and haplotype structures can vary amongst different ethnic groups. Therefore, other markers in the same candidate gene in linkage disequilibrium with *IL8/CXCL8* –251 SNP should be analysed to confirm the results, as well as the combination of these polymorphisms with others in different genes, which may also be important to define the role of these in the pathogenesis of AP.

Conclusion

The molecular epidemiological evidence from this study suggests that *IL8/CXCL8* –251 T and A alleles, respectively, may contribute to develop acute suppurative and chronic nonsuppurative AP. This may be due to differences in regulation of *IL8/CXCL8* expression at transcriptional level. Thus, individuals that carry the *IL8/CXCL8* –251 T allele would produce higher amounts of *IL8/CXCL8* than those who carry the *IL8/CXCL8* –251 A allele and, as a result, the chemotaxis of neutrophils to the infected dental pulp will be increased, which consequently favours an ASAP rather than the chronic type of AP.

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