INTERLEUKIN-6 GENE POLYMORPHISM MODULATES THE RISK OF PERIODONTAL DISEASES

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Gingivitis and periodontitis are the two main periodontal diseases. Both are characterized by inflammation of the tissues surrounding the teeth but while tissue damages observed in gingivitis are mild and reversible, destruction caused by periodontitis is deeper and irreversible. Periodontal diseases and levels of degeneration of tissues surrounding teeth depend on several interacting endogenous and exogenous factors. Polymorphisms of genes encoding molecules that modulate the immune response and tissue homeostasis are the main causes of individual susceptibility to periodontal diseases. The aim of this study was to investigate IL6, IL10 and VDR gene polymorphisms in a large number of subjects affected by either gingivitis or chronic periodontitis. The sample included 750 Italian patients. We found that the rs1800795 SNP located in the IL6 gene promoter was strongly associated with the occurrence of both gingivitis and periodontitis. Indeed, homozygous individuals with variant allele appeared less-susceptible to both gingivitis OR=0.47 (95% C.I. 0.27-0.82) and periodontitis OR=0.36 (95% C.I. 0.21-0.64). No evidence of association between periodontal diseases and IL10 or VDR polymorphisms was obtained. This data confirmed the role of IL6 in susceptibility to periodontitis among the Italian population. The evidence that IL6 polymorphisms are also involved in gingivitis has implications in periodontal disease pathogenesis and reduces the appeal of IL6 as a periodontitis biomarker.

A normal periodontium provides the support for teeth attachment and function. It consists of several highly specialized tissues and anatomical components, including gingival with its junctional epithelium, periodontal ligament, cementum and alveolar bone.

Periodontal disorders are extremely frequent in all populations although prevalence increase with poor oral hygiene and malnutrition (1). Gingivitis, the mildest form of periodontal disease, is a rapidly inducible and reversible inflammatory affection of the gingiva, mainly caused by accumulation of bacterial biofilm. The combination of bacterial infection and persistent inflammatory response could be responsible for the progressive destruction of periodontal tissues characterizing chronic periodontitis (1). Gingivitis lesions do not necessarily progress to periodontitis. The proportion of gingival lesions converting to periodontitis and the factor promoting this conversion has not been well understood. However, longitudinal studies in humans indicate that gingivitis represents not only

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the precursor of periodontitis but also a clinically relevant risk factor for disease progression and tooth loss (2).

Several environmental and genetic susceptibility factors concur to the aetiology of periodontitis. Both the total amount of bacteria in the periodontal pockets as well as its specific composition can well differentiate healthy and periodontitis patients (3).

Specifically, bacterial species such as *Porphyromonas gingivalis, Treponema denticola*, and *Tannerella forsythia* were consistently found associated with periodontitis (4, 5). Additional recognized risk factors of periodontitis are tobacco smoking, alcohol consumption, race-ethnicity, socio-economic status and systemic conditions such as diabetes, osteoporosis, malnutrition and stress (1, 6). Familial aggregation studies suggested that genetic factors may be relevant in susceptibility to early onset of periodontitis (7), while relevance of genetic contribution to chronic periodontitis is still debated (8, 9). Most of the associated studies that aimed to identify gene polymorphisms involved in periodontitis, focused on candidate genes selected for their role in immune response modulation and tissues degeneration (10). Recently, genome-wide association analyses were carried out to identify novel genetic factors involved in periodontal disease (11, 12).

However, both approaches were not completely successful since confirmation studies often failed to replicate earlier results. Possible explanations could account for the conflicting results including genetic heterogeneity among population. Indeed, the contribution of a gene polymorphism could greatly differ in different populations because of a different genetic background, socio-economical status or because allele frequency variation based on population. On the other hand, the fairly small sample size of most of the published investigation, generating both false-positive and false-negative results, increased confusion and limited success rate in identifying genetic factors for periodontitis (10).

This study involved 750 Italian patients and was aimed to verify whether Interleukin-6 (IL6), Interleukin-10 (IL10) and Vitamin D Receptor (VDR) gene polymorphisms were susceptibility factors of periodontal disease. The study attempted to replicate previous data obtained with an independent sample of the same population, where single nucleotide polymorphisms (SNPs) of IL6 and IL10 were found to increase the risk of developing periodontitis in Italians (13). The present study also explored the role of polymorphisms in gingivitis.

Polymorphisms of IL6 and IL10 were found associated to periodontal disease in various populations.

MATERIALS AND METHODS

Sampling and genotyping

A total of 750 patients participated in the study. Patients were randomly selected over a period of 12 months according to the following criteria: a 20-year-old age limit; all individuals had to have good general health. Exclusion criteria included diabetes, hypertension, rheumatoid arthritis, depression-anxiety and obesity as well as heavy exposure to risk factors such as alcohol consumption and smoking. Patients had not had periodontal treatment in the previous 2 months.

Case definition was performed according to the Periodontal Screening and Recording (PSR) system. Gingival bleeding and pocket probing depth were monitored by a specific WHO probe and scored using the basic method indicated by the WHO oral health survey (14). Basically, the mouth was divided into sextants for the PSR examination. The probe examines each tooth circumferentially in the examined sextant. The clinician only observed the position of the color-coded reference marking in relation to the gingival margin and the presence of furcation invasion, mobility, mucogingival problems or recession. Only the highest code obtained was recorded for each sextant in the mouth.

The American Dental Association and the American Academy of Periodontology suggest that all routine dental examinations include a screening examination using PSR to identify patients who need a comprehensive periodontal assessment. The results of this screening examination are used to separate patients into two broad categories: those who have periodontal health or gingivitis, and those who have periodontitis. These two categories were the main subject of the investigation, the control group of patients with low PSR in all sextants (PSR code 0, 1 or 2), and a second group of patients with evidence of periodontitis in one or more sextants (code 3, 4 or *). In addition, healthy patients with no periodontal disease (code 0) were compared to patients with plaque-associated gingivitis (code 1 or 2).

The study was approved by the Ethics Committee and written informed consent was obtained from enrolled individuals.

After the removal of supragingival biofilm, a sample of the periodontal pocket microbiota was taken from a single site using sterile paper probes, among sites having the highest score, or randomly in the case of healthy patients. Specimens were processed to extract and purify DNA through a silica spin-column (Sigma-Aldrich, St. Louis, MO, USA).

The following polymorphisms were investigated: the XR_108749.1:n.50-321G >C (rs1800795) at IL6 locus, the NG_012088.1:g.3943A >G (rs1800896) and NG_012088.1:g.4433A >C (rs1800872) at IL10 locus, and the NM_000376.2:c.1056T >C (rs731236) at VDR locus. Single nucleotide polymorphisms (SNP) were selected to replicate results obtained in a previous investigation on the same population (PMID: 23814583). Genotyping were performed using ABI PRISM 7500 Sequence Detection System and TaqMan chemistry according to manufacturer's protocols (Life Technologies). Genotypes were scored by researchers in a blind system with no information about the clinical data associated with samples.

Statistical analysis

The distribution of genotypes in patient and control groups was tested for deviations from the Hardy-Weinberg equilibrium using Pearson's χ^2 test. Genetic association was investigated by both allelic and genotypic tests with a likelihood ratio approach using Unphased software v3.1.5 within a Windows Vista operative system. (15, 16). Odds Ratios (ORs) were calculated in order to evaluate the level of association of the rare allele carriers as well as heterozygote and homozygote individuals. Haplotype association was evaluated for IL10. A global test of association was performed which tested whether any haplotype was associated with the disease. A specific association test for each haplotype was also performed. Logistic regression analysis was performed to evaluate association levels corrected by patients' age and gender covariates.

RESULTS

Of the 750 patients, 215 were diagnosed as healthy, 250 affected by gingivitis, and 285 affected by periodontitis. Mean age was similar for control and gingivitis patients, 48 years (SD=15) and 47 (SD=15) respectively, while it was higher for periodontitis patients 56 (SD=13), P value>0.001.

A total of 2892 genotypes were obtained at four polymorphic loci. Genotyping success rate was 99% for rs1800795 and rs1800872 and 94% for rs1800896 and rs731236. Genotype frequency distribution of all polymorphisms in both groups was in agreement with the Hardy-Weinberg equilibrium law.

A comparison of the allelic frequencies observed in the control, gingivitis and chronic periodontitis patients are shown in Table I. A significant difference between control and periodontal disease groups was observed for the IL6 polymorphism rs1800795 while the variant allele C was less frequent in both gingivitis and periodontitis patients (P value 0.008 and 0.0009 respectively). Homozygous individuals with variant allele at IL6 polymorphisms appeared less-susceptible to both gingivitis OR=0.47 [95% confidence interval (C.I.) 0.27-0.82] and periodontitis OR=0.36 (95% C.I. 0.21-0.64). No evidence of association between periodontal diseases and IL10 or VDR polymorphisms was obtained.

Since both median age and gender ratio differed in periodontitis group, logistic regression analysis was used to calculate the level of association with IL6 polymorphisms considering these covariates. The corrected OR were 0.76 (95% C.I. 0.51-1.2) and 0.32 (95% C.I. 0.18-0.59) for heterozygous and homozygous patients, respectively.

Haplotypes analysis of IL10 polymorphisms did not show any evidence of association with gingivitis (global P value=0.26) nor periodontitis (global P value=0.41).

DISCUSSION

Previous investigations exploring genetic susceptibility factor of periodontitis provided conflicting data. This is not surprising since several interacting risk factors contribute to the aetiology of this complex disease (1, 6). A number of investigations supported association with gene polymorphisms of inflammation modulators, however there is still no agreement regarding the genetic basis of periodontal diseases. Conflicting reports of genetic association may arise because of the different attributable risk level of a specific factor in different ethnicities as well as to the small sample size that may increase both false positive and false negative results of associated studies. Another source of variability could be related to the study design due to different studies focused on specific phenotypes such as aggressive periodontitis, chronic periodontitis or response to treatment. In such a complex scenario, every finding should be replicated

Table I. *Association analysis between selected gene polymorphisms and ocurrence of gingivitis and periodontitis.*

Gene	SNP-ID	Group	Observed genotypes			MAF	Association test		
							Allelic P	OR heterozygous	OR homozygous
			GG	**GC**	**CC**				
IL6	**rs1800795**	healthy	77	92	42	0,42	ref.	ref.	ref.
		gingivitis	110	106	28	0,33	**0,008**	0.81(0.54-1.2)	**0.47(0.27-0.82)**
		periodontitis	131	127	26	0,32	**0,0009**	0.81(0.55-1.2)	**0.36(0.21-0.64)**
			AA	**AG**	**GG**				
IL10	**rs1800896**	healthy	76	87	38	0,41	ref.	ref.	ref.
		gingivitis	96	101	29	0,35	0,11	1.5(0.87-2.67)	1.6(0.94-3.0)
		periodontitis	117	120	43	0,37	0,24	1.2(0.73-2.0)	1.4(0.81-2.3)
			CC	**CA**	**AA**				
IL10	**rs1800872**	healthy	114	83	16	0,27	ref.	ref.	ref.
		gingivitis	122	105	19	0,29	0,49	1.2(0.81-1.74)	1.1(0.54-2.3)
		periodontitis	143	109	27	0,29	0,44	1.0(0.72-1.5)	1.3(0.69-2.6)
			TT	**TC**	**CC**				
VDR	**rs731236**	healthy	72	85	44	0,43	ref.	ref.	ref.
		gingivitis	81	103	44	0,42	0,73	1.1(0.7-1.7)	0.89(0.52-1.5)
		periodontitis	98	137	44	0,40	0,71	1.2(0.79-1.8)	0.73(0.44-1.2)

SNP-ID: Single nucleotide polymorphism identification number at NCBI SNP data base; MAF: Minor Allele Frequency; Allelic P: P value of allelic association test.

through an independent study.

A previous study of a sample of Italian ancestry population reported evidence of association between periodontitis and IL6 polymorphisms, as well as suggestive association with IL10 and VDR polymorphisms. In this study we reported the results of a replication study performed with 750 Italian patients. The study was aimed to test genetic association between IL6, IL10, and VDR polymorphisms and chronic periodontitis and also investigated their role in gingivitis. The main finding was that an IL6 gene promoter variant influenced the risk of periodontitis in the Italian population. The variant C allele of rs1800795 (also named IL6 -174G>C) was confirmed to be more frequent in healthy patients than those with periodontitis [OR=0.47 (95% C.I. 0.27-0.82) for homozygotes]. Interestingly, a similar level of association was observed for gingivitis patients [OR=0.36 (95% C.I. 0.21-0.64) for homozygotes].

The PSR system was used to evaluate periodontal disease in this investigation. This rapid and non-invasive measure of periodontal status is useful for case definition. However, it cannot be considered a comprehensive examination able to produce a precise diagnosis.

Nonetheless, PSR demonstrated a high predictive potential compared to current periodontal disease definitions as outlined by the 1999 International Workshop Classification of Periodontal disease (17, 18).

Il-6 is an important modulator of inflammatory

response in periodontal tissues that could also activate osteoclasts and induce differentiation of B-cells acting synergistically with other factors like IL-1 and TNFa (PMID: 9655045). In comparison to control group, a higher level of IL-6 was detected in crevicular fluid of periodontal disease group; specifically IL-6 was significantly higher in aggressive and chronic periodontitis patients (19). Increased expression was found in gingivitis, but the difference was not statistically significant (19). Considering that the rs1800795(C) allele is generally associated with lower levels of IL6 (20), the data appear to agree with our results and with our data obtained through a Brazilian population sample (21). In this study the higher expression of IL6 positively correlated with clinical attachment loss and the rs1800795 CC genotype was more frequent in control group (21) whereas the CC genotype that had an unusually higher frequency in a sample of German population, appeared associated with an elevated risk of developing periodontitis (22). Rs1800795 SNP tends to be quite polymorphic in Caucasians while Asian and African populations are almost monomorphic for the G allele.

Interestingly, the severity of gingivitis resulted associated with polymorphisms of IL6 gene in a Russian male population (23). These data appear to indicate that the rs1800795 in IL6 promoter could modulate IL-6 expression in periodontal tissues and carriers of the variant allele could be protected from periodontal diseases, including both gingivitis and chronic periodontitis. This information should be considered seriously in planning future studies. A control sample to investigate the role of IL6 in periodontitis should include only healthy individuals, as a mixed control sample which include healthy and gingivitis patients could significantly reduce the success of the study.

The rs1800795 SNP has been found to be associated to several diseases including heart disease, Kaposi's sarcoma, type-2 diabetes, stroke, obesity, Hodgkin's lymphoma, sudden infant death syndrome, cancer, endometriosis, and hypertension (24-29).

In regards to IL10 and VDR polymorphisms, the present study did not find any evidence of association with periodontal diseases. This result does not rule out a possible role of IL10 variant in periodontitis. (13, 30).

In conclusion, the present study confirmed the strong association between the IL6 polymorphism rs1800795 and periodontitis in Italian population that was found in two independent studies. Moreover, the same level of association was found for gingivitis. This data should be carefully considered when planning future studies or looking for possible diagnostic application.

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