Research Paper

Cytokine Gene Polymorphisms Associate with Microbiogical Agents in Periodontal Disease: Our Experience

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Abstract

Periodontics has evolved from a simplistic model to a more complex interplay between infection and host response. Genetic factors have been a new addition to the list of risk factors for periodontal diseases. The processes leading to destruction and regeneration of the destroyed tissues are of great interest to both researchers and clinicians.

The selective susceptibility of subjects for periodontitis has remained an enigma and wide varieties of risk factors have been implicated for the manifestation and progression of periodontitis. Emerging pathway models suggest that gene-environment interactions are etiologically important in disease pathogenesis. The current practical utility of genetic knowledge in periodontitis is limited.

Allelic variants at multiple gene loci probably influence periodontitis susceptibility. The pro-inflammatory cytokine interleukin-1 (IL-1) is a key modulator of host responses to microbial infection and a major modulator of extracellular matrix catabolism and bone resorption, and polymorphisms in the IL-1 gene cluster have been associated with an increased risk of developing severe adult periodontitis.

The aim of this study was to test if polymorphisms of genes of IL-1α^{+4845} and IL-1β^{+3954} were linked with periodontitis, in a case-control study population, delimited to a specific geographic area, in association with microbiological findings.

The polymorphisms observed in IL-1α^{+4845} and IL-1β^{+3954} single nucleotide polymorphisms (SNPs), was significantly different among the study groups (healthy controls, mild, moderate and severe periodontitis with $p<0.05$, d.f.=1.

We found a significant correlation between the severe form of periodontitis and the presence of composite genotype ($p < 0.05$, d.f.=1, calculated among healthy vs. severe). Furthermore a statistically significant association between the presence of bacteria and periodontitis was detected ($p<0.05$, d.f.=1). In the current investigation findings were concordant with literature observations.

Key words: Periodontal disease, Single nucleotide polymorphisms, Interleukin-1, Genetic factors, Oral Microbiology.

Introduction

Inflammation is clearly a central component of many chronic diseases. Cytokines are regulatory low-weight proteins acting as mediators into genesis and control of immune and inflammatory response.

Interleukin-1 (IL-1) is a proinflammatory cytokine and includes two different but functionally similar molecules: IL-1α e IL-1β, genes which are all located on the long arm of chromosome 2 (region 2q13–14). [1-3]

IL-1 play an important role in immune regulation and inflammatory processes by inducing expression of many effector proteins, e.g. cytokines/chemokines, nitric oxide synthetase (NOS) and matrix metalloproteinases (MMPs) [1,2].

IL-1α is mainly produced by keratinocytes of junctional epithelium or pocket epithelium, is a mediator of local inflammation and regulates intracellular events. [1]

IL-1β is primarily produced by activated macrophages and fibroblasts that release this extracellular protein. [1]

IL-1 upregulates complement system, Fc-receptor on polymorphonuclear neutrophils and monocytes, and the expression of adhesion molecules upon fibroblast and leukocytes. [1, 4]

It induces the expression of homing receptors into the extracellular matrix for leukocytes and bone resorption by stimulating the proliferation and differentiation of osteoclast progenitors and activating formed osteoclast indirectly. [5]

It increases the production of MMPs, prostaglandins and IL-1 itself by macrophages, fibroblast and polymorphonuclear neutrophils. [5,6]

It upregulates the expression of major histocompatibility complex molecules (MHC) upon B and T cells facilitating their activation, clonal expansion and immunoglobulin production. [6]

Excessive and/or dysregulated activity of these mediators is associated with tissue destruction and therefore the synthesis, secretion and biological activity of IL-1 cytokines have been identified as therapeutic targets for common inflammatory disorders such as rheumatoid arthritis (RA), diabetes and periodontal disease [7-9].

Increase of IL-1 gingival concentration in subjects with periodontal disease and its reduction after periodontal treatment was demonstrated. [10,11]

Periodontal disease (PD) is characterized by loss of connective tissues within the periodontium and destruction of (alveolar) bone support. [12-15]

The most prevalent form of PD is chronic periodontitis, which is an inflammatory disease initiated by pathogenic bacteria in the subgingival plaque biofilm. [12]

Furthermore, a prominent, localized inflammatory cell infiltrate involving neutrophils, monocytes and both T and B lymphocytes is another characteristic these cells are further sources of MMPs and the cytokines that regulate them.[5,6]

Single nucleotide polymorphisms (SNPs) into the IL-1 promoter region could modulate this cytokine function that acts a fundamental role into the pathogenesis of PD, by affecting the susceptibility and severity of the affection. [16-18]

The composite IL-1 genotype, known as periodontitis-associated genotype (PAG), obtained by the combination of the two rare alleles at the above mentioned SNPs, has been extensively investigated with respect to the form of periodontal disease (chronic *vs* aggressive), smoke, ethnic group, oral microbial pathogens present in the plaque biofilm and other periodontal clinical parameters related to inflammatory status or to the progression of the disease. [16,17]

The aim of this study was to test if polymorphisms of genes of IL-1α^{+4845} and IL-1β $^{+3954}$ were linked with periodontitis, in a case-control study population, delimited to a specific geographic area, in association with microbiological findings.

Patients, Materials and Methods

All subjects were chosen among current and permanent residents of Bari area (Italy).

Apulia (Italian: Puglia) is a Region in Southern Italy. The Region comprises 19,345 square kilometers (7,469 sq mi), and its Population is about 4.1 million Density 210/km2 (540/sq mi). Its capital city is Bari.

Population proportionate technique was employed for sample size estimation. The city is divided in 10 quarters. The average age of Bari residents is 42 compared to the Italian average of 42. The present study includes only resident form 4 generations in the quarter of Old Town that is the one with less heterogeneity of population. According to population census 2011, city itself has a population of about 320.000, over 116 km², the Old Town in an area of around 0,4 square Kilometers, have a population of around 6000 people, of which the 26-56 old adults constitute 33%. With 95% confidence level, the estimated sample size was 99.

This work was conducted according to the recommendations of the Declaration of Helsinki; all patients received informed consent.

The study included as described in Table 1 and Table 2, 195 unrelated patients, recruited only from private dental practice.

All subjects were in good general health conditions.

To identify suitable participants, patient referral records, including a full-mouth series of peri-apical radiographs, were screened.

Complete examinations of soft and hard oral tissues were performed on all patients.

Exclusion criteria from the study were: diseases of the oral hard or soft tissues except caries and peri-

odontal disease; presence/use of orthodontic appliances; need of pre-medication for dental treatment; use of antibiotics for the preceding 6-month period; pregnancy or lactation; inability or unwillingness to sign informed consent; history of diabetes; HIV positive; immunosuppressive chemotherapy; history of any disease known to severely compromise immune function.

Table 1. Gender characteristic and data of Patients with Periodontal Disease and Control Subjects.

		Cases		*Controls*	
Age (years)	54.25 (mean)	32 to 56 (range)	48.15 (mean)	26 to 56 (range)	
Male	52	52.5%	49	51%	
Female	47	47.5%	47	49%	
Smokers	33	33.3%	24	25%	
No smokers	66	66.7%	72	75%	

Table 2. Cases population divided by periodontitis severity.

	Subjects	%
Mild periodontitis (Group B)	40	40.4
Moderate periodontitis (Group C)	39	39.4
Severe periodontitis (Group D)	20	20.2
Total	99	100

No subject with localized periodontitis or acute necrotizing ulcerative gingivitis was included in the study.

All patients were interviewed and smoking habits were recorded. They were subjected to a clinical examination of the periodontal tissues.

The parameters investigated were: plaque accumulation (0/1/2), overt gingivitis (0/1), bleeding on probing (0/1), and suppuration (0/1).

A manual periodontal probe was used to assess the attachment loss which was measured at six sites per tooth at all teeth excluding third molars.

Regarding these parameters, the cases were divided into four groups:

(A) Healthy controls patients;

(B) With mild periodontitis (plaque accumulation, overt gingivitis, attachment loss ≤ 4 mm with or without bleeding);

(C) Moderate periodontitis (bleeding and/or attachment loss between 4-6 mm);

(D) Severe periodontitis (bleeding or suppuration and attachment loss > 6 mm).

Periodontally intact subjects were qualified as controls if they displayed: no sites with probing depth exceeding 3 mm and no more than 4 sites with bleeding on probing.

Our study recruited only Italian Caucasian subjects, and none of them had a history of diabetes mellitus or current manifestation of systemic diseases, which correlates with destructive periodontal disease.

Radiographic evaluation included Orthopantomography and 21 intraoral radiographs.

All data from clinical and radiographic evaluation were collected into a dedicated periodontal folder.

The periodontal probe (PCP15 –HuFriedy, Chicago, IL, USA) was inserted parallel to the vertical axis of the tooth and 'walked' circumferentially clockwise around each surface of the tooth, to detect the area of deepest penetration [19].

Genomic Analysis

Analysis of genetic polymorphisms

Analysis of blood samples was done in a blinded fashion.

Five milliliters of venous blood was drawn from the antecubital vein and transferred to a vacutainer containing ethylenediaminetetraacetic acid (EDTA; 3%) to prevent coagulation and stored at −20°C.

Genomic DNA was extracted by the salting out method [20]: DNA was aliquoted in distilled water and the stocks were stored at 4°C; DNA integrity was also tested, by running genomic DNA through 0.8% agarose gel, and visualized with ethidium bromide.

We tested the following variants by using primer concentrations, optimized thermocycling conditions and gel compositions as already published in the cited literature: IL-1A^{+4845} [21], IL-1B^{+3954} [22].

The genomic DNA regions were amplified using PCR primers annealing to positions flanking the polymorphisms. IL-1α $^{+4845}$ G>T(rs17561), and IL-1β $^{+3954}$ C>T (rs1143634) with the primer sequences and PCR conditions given in Table 3, using a Q-PCR thermocycler.

Table 3. The primer sequences and the genotyping methods for IL-1α and IL-1β polymorphisms.

LOCUS AND PRIMER SEQUENCES	ANNEALING TEMPERATURE (°C)	PCR AMPLICON SIZE (BP)
IL-1α^{+4845}	56	229
F:5′ATGGTTTTAGAAATCATCAAGCCTAGGGCA3′		
R:5′AATGAAAGGAGGGGAGGATGACAGAAATT3′		
IL-1β^{+3954}	58	194
F: 5′ CTCAGGTGTCCTCGAAGAAATCAA 3′		
R: 5′ GCTTTTTTGCTGTGAGTCCCG 3′		

For a more accurate identification of SNPs, we purified 2–3 µl of some PCR products with Exonuclease I and Alcaline Phosphatase USB (Amersham Pharmacia Biotech, Cologno Monzese, Italy) enzymes; then they were sent for sequencing with a DNA Analyzer (ABI PRISM 3700 Applied Biosystems, Monza, Italy). For all markers, primers were from MWG Biotech (Firenze, Italy).

DNA Sequencing was performed by modified dideoxy mediated chain-termination method, using the ABI Prism (Applied Biosystems, USA) 3130 genetic analyzer automated DNA sequencer and ABI Prism Big Dye Terminator v3.1 cycle sequencing kit.

Accuracy of the method was confirmed by reanalyzing mutant samples, and representative samples of the genotypes of each polymorphic loci was sequenced to confirm the mutations detected.

MicroDent test

Subgingival microbial species were determined in each plaque sample using a PCR technique (micro-IDent® *plus 11* test, Hain Diagnostika Ltd. - Nehren, Germany), according to the manufacturer's recommendations. [23-26]

The micro-IDent® *plus 11* test is a fast and reliable DNA hybridization method to identify microbial subgingival species.

During the clinical examination, the periodontal plaque samples (PS) were collected as follows: the supragingival plaque was removed with an ultrasonic device and with gentle rotary brushing from central lower incisors and mandibular second molars (caries-free periodontal sites with the deepest, inflamed periodontal pockets and attachment loss).

The periodontal sites to be sampled were air-dried and isolated with cotton rolls, and two sterile paper points ISO size 40 (Dentsply Maillefer, Ballaigues, Switzerland) were inserted into periodontal pocket [27] after 1 min, the paper points were removed, pooled and frozen immediately at -80 °C in a separate sterile 2-mL cryotube filled with 1 mL of sterile phosphate-buffered saline (PBS, 50 mmol L^{-1} potassium phosphate, 150 mmol L^{-1} NaCl, pH 7.2.

For the rapid manual isolation of genomic bacterial DNA from patient specimen we used GenoLyse® kit (Hain Diagnostika Ltd. - Nehren, Germany), according to the manufacturer's recommendations.

After PCR amplification, evaluation of the results was performed with a specific reading card that allows to immediately identifying the microbial species.

Statistical Analysis

Statistical analysis of data was performed using the NCSS8¸ Statistical Software. The Chi-square test was used to compare the genotype distributions between the control and PD groups (d.f.=1). A p-value of < 0.05 was considered significant.

Results

The present study determined the frequencies of the two IL-1 polymorphisms, IL-1α^{+4845} and IL-1β^{+3954} in a sample group of 195 individuals.

Sixty-four subjects were positive for IL-1α^{+4845} genotype (33 %), with a major frequency of positive subjects observed among the group B subjects and the least frequency observed among the healthy subjects. (Table 4)

This difference achieved statistical significance because p < 0.05 at d.f.=1.

Similar trend was noticed for the SNPs of IL-1β^{+3954} gene, with the frequency progressively increasing from group A through group C, B, to group D, which was significant at p<0.05, d.f.=1. (Table 5)

The combined presence of both the IL-1α and IL-1β composite genotype, dived for severity of disease was studied. (Table 6)

This difference brought out a significant correlation between the severe form of periodontitis and the presence of composite genotype (p < 0.05, d.f.=1, calculated among healthy vs. severe).

Furthermore, microbiological data were available for all the subgingival plaque samples collected from all cases and controls. (Table 7)

As expected a statistically significant association between the presence of bacteria and periodontitis was detected (p<0.05, d.f.=1).

Table 4. Distribution of IL-1 positive genotype polymorphisms in periodontitis (divided for disease severity) and controls.

	Severe periodontitis	Moderate periodontitis	Mild periodontitis	*Controls*	*Total*
IL-1α (+)	7	20	21	116	64
IL-1α (–)	13	19	19	80	131
Total	20	39	40	96	195

Table 5. Distribution of IL-1β genotype polymorphisms in periodontitis (divided for disease severity) and controls.

	Severe periodontitis	Moderate periodontitis	Mild periodontitis	*Controls*	*Total*
IL-1β (+)	61	5	6	06	23
IL-1β (–)	14	134 1	34	90	173
Total	20	39	40	96	195

Table 6. Distribution of IL-1 composite genotype polymorphisms in periodontitis (divided for disease severity) and controls.

	Severe periodontitis	Moderate periodontitis	Mild periodontitis	Controls	Total
IL-1α (+)/IL-1β (+)	3	3	2	2	10
IL-1α(+)/IL-1β(−)	4	17	19	14	54
IL-1α(−)/IL-1β(+)	3	2	4	4	13
IL-1α(−)/IL-1β(−)	10	17	15	76	118

Table 7. Pathogens presence in periodontitis (divided for disease severity) and healthy controls.

	Severe periodontitis	Moderate periodontitis	Mild periodontitis	Controls	Total
Bacteria (+)	20 (100%)	39 (100%)	40 (100%)	50 (52%)	149
Bacteria (−)	0 (0%)	0 (0%)0	0 (0%)	46 (48%)	46
Total	20 (100%)	39 (100%)	40 (100%)	96 (100%)	195

The most frequent pathogens found in the subgingival plaque of patients positives for the presence of bacteria were Fusobacterium nucleatum (27%), Campylobacter rectus (20%), Eikenella corrodens (20%), Tannerella forsythia (2%), Treponema denticola (6%), Parvimonas micra (7%),, Porphyromonas gingivalis (10%), and Prevotella intermedia (8%). (Figure 1)

When the allele distribution was compared between cases and healthy controls, statistically insignificant values were obtained. (Table 8)

The heterozygous genotypes (GT) and (CT) are more frequent respectively in, IL-1α^{+4845} and IL-1β^{+3954}, for both cases and controls.
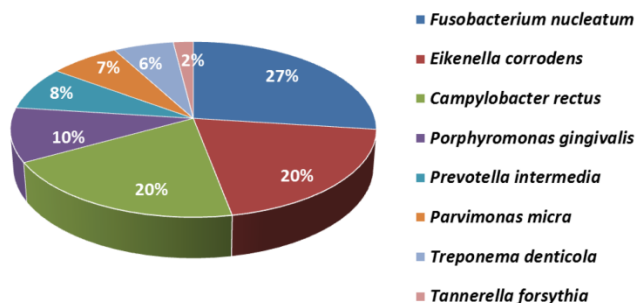

Figure 1: Pathogens found in the subgingival plague.

Table 8. Summary of allele and genotype distribution in cases and healthy controls.

Locus	Genotype			Allele		*P*-value
IL-1α(+)	**GG**	**GT**	**TT**	**G**	**T**	
Cases	18	28	2	60	36	0.19
Controls	4	11	1	24	8	
IL-1β(+)	**CC**	**CT**	**TT**	**C**	**T**	
Cases	7	9	1	28	6	0.93
Controls	2	4	0	10	2	

In the current investigation findings were concordant with literature observations. [21-22].

However, contradictory results were obtained in a recently published data [28] where they have found that for IL-1β^{+3954} CC genotype was the more frequently genotype identified in cases than in controls.

Discussion

In the present work, we have directed our effort primarily on the study of a homogeneous population from both ethnic and clinical points of examination.

This approach, given the small extent of our region, reduces the possibilities for establishing large sample sizes, but the homogeneous population has fewer problems with population stratification.

A second limitation of the present study is represented by the limited size of case samples.

Current evidence from complex diseases (with large heritable components) indicates that the predicted odds ratios for many genes may be 1.5 or less and, to identify such associations, as a general rule at least 100 cases and controls should be genetically characterized. [29, 30]

The subjects may not be representative of the true disease distribution because of recruitment from a clinical practice.

This phenomenon is known as the "clinicians' illusion," where the identified cases among the subjects are likely to be more numerous and severe than cases identified from a general representative population sample. [31]

The sample is very small as to provide for any generalized conclusions based on the results that apply to the whole population.

Due to the phenomenon of genetic heterogeneity, the periodontal phenotype may be due to several different genotypes. [21-22, 32-33]

The clinical categorization of PD has been different in different studies due to changes in classification and nomenclature.

Since many risk factors contribute to PD, the ideal method of studying genetic relationship would be to start before the disease sets in and following it up through the natural history, i.e., to establish the genotypes required first and then follow it up as the subject is exposed to various predisposing and risk factors.

Also, as recommended, further researches are needed to validate the biologic basis for genetic susceptibility testing, to evaluate the ability of IL-1 genotypes to predict disease initiation and to evaluate the effectiveness of IL-1 genotyping in making diagnostic or treatment intervention strategies, especially in dental "new age" [34] tissue engineering approach [35-40].

Acknowledgement

Authors' contributions

DdV supervised and coordinated the research. SS, FI and FP revised it critically for important intellectual content and gave final approval of the version to be published. AB made substantial contributions to conception and design and edited the manuscript. SC assisted the clinical procedures and selected the patients performing all the clinical measurements and the database. GD was responsible for bibliographic research. RM and FMC were responsible for the statistical elaboration and graphics layout of the manuscript.

Competing Interests

The Authors declared that no competing interest exists. None of the funding sources had any role in the design and conduct of the study. All Authors reviewed the paper, and read and approved the final manuscript.

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