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Research Article

THE EFFECT OF SMOKING ON IL-6 POLYMORPHISMS IN PATIENTS SUFFERING FROM CHRONIC PERIODONTITIS

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ABSTRACT

***Background & Objectives*:** Periodontitis is a multifactorial, inflammatory disease of supporting structures of the tooth. The complex pathogenesis of periodontitis implies the involvement of a susceptible host and a bacterial challenge. Many studies have provided a valuable contribution in understanding the genetic basis of periodontal disease, but the specific candidate genes of susceptibility are still unknown. The genome-wide studies and screening of Single Nucleotide Polymorphisms (SNPs) have yielded new genetic information but without a definitive solution for the management of periodontal disease.

***Methods*:** The present study was undertaken with the aim to evaluate the association of SNPs of IL-6 gene with Chronic Periodontitis in smokers and nonsmokers. A total of 60 subjects aged between 25-60 years were selected for the study. They were divided into following groups as **GROUP I** Periodontally and systemically healthy individuals, **GROUP II** Chronic periodontitis subjects who were non-smokers and **GROUP III** Chronic Periodontitis subjects who were smokers**.** The clinical parameters: Plaque Index (PI), Gingival Index (GI), Probing Pocket Depth (PPD) and Clinical Attachment Level (CAL) were recorded and Blood samples were taken for genotyping.

Results: The study thus demonstrated that for SNP IL-6 (rs 1800795), genotype GG (homozygous wild type) was significantly associated with Chronic Periodontitis. It also showed that there is an increased risk of association of disease severity with GC (heterozygous mutant) genotype i.e., an increased carriage rate of genotype GC was evident with the increase in severity of CP. Thus, highlighting an increase susceptibility to CP due to this gene polymorphism. There was no correlation with smoking in Test groups (II & III) seen, hence proving that the genotype frequencies are dependent on the severity of the disease rather than the risk factors involved.

INTRODUCTION

Chronic Periodontitis is known to be aninflammatory and a complex multi-factorial disease affecting the periodontium and resulting in its destruction if timely intervention is not done. Genetic variance in chronic periodontitis can occur which is complex and involves host-gene-environment interactions.

The genetic polymorphisms in some situations can cause a change in genetic protein or its expression possibly resulting in alterations in innate and adaptive immunity and may thus be deterministic in disease outcome; making a person susceptible or sometimes resistant against the disease[1].

Interleukin-6 is a pleiotropic cytokine produced by many cell types such as stimulated monocytes, fibroblasts, endothelial cells and T and B lymphocytes[2]. It is not expressed constitutively, but is highly inducible and is produced in response to a number of inflammatory stimuli such as IL-1, tumour necrosis factor (TNF)-a, bacterial products and viral infection[3]. IL-6 has diverse functions, including differentiation and/or activation of macrophages and T cells, growth and differentiation of B cells, stimulation of haematopoiesis and neural differentiation[4]. It is also a potent stimulator of osteoclast differentiation and bone resorption[5] and an inhibitor of bone formation[6]. With regard to periodontitis, IL-6 is expressed by a variety of cells in the periodontal lesion which along with IL-1 and TNF-a, enhances bone resorption[7].

Elevated levels of IL-1 β and IL-6 have been shown to be induced by periodontal pathogens and are correlated with the continuous tissue destruction observed in periodontitis[8]. This study describes the structure and organization of the different transcription initiation sites for the expression of IL-6 gene in the blood samples of the patients and the role of smoking on these genotype frequencies.

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MATERIALS AND METHODS

Study Design: This study was conducted in the Department of Periodontics, BBD College of Dental Sciences, Lucknow, Uttar Pradesh. The aim of the study was to establish an association between Chronic Periodontitis and IL-6 gene in smokers and non-smokers respectively. A total of 60 subjects reporting to the OPD of Department of Periodontics aged between 25-60 years were selected for the study. The study confirmed to the ethical guidelines of the Helsinki Declaration and was evaluated and approved by the Institutional Ethical Committee. A written informed consent was obtained from all subjects participating in the study. The patients were selected based upon the following inclusion and exclusion criteria.

Inclusion Criteria

1. Patients within the age group of 25-60 years.
2. Cigarette & beedi smokers.
3. Patients having chronic periodontitis defined as having minimum of 20 remaining teeth, with periodontal disease as evidenced by at least 4 tooth sites with probing pocket depth (PPD)>4mm, clinical attachment level (CAL) > 2mm; and radiographic evidence of bone loss>2mm from the cementoenamel junction (CEJ).
4. Patients who had smoked an average of >10 cigarettes/day for >2 years.
5. Patients who were cooperative and committed to maintain oral hygiene. 6. Patients with no contraindication to periodontal therapy.

Exclusion Criteria

1. Patients suffering from chronic systemic illness like diabetes, hypertension, active infection etc.; and taking medication for the same.
2. Patients suffering from aggressive periodontitis, periodontal abscess, necrotizing ulcerative gingivitis or periodontitis.
3. Patients who have undergone any periodontal treatment or antibiotics within the preceding 6 months.
4. Tobacco chewers.

Patients who fulfilled the criteria were enrolled in the study and divided into the following groups: **Group I:** 20 Periodontally healthy individuals. **Group II:** 20 Non Smokers suffering from Generalised Chronic Periodontitis. **Group II:** 20 Smokers suffering from Generalised Chronic Periodontitis.

Clinical Parameters

Clinical parameters were recorded and blood samples were collected. These blood samples were then subjected to genotype analysis. The following clinical parameters were recorded:

1. Plaque Index (Sillness & Loe 1964)[9]
2. Gingival Index (Loe & Sillnes 1963)[10]
3. Probing Pocket Depth (PPD)
4. Clinical Attachment Level (CAL)

Probing pocket depth (PPD) and clinical attachment level (CAL) were measured at four surfaces of all teeth (mid-buccal, mid-lingual, mesial and distal inter-proximal sites) to the nearest mm, using UNC-15 probe, having markings from 1 to 15 and colour coded at an interval of 5, 10 and 15.

Smoking Status

20 patients suffering from chronic periodontitis and were current smokers (person who has smoked >100 cigarettes in their lifetime and who at the time of the study reported that he was smoking) were recruited in the study. The smoking status was assessed by means of a questionnaire asking them of the number of cigarettes smoked per day and number of years of smoking[11].

Accordingly pack years were calculated as:

$$\text{Pack years} = \frac{\text{Number of cigarettes per day X number of years smoked}}{20}$$

This standard formula is based on 20 cigarettes per pack. For variable number of cigarettes in a pack (eg. 10) the formula has been modified as:

Number of Pack Years = (Packs smoked per day) x (years as a smoker)

Quantification of pack years gives the degree of tobacco exposure and is closely related to the risk of disease.

Blood Sampling and Storage

5ml of IV blood was collected from all the subjects by venipuncture in the anticubital fossa. The blood samples were collected in sodium EDTA vacutainer and stored at 4^0C. It was shaked thoroughly to avoid clot formation.

Laboratory Analysis: Samples were analyzed at Genotoxicity Laboratory of Toxicology Division at Central Drug Research Institute, Lucknow, for genotyping.

Steps of Genotyping: All the blood samples were analyzed in three steps of genotyping, which are as follows:-

1. DNA isolation from blood
2. Selection of single nucleotide polymorphism (SNP)
3. Polymerase Chain Reaction (PCR) amplification
4. Restriction Fragment Length Polymorphism (RFLP)

After the DNA Isolation of IL-6 gene at RS1800795 in chromosome 7 at location 22727026 in the coding region from cytosine → guanine. Each DNA sample was amplified by PCR with specific primers and was analyzed for rs1800795 polymorphism in the IL-6 gene.PCR product target specific primers were designed for the SNPs of genes selected for the present study using Primer Select module of Laser gene v6.0 software (DNA Star) and synthesized commercially. Specific primer and temperature conditions are used for each Single nucleotide polymorphism, **IL-6 (C-> G):** Forward Primer: 5-CAGAAGAACTCAGATGACTG-3' and Reverse Primer: 5-GTGGGGCTGATTGGAAACC-3´.

Restriction Fragment Length Polymorphism (Rflp) makes use of different endonuclease enzymes and their high affinity to specific restriction site. Digestion by specific restriction enzyme render fragments of different lengths which are determined by gel assay, to ascertain presence of Single Nucleotide Polymorphism (SNP).

Table A Details of Restriction enzyme used and length of fragments generated upon Restriction digestion

Single nucleotide polymorphism	Restriction Enzyme	Restriction fragment lengths of genotypes		
		Homozygous Wild type	Heterozygous Mutant	Homozygous Mutant
IL-6 (Rs1800795)	N1aiii	173	173+122+51	122+51

Where, C, T, A and G respectively denotes nucleotides cytosine, thymine, adenine and guanine & Bp denotes Base Pair

Use of agarose gel electrophoresis was required for quantification and analysis of quality of the DNA, PCR, RFLP products, were necessary to ascertain the approximate quantity obtained and the suitability of sample.

RESULTS

The present study results revealed that:

Table B Demographic characteristics three groups

Demographic characteristics	Group I (n=20) (%)	Group II (n=20) (%)	Group III (n=20) (%)	F/χ^2 value	p value
Age (yrs):					
Mean ± SD	37.50 ± 9.57	37.30 ± 7.91	37.45 ± 7.42	0.003	0.997
Range (min-max)	26-58	25-50	29-52		
No. of teeth present:					
Mean ± SD	30.10 ± 1.55	29.05 ± 1.70	27.85 ± 1.95	8.34	0.001
Range (min-max)	28-32	26-32	24-31		
No. of year smoked:					
Mean ± SD	NA	NA	7.75 ± 5.17	NA	NA
Range (min-max)			1-18		
Pack year (no):					
Mean ± SD	NA	NA	1.92 ± 1.42	NA	NA
Range (min-max)			0.2-4.5		

NA: not applicable

DISCUSSION

Periodontitis is a chronic inflammatory condition of the periodontium that is initiated by accumulation of bacterial plaque on tooth surface adjacent to the gingival tissues. The specific bacterial component accounts a relatively small proportion (approximately 9-16%) which leads to breakdown of periodontal tissues.[12] This suggests that susceptibility to periodontitis may be significantly modified by the host responses to bacterial plaque[7,8,12]. The role of genetic factors in modifying the host responses to bacterial plaque has also emerged as a field of prime importance [12] Human genetic variants are found most commonly in Single Nucleotide Polymorphism (SNP's) and brings about the deletion and insertion mutations with adenine, thymine, guanine and cytosine nucleotides.

The frequency of appearance of a particular SNP (related to disease or susceptibility to disease) in a particular population is of great importance[15]. A pathological SNP can segregate within the particular population and make individuals of the population susceptible to the disease.[16] These polymorphisms may cause a change in the encoded protein, or its expression, possibly resulting in alterations in innate and adaptive immunity, and may thus be an important determinant in disease outcome [18]. One of the well-studied cytokine genes whose variants are associated with periodontal disease occurrence is Interleukin-6. IL-6 is locally produced in inflamed tissues following cellular activation by bacterial lipopolysaccharide (LPS) or other cytokines such as IL-1 or tumour necrosis factor (TNF)-α[19].

Table C Periodontal parameter level (Mean ± SD) of three groups

Periodontal parameters	Group I (n=20)	Group II (n=20)	Group III (n=20)	F value	p value
Plaque index	1.06 ± 0.14	1.58 ± 0.13	1.77 ± 0.15	134.48	<0.001
Gingival index	1.01 ± 0.18	1.39 ± 0.14	1.07 ± 0.14	36.32	<0.001
Pocket probing depth (mm)	1.76 ± 0.32	2.66 ± 0.36	2.97 ± 0.47	51.78	<0.001
Clinical attachment loss (mm)	2.77 ± 0.87	4.06 ± 1.18	4.67 ± 0.38	24.45	<0.001

Table D Distribution of Il-6 gene polymorphism of three groups

Il-6 gene polymorphism	Group I (n=20) (%)	Group II (n=20) (%)	Group III (n=20) (%)	χ^2 value	p value
Genotype:					
CC	5 (25)	0 (0)	0 (0)		
GC	13 (65)	18 (90)	20 (100)	13.53	0.009
GG	2 (10)	2 (10)	0 (0)		

- Among the Periodontal Clinical Parameters, the Plaque Index showed a significant increase in Group III (smokers suffering from chronic periodontitis) and Gingival Index showed a significant increase in Group II (non-smokers suffering from chronic periodontitis). Further, the increase shown by Pocket Probing Depth & Clinical Attachment Loss in Group III was significant when compared between the Groups.
- There was no correlation to smoking seen when evaluating the association of SNP's of IL-6 with Chronic Periodontitis. The difference in genotype frequencies was insignificant between Groups II & III.

IL-6 has diverse functions, including differentiation and activation of macrophages and plays a role in the transition between acute and chronic inflammation through the recruitment, activation and clearance of individual leucocyte subpopulations (i.e. neutrophils, monocytes and macrophages).[21]

The control of IL-6 levels plays a crucial anti-inflammatory role in both systemic and local inflammatory responses[23]. It has been widely investigated *IL6*174G>C polymorphism is associated with periodontopathogens detection, variations in severity of inflammation, tissue destruction, and attachment loss in periodontal disease.[17,18,21,22,24]

Second to bacterial plaque, cigarette smoking is the strongest and modifiable risk factor for periodontal disease.[23] Smokers harbour a higher prevalence of potential periodontal pathogens.[9] Smoking impairs various aspects of the innate and adaptive immune responses, including neutrophil function, antibody production, fibroblast activities, vascular factors and inflammatory mediator production.[10]

In this study, the IL-6 gene (gene name INTERLUKIN-6, chromosome 7), with polymorphism at Rs1800795 was selected. It had the presence of 427 base pairs with Forward Primer sequence being: 5-CAGAAGAACTCAGATGACTG-3' and Reverse Primer sequence being: 5-GTGGGGCTGATTGGAAACC-3 on one or both alleles. This polymorphism lies inside the protein coding region of the IL-6 gene (bases) and is located within the N1aiii site within bases 173,122, 51, allowing deferential restriction enzyme digestion fragments of the genomic DNA in this gene region.

Genetic association study for one population/ ethnic group may not hold true for another, as carriage rate of genes varies in different ethnic groups.[16] The present investigation was aimed to study the association of SNPs of IL-6 gene with Chronic Periodontitis in Indian population and also to find a genetic association with the disease severity and smoking.

The homozygous mutant genotype taken for the study was CC which was the protector gene. Heterozygous mutant genotype was CG which was the carrier gene and wild type was GG which was the diseased gene.

Male patients were enrolled because the factor of sex affects the severity of periodontal disease, showing that male smoker patients have 17% higher recession, 6% greater probing depth and 8% more clinical attachment loss than females, as stated by Wattles J *et.al* in 1993,[24] also, according to the trend, females do not show a higher probability of smoking . Individuals smoking >10 cigarettes/day for >2 years were included. Tobacco has its effect on periodontal tissues over a certain threshold. As the consumption increases so does the severity of periodontal disease, although the effect seems to clinically evident from >10 cig/day as stated in a study by Machtei EE *et. al* in 1995.[25] The history of smoking was taken by means of a questionnaire in the case history sheet and accordingly pack years were calculated by the formula given by Grossi et al.in 1994.[11]

Higher plaque accumulation in group II and III could be attributed to poor oral hygiene, pain and bleeding while brushing and improper brushing technique. Smoking may sometimes be associated with a psychological aspect especially deprivation, wherein oral hygiene measures are not performed properly leading to plaque accumulation. There was a statistically significant increase in Group III when compared with Groups II as smoking creates an environment that favours the colonization of anaerobic pathogens and smokers were also associated with poorer oral hygiene.[26]

Group II showed a statistically higher GI as compared to Group III .The gingival inflammatory response is dampened in smokers as compared to non-smokers, evidenced by fibrotic appearance of the tissue fewer sites that bleed upon probing. The hemorrhagic responsiveness in patients with different levels of periodontal disease was found to be suppressed in smokers, while in non-smokers an exponential hemorrhagic response to increasing plaque levels was suggested. When looking at the gingival vessel density smokers have a higher proportion of small blood vessels and a lower proportion of large blood vessels compared to non-smokers.[27]

PPD & CAL were significantly higher in Group III. Neutrophils migrating in the inflamed tissues release proteolytic enzymes such as matrix metalloproteinases, elastases and collagenases and inflammatory mediators which lead to localized destruction of supporting periodontal tissues. Cigarette smoking tilts the balance even further away from the protective functions of neutrophils and towards greater destructive activity. These cells release potentially destructive oxidative burst products, such as superoxide and hydrogen peroxide, triggering destructive processes thus bringing about the destruction of periodontal tissues[28].

Group I showed an increased presence of CC genotype which is the protector gene. Also there was presence of GC genotype which confirmed the presence of carriers in Group I, even though the levels of IL-6 were low. Thus, these patients were either passive smokers or were suffering from gingivitis.[28] Passive smokers (individuals exposed to tobacco smoke in the house and/or workplace) are seen to alter the results of the study.[28]

Highest serum IL-6 levels were observed in subjects in Groups II & III, especially those carrying the GG genotype. The GG genotype is the diseased allele which increases in subjects with chronic periodontitis. This diseased genotype was seen in participants of Group II & III which included the non-smokers & smokers suffering from chronic periodontitis. These subjects were more than 45 years of age and had pocket depths more than 6mm, thus confirming that the genotype frequencies are dependendent on the severity of the disease and not the smoking status of the individual.[17,32,33]

The presence of GC genotype and increased levels of IL-6 were seen in subjects who were less than 45 years and had pocket probing depth less than 6 mm, thus they were not suffering from severe periodontitis but from moderate periodontitis.[17,33] Tervonen T *et.al* in 2007[33] concluded that the presence of GC genotype increases the susceptibility for developing the disease. This genotype is dominant in mild to moderate cases of periodontitis having pocket depth ≤ 6 mm. With the increase in age (>50 years) there is a higher probability of GG genotype, thus age also plays a significant role.[33]

The study showed individuals of Group II & Group III with a decrease in CC genotype due to present of the periodontitis. The protector gene gets altered into either the carrier gene (GC) or the diseasesd gene (GG).[31]

On comparing the genotype frequencies of Groups II & III there was statistically insignificant difference observed, hence proving that the genotype frequencies are dependent on the severity of the disease rather than the risk factors involved[32]. Risk factors may only play a role in increasing the severity of the disease, however they have no role in influencing the genotype frequencies of the individual.

In the present study, a significantly higher frequency of heterozygous genotype IL-6 -174 (G/C) was seen compared to the periodontally healthy controls. Thus, IL-6 174 G/C

genotype may be considered a risk genotype for periodontitis susceptibility.[30]

CONCLUSION

It can be concluded that no correlation with smoking was seen in the genotype frequencies hence proving that the genotype frequencies are dependent on the severity of the disease rather than the risk factors involved.

Genetic Polymorphism may vary among different ethnic populations. The total cases showed a significantly higher frequency of heterozygous genotype IL-6 -174 (G/C) compared to the periodontally healthy controls. Thus, IL-6 -174 G/C genotype may be considered a risk genotype for periodontitis susceptibility.

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