MICROBIOLOGICAL STUDIES RELATED TO GENETIC POLYMORPHISM OF INTERLEUKIN -1(α AND β) IN EGYPTIANS WITH PERIODONTITIS

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ABSTRACT

Genetic polymorphism for cytokines has been investigated as potential genetic markers for periodontitis. The aim is to determine the distribution of genetic variation in IL-1 gene family IL-1α (+4845) and IL-1β (+3953) as potential genetic marker for periodontitis among normal Egyptian individuals taking into account the association between the periodontal status and periodontopathic organisms as an additional variable.

Thirty normal Egyptian volunteers (between the ages 17-35) constituted the sample of this study. Dental examination was performed for each individual, in order to determine periodontal status and record Gingival-Index(GI) scores. Samples were categorized according to their Gingival-Index(GI) scores (good GI and fair GI). For each subject, IL -1α and IL-1β loci were genotype by PCR amplification, followed by digestion with restriction enzymes and gel electrophoresis. Certain micro-organisms were identified using both selective media and API test to establish their DNA-capture on Whatman FTA cards using PCR.

In the first group (the Good GI score group; 14 individuals), 85.7% were healthy, 7.14% had initial periodontitis and 7.14% had moderate periodontitis. Genetic polymorphism of IL-1 showed that 25 % of them were genotyped positive for IL-1α (+4845) and 6.25% were positive for IL-1β (+ 3953).

The second group (the Fair GI score;16 individuals) showed initial periodontitis in 18.75%, moderate periodontitis in 31.25% and severe periodontitis in 50%. 35.72% of them were IL-1α genotype positive and 7.4% were genotype positive for IL-1β (+ 3953). The study clarified that the distribution of IL-1α (+4845) and IL-1β (+3953) composite-genotype among the total studied samples were 30% and 6.66%, respectively. Results revealed that the percentages of certain bacteria, present in the oral cavity of Good and fair GI score groups, were respectively as follow: Actinobacillus actinomycetemcomitans, 7.14% and 6.25%, Porphyrmonas gingivalis in 7.14% and 18.75% and Prevotella intermedia 0% and 6.25%. In addition DNA was stable and could be captured on FTA cards: The results of identifying these microorganisms using PCR (after collecting samples on FTA card (Flinders Technology Associates) was found similar to the ordinary microbiological techniques.

Data of the present study provided evidence that polymorphism in genes of IL-family are associated with those suffering from periodontitis, which brings into question the usefulness of detection of the composite genotyping of IL-1α (+4845) and IL-1 β (+3953) alleles, as a reliable method for determining the susceptibility of patients to periodontitis .It would also be of interest to evaluate the role of other potential candidate genes as contributors to periodontitis by studying more cases. Presence of different microorganisms may be a variable parameter in causing periodontitis. Our results recommended the use of FTA cards as it establishes a new surveillance tool for molecular techniques .It constitutes a significant improvement in the collection of samples and their transport (especially from remote areas of the world to centralized laboratories).

INTRODUCTION

Inflammatory and immune processes operate in the gingival tissues to give protection against local microbial attack, and prevent microorganisms from spreading or invading the tissues (Page, 1991). It has been shown that some individuals carry polymorphisms of host response genes, that code for hypersecretion of certain cytokines in response to noxious stimuli (Lynch et al., 1994).

The diagnosis of moderate to severe periodontitis is a simple clinical process. However ,there is currently no known mechanism for determining which patients with no or mild periodontitis will respond to bacterial plaque, with progression to a more severe periodontitis that demands more extensive therapy .The lack of reliable markers for

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patient susceptibility to severe periodontitis has prevented the early identification of those at most risk and has prevented delivery of therapy appropriate for the degree of the risk. Moreover, the development of new therapeutics has been hampered by clinical trials in which only a small number of enrolled subjects exhibit disease progression, and by lack of clarity as to which segments of the population would benefit from more complex therapies (Kornman ,et al.,1997).

As our understanding of human genetics increases, it is apparent that host response to microbial infection, inflammation, and tissue destruction and repair, are all mediated by a complex of gene-gene and gene-environment interactions. Genetic variance may be, thus, due to combined effect of multiple, normally existing, and functional polymorphisms of genes. Functional polymorphisms are genetic variants that exist at a relatively high frequency, in the general population (>1%) (Malo and Skamene, 1994) .These gene variants may slightly alter the function of the gene product. Such variants usually do not cause disease per se, but it is possible that specific combinations of functional polymorphisms at a number of different genes may significantly alter an individual risk to develop a certain disease phenotype. This may be particularly true for gene products that are important modulators of gene- environmental interactions (Malo and Skamene, 1994).

A functional polymorphism of interleukin family gene has been proposed to be a risk factor for periodontitis, where IL-1 plays a pivotal role in the inflammatory cascade of the immune response to microbial challenge, and it was showed that its level increases with periodontitis, (Walker et al., 2000).

Genetic polymorphisms of cytokines have been investigated as genetic markers for periodontitis (Lopez et al., 2005). Furthermore, Shapira et al., (2005) suggested that the production of inflammatory mediators by inflammatory cells could be affected by different genetic traits.

Lopez(2000) reported that Actinobacillus actinomycetemcomitans, Porphyromonas Gingivalis, and Prevotella intermitis were the major periodontal bacteria species in most forms of progressive periodontitis in his study .He added that the occurrence of periodontal pathogens appears to be different in subjects of different ethnic origins, and that geographical factors may influence the distribution of these species. Umeda et. al., (1998) and Darout et al., (2002) have emphasized detection of many pathogenic bacteria causing periodontitis from saliva.

Dried blood spots (DBS), on filter paper have been used worldwide for the neonatal screening of congenital disorders (Guthrie, R. 1992). Recently, FTA cards (Flinders Technology Associates) was introduced to detect bacterial DNA or viral RNA from different biological samples as blood, saliva, tissues (Burgoyne, 1996). It is a cotton-based cellulose paper, impregnated with anionic detergent and buffer, that provide chelating and free radical-trapping properties. FTA paper contains reagents designed to adapt the storage, transport and integrity of samples so as to kill or inhibit saprophytes, during drying or bouts of high humidity, (Burgoyne, 1996). Moreover, it contains lyophilized chemicals that lyses many types of bacteria and viruses. Most cell types are lysed during contact with FTA, including white blood cells (Devost and Choy, 2000) and bacteria (Lampel,etal.,2000).Viruses are also inactivated ,leaving the nucleic acids suitable for molecular identification(Katz 2002). Therefore, the aim of this study was to determine the distribution of genetic variation in IL-1 gene family [(IL-1 α (+4845) and IL-1β (+3953)] as potential genetic markers for periodontitis among Egyptian individuals, taking into account the association between genetic variability and the periodontal status and periodontopathic organisms as an additional variable

2. METHODOLOGY AND SAMPLING

2.1 Subjects

Thirty normal Egyptian volunteers represented the sample of this study; their ages being between 17-35 years. They were subjected to a full mouth examination, to detect periodontal status and were submitted to gingival evaluation using Gingival Index according to Löe and Silness (1963).

They were divided into two groups according to Gingival Index scores (good GI scores group and fair GI scores group). Scoring of Gingival Index was done according to the following criteria:
0 = Normal gingiva
1 = Mild inflammation – slight change in color, slight edema. No bleeding on probing.
2 = Soft debris covering more than one third but not more than two third of the exposed tooth surface

3 = Soft debris covering more than two thirds of the exposed tooth surface.

N.B. The surface area covered by debris is estimated by running the side of the tip of an explorer across the teeth surface. The following examinations of individuals in this study were conducted:

i. Periodontal examination: Probing pocket depth, bleeding on probing, clinical attachment loss and gingival recession (A North Carolina probe was used in the examination).
ii. Clinical attachment levels were measured with a manual probe on six locations around each tooth, through investigation and evaluation of bleeding disorders and family history of cardiovascular disease or diabetes mellitus was done.
iii. The routine professional tooth-cleaning procedures were identified

2.2 Samples

From each case, the following samples were studied:

2.2.1. Saliva samples

a. 2ml of saliva was collected in 2ml transport media for detection of certain species of periodontopathic bacteria (A. actinomycetemcomitans, P.gingivalis and Prevotella intermedia) and identification using standard techniques.
b. Aliquots of samples (0.5ml) were blotted onto Whatmann FTA cards (1 cm in diameter) and allowed to air dry at (20 to 25°C). Samples after being dried were ready to be subjected to PCR reaction, or stored at an ambient temperature in envelopes with silica gel desiccant till use.

2.2.2. Blood samples

5ml Venus blood was collected from each case in order to detect IL -1 polymorphism using PCR technique.

2.3 Methods

2.3.1. Analysis of Interleukine (IL- 1 α and IL-1β) genetic polymorphism

This was done through PCR technique, followed by use of restriction enzymes for genotyping. The following steps were obeyed:

DNA extraction: DNA was extracted according to the instructions in the kit used (QIA amp DNA Mini kit QIA En, Ltd, UK " (genomic DNA purification kit)

Genotyping technique: 2-IL-1α(+4845): The primers used were:

Oligonucleotide primers 5' ATG GTT TTA GAA ATC ATC AAG AAT AGG GCA-3';5'-AAT GAA AGG AGG GGA GGA TGA CAG AAA TGT -3' (Cited after Walker,etal.,2000).

The technique:
PCR using Taq polymerase was carried out in 50µl reaction mixture containing 0.4Mµ primers under the standard buffer conditions. Cycling was performed as follows: 94°C, 1 minute;35 cycles 94°C, 1 minute; 56°C 1 minute; 72°C 2 minutes; one cycle 72°C 5 minutes. After the cycling period 23 µl of amplicon was digested with 2-5U Fnu 4HI yielding 2 fragments of 124 bp and 29 bp in subjects homozygous for allele "1", and one fragment of 153 bp in subjects homozygous for allele "2" . Individual bands were visualized by electrophoresis through 3.5 % agarose and ethidium bromide staining.
IL -1ß (+3953):

The primers used were:

Oligonucleotide primers 5'-CTC AGG TGG TGT CCT CGT AGAAAT CAA A-3'; 5'GCT TTT TTG CTG TGA GTC CCG -3' (Cited after Walker, et. al.,2000).

The technique:
PCR using Taq polymerase was carried out in 50µl reaction mixture containing 0.4Mµ primers under the standard buffer conditions. Cycling was performed as follows: 95°C, 2 minutes; 35 cycles 94°C, 1 minute; 53°C, 1 minute; 72°C, 1minute. After cycling period 23µl of amplicon was digested with 2-5U Taq 1 restriction endonuclease at 65°C for 2 hours yielding 2 fragments of 97bp and 85bp. Individual bands were visualized by electrophoresis through 3.5% agarose and ethidium bromide staining.

2.3.2 Microbiological Study:

Bacterial culturing technique:

The following media were used as instructed:
(ETSA)Trypticase soy agar, (TSBV) Tryptic soy

serum agar with bacitracin(75µg/ml)-vancomycin(5µg/ml) for isolation of A. actinomycetemcomitans and KVLB-2 (Kanamycin 75µg/ml, vancomycin 2µg/ml-laked blood agar) for isolation of Polphyromonas Gingiralis spp. and Prevotella intermedia spp

Procedure:
Samples were diluted in tenfold steps with repeated homogenization on vortex mixer at a maximal setting for 10 seconds between successive dilutions. Aliquots of 0.1 ml of the dilution were spread on each of freshly prepared plates of the last mentioned media. TSBV plates were incubated in 5% CO_2 incubator for five days at 37° C.ETSA and KVLB-2 plates were incubated at 37°C for 5-7 days in an anaerobic chamber (5% CO_2+10% H2+ 85% N2); black pigmented colonies were suspected to be isolated .Representative colonies of these types were subcultured on ESTA and incubated for 4-7 days.

Characterization and Identification:

Gram stain reaction, phase contrast microscopy for registration of motility, biochemical reactions agar fermentation pattern, and fluorescence in long-wave ultra violet light, as well as characterization and identification based on colony and cellular morphology.

Confirmation had taken place by using API system (La Balme Les Grottes 38390, Montalieu, Vercieu,France), which is fermentative 29. Biochemical standardized enzymatic reactions depend on the biochemical properties of the tested anaerobic microorganisms.

Polymerase chain reaction (PCR) for identifying certain bacterial strains:

1- Oligonucleotide primers used :
For analysis of A. actinomycetemcomitans , the following sequences of the forward and reverse primers were LKT2 (5- GGA ATT CCT AGG TAT TGC GAA ACA ATT TGATC -3) and LKT3 (5- GGA ATT CCT GAA ATT AAG CTG GTA ATC -3) .

Primers used for P.gingivalis analysis were FIM1 (5- ATA ATG GAG AAC AGC AGG AA -3) AND FIM2 (5- TCT TGC CAA CCA GTT CCA TTGC -3).

PCR using the last mentioned primers were described by Riggeno et al (1996). They were determined by a distinct band on 1.5 % agarose at 262 bp in size for A. actinomycetemcomitans and 131 bp for Polphyromonas Gingiralis. Primers used for Prevotella intermedia were (5- CCT AAT ACC CGA TGT TGT CCA CA -3) Pi -1 and (5- AAG GAG TCA ACA TCT CTG TAT CC-3) Pi2.These primers were described by Riggeno et al., (1998), which were determined by a distinct band on 1.5% agarose at 855 bp in size

DNA extraction and PCR procedure:

Samples on filter paper were air dried. 3 disks were punched and washed with FTA purification reagent twice. Used reagents were discarded after each wash. The samples were washed again with TE buffer (10 mM tris, 0.1 mM EDTA, PH 8), and the used buffer was discarded after each wash. Discs were dried and directly subject to PCR step. For detection of Actinobacillus actinomycetemcomitans and Polphyromonas Gingiralis, PCR amplification was carried out in a reaction volume of 100 µl contained 50 p mol of forward and reverse primers, 0.2 mM each of 4 deoxynucleoside triphosphatases, 2 units of the thermophilic DNA polymerase,1x buffer (10 mM tris-HCL PH 8.8 ,50 mM KCL, 0.1 % Triton x-100 and, 2.5 mM of MgCl2 (in reaction for A. actinomycetemcomitans and 1.5 mM in reaction with Polphyromonas Gingiralis and 2 units of Dynazyme Taq polymerase. PCR was carried out at 95°C for 5 min for one cycle, followed by subsequent 33 cycles of denaturation at 95°C for 1min, annealing at 55°C for 1 min and extension at 72°C for 1 min. The final extension was carried out at 72°C for 10 mins. For Prevotella intermedia, PCR amplification was carried out by the same composition of the last mentioned reaction mixture, but it was carried out at 94°C for 5min for one cycle, followed by subsequent 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1min, and extension at 72°C for 1.5 min. The final extension was carried out at 72°C for 10 min .

2.4 Results

Results are recorded in tables 1,2 and 3, to illustrate the relation between the periodontal status and IL-1 polymorphism genotype composite, as well as, the periodontal status and the distribution of certain periodonto-pathic micro-organisms.

Table 1, shows that among the examined individuals; group 1 (with good GI score; composed of 14 individuals) 85.72% were healthy, 7.14% had

Table - 1: Distribution of the samples according to mean gingival index scores and disease categories

Disease categories*	Gingival index score - groups		Total (n= 30)
	Good	Fair	
Healthy	12/14(85.72%)	0/16 (0%)	12/30(40 %)
Initial periodontitis	1/14(7.14%)	3/16(18.75 %)	4/30(13.33 %)
Moderate periodontitis	1/14 (7.14%)	5/16 (31.25%)	6/30(20 %)
Severe periodontitis	0/14 (0%)	8/16(50 %)	8/30(26.66 %)

*According to periodontal examination mentioned

initial periodontitis, 7.14% had moderate periodontitis, and none of them suffered from severe periodontitis. On the other hand, the group 2 (with fair GI score; were16 individuals) showed that none of them were healthy, 18.75% had initial periodontitis, 31.25% were moderate and 50 % had severe periodontitis

Table 2 reveals that only 11 out of the 30 subjects (36.66 %) carried the composite IL -1 genotype consisting of both IL-1α(+4845) and IL-1 β(+3953); 31.25% from group 1 individuals (those with good GI score) & 42.86% from group 2 individuals (those with fair GI score).

IL -1 α (+4845) polymorphism was carried by 30 % (9/30) of all subjects tested; where 25% of them were carried by group 1 and 35.72% from group 2 .

IL-1β(+3953) polymorphism was less; only 6.66 % (2/30) of all subjects carried this marker. One individual from each group (1&2) carried IL-1β(+3953) polymorphism, representing a percentage of 6.25% & 7.14% respectively. According to the distribution of the cases in table 2: IL -1 a (+4845) polymorphism was found to be most frequent in the studied subjects than IL-1β(+ 3953) . Composite IL -1 genotype, was also more frequent among individuals with fair GI score than those with good GI score, which may be considered a risk factor for severe periodontitis.

Table 3 presents the percentage of certain bacterial colonization in the oral cavity of the tested subjects. Actinobacillus actinomycetemcomitans percentage was nearly similar among good and fair score groups (7.14 % and 6.25%), whereas, Porphyrmonas gingivalis showed different percentages (7.14% in good and 18.75%in fair GI score groups). Prevotella intermedia were not isolated from good GI score group (0%) and could be isolated from one subject (6.25%) of all individuals with the fair GI score group.

In addition, microorganisms isolated using ordinary culture technique, were readily identified from FTA cards using PCR i.e. bacterial DNA was stable in the dried condition and could be recovered from the FTA cards.

2.5 Discussion & Conclusions

Microbial complexes colonizing the subgingival area, can provide a spectrum of correlations with the host, ranging from beneficial effect, in which the organisms prevent disease, to harmful effect where the organisms cause disease. At the pathogenic end of the spectrum, it is conceivable that different

Table - 2: Distribution of IL-1α (+4845) and IL-1 β(+ 3953) composite genotype among the studied subjects

Studied groups	IL-1 genotype		Total +ve IL -1 Genotype	Total -ve IL -1 Genotype
	IL-1α	IL-1β		
Good(GI)gp. group (1)	4/16(25%)	1/16(6.25%)	5/16(31.25%)	11/16(68.75%)
Fair (GI)gp. Group (2)	5 /14(35.72%)	1/14(7.14%)	6/14(42.86%)	8/14(57.14%)
Total studied sample	9/30 (30%)	2/30 (6.66 %)	11/30(36.66%)	19/30(63.33%)

Table 3: Distribution of bacterial species in the two studied groups (good and fair gingival index scores groups)

Bacterial species	Good GI score group Group (1)	Fair GI score group Group (2)
G-ve facultative rods *A. actinomycetemcomitans*	1/14(7.14%)	1/16(6.25%)
G –ve anaerobes: *Performonas gingivalis* *Prevotella intermedia*	1/14(7.14%)	3/16(18.75%)
	0/14 (0%)	1/16(6.25%)

correlations exist between pathogens. Pathogenicity could be enhanced in either an additive or a synergistic fashion, (Simonson et al., 1992).

The different members of the bacterial species possess a large number of virulence factors with a wide range of activities. These variable activities enable the organisms to colonize the oral cavity, invade periodontal tissues, evade host defenses, initiate connective tissue destruction and interfere with tissue repair, (Wilson and Henderson, 1995). Zambon (1996) added that they produce collagenase, an array of proteases (including those that destroy immunoglobulines) endotoxins, fatty acids, ammonia, and hydrogen sulphite and /or indole. These virulent enzymes induce inflammation, or form an immune reaction to waste products or to the lipopolysaccharide outer membrane component of Gram-negative organisms. This reaction leads to the change from a clinically healthy situation to a clinically inflamed state (gingivitis or periodontitis), as reported by Haffajee and Socransky(1994) and Takeuchi,et al., (2001). It is known that the nature of the host-response is affected primarily by genetic and environmental factors. The host response is essentially protective in nature, but both the under-activity (hypo-responsiveness) and the over-activity (hyper-responsiveness) aspects of the host response, can result in enhanced tissue destruction (Preshaw, et. al., 2004).

In this regard, our results showed that (36.66 %) carried the composite IL -1 genotype consisting of both IL-1α(+4845)and IL-1β(+3953). In addition, IL -1α(+4845) polymorphism was carried by 30 %, and IL-1β(+3953) polymorphism was carried by 6.66 % of our study population i.e. IL-1α (+4845) polymorphism was most frequent in our sample than IL-1β(+3953).This finding was in accordance to the finding of Gary et al., (2000) that IL-1β(+ 3953) polymorphism was much rare with only 3.3 % (10/300) in their study on Chinese population. Whereas the findings of walker et al., (2000) suggested that IL-1β(+3953) polymorphism was the most prevalent allele in the general African-American population in Western North Carolina, in a study of 37 individuals .

The prevalence of IL-1genotype positive subjects in other ethnic populations, has been reported around 30%among North European Caucasians (Engebretson et al., 1999 & Lang et al., 2000). It was 26% in a Hispanic Mexican population (Caffesse et.al., 2002). A higher percentage (38.9%) was observed by Mary et al., (2001) in European heritage. A high percentage of their presence was moderately high in our results (table 2).

Prevalence of the total IL-1 (IL- 1α and IL-1β) polymorphism in subjects of our results with fair GI score (42.86%) was more than in those with good GI (31.25%), which indicated that there is an evidence of a linkage between severity of periodontal disease and IL-1 gene expression. These results coincide with the findings reported by Di Giovine et al.,(1996) ,Salvi et al., (1998), Mary et al., (2001) and Laine et. Al., (2002).They agreed that individuals carrying this allele polymorphism are at greater risk for developing severe periodontitis.

In our study, there was no relation between the presence of A. actinomycetemcomitans and disease, since the percentage of its level was nearly the same among those groups, with fair and good GI scores groups. Albander et al., (1998) reported the same conclusion, and Ooshima etal., (2003) also reported that they considered A. actinomycetemcomitans a common member in children. In addition, the low level of isolating Porphyrmonas gingivalis and Prevotella intermedia from the studied subjects in both groups, showed that their presence was not related to the periodontal status. Schroeder and Lisgarten (1997) suggested that the continuous presence of such

large numbers of bacteria probably account for varied host-defense mechanisms against bacterial invasion and growth that can be found in the gingival tissues. Marsh and Martin (1999), supposed that the variations may exist due to many parameters influencing bacterial colonization and survival. These parameters greatly differ in the concentration of O_2, nutrients, PH, and metabolic products that cause local environmental heterogeneity. Laine etal., (2001), found that there is no relation between presence of A. actinomycetemcomitans and Porphyrmonas gingivalis in subjects with the genotype positive IL-1, Agerbaek et. al.,(2006) also stated that IL-1 gene negative subjects had a higher total bacterial load. On the other hand, Socransky et al.,(2000) concluded that the proportion of IL -1 genotype positive subjects that exhibited mean counts of specific subgingival species above selected thresholds was significantly higher than the proportion of genotype negative subjects. These findings cannot be exactly noted with our samples due to small percentage of microbial profile isolated from subjects who may be regarded as transient organisms. Saliva was used by different authors in order to isolate periodontopathic bacteria Darout et. al., (2002) and Umeda, et. al., (2004) .Some workers reported that the variations in some parameters presented in saliva, and PH may aid or suppress periodontal diseases [Maglis, et. al.,(1989), Sculley and Langley–Evans (2002) and Diab-Ladki,et al., (2003)].

Bacterial DNA was stable upon using FTA cards in the dried condition and could be analyzed by PCR. Elizabeth et. al., (2006) stated that FTA cards are a robust DNA collection method and generally produce DNA suitable for PCR, more reliably than buccal smear, as well as, analysis by nucleic acid amplification techniques, even when freezing conditions are not available.

3. CONCLUSIONS

Data of the present study provided evidence that polymorphism in genes of IL-family are associated with those suffering from periodontitis, which bring into question the usefulness of detection of the composite genotyping IL-1α (+4845) and IL-1β(+ 3953) alleles as a reliable method for determining the susceptibility of patients to periodontitis. It would also be of interest to evaluate the role of other potential candidate genes as contributors to periodontitis by studying more cases. Presence of different microorganisms may be a variable parameter in causing periodontitis. Our results recommended the use of FTA cards as it establishes a new surveillance tool for molecular techniques .It constitutes a significant improvement in the collection of samples and their transport (especially from remote areas of the world to centralized laboratories).

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