Tumor Necrosis Factor-Alpha Gene Polymorphisms in Iraqi patients with Chronic Periodontitis

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Abstract

Background: Chronic periodontitis is an inflammatory disease of bacterial etiology that results in the destruction of tooth supporting tissues, tooth mobility, and tooth loss. The inflammatory response of the periodontal tissues to infection is influenced by environmental and genetic factors. The polymorphism of tumor necrosis factor-alpha (TNF-α) has been reported to influence the expression of TNF-α, thereby playing a role in the pathogenesis of periodontitis.

Objective: To study the genotyping of tumor necrosis factor-α at position (-308) and to find out whether any associations exist between the severity of periodontitis and the gene polymorphisms.

Patients and Methods: The study groups included 50 patients with chronic periodontitis and 20 healthy controls with clinically healthy periodontium with an age range of 25-50. Everyone were analyzed for polymorphism of TNF-α gene at position (-308). Periodontal parameters used in this study were plaque index (PLI), gingival index (GI), probing pocket depth (PPD), clinical attachment level (CAL) and bleeding on probing (BOP). Five ml of venous blood was collected from all patients and controls. DNA was extracted from blood samples, and then the results of electrophoresis of polymerase chain reaction (PCR) products for this cytokine were subjected for sequencing and to locate the positions of possible mutations.

Results: The results of sequencing for the tumor necrosis factor-α gene showed higher frequency of mutations in patient samples as compared to healthy control samples. A highly significant difference was found in the frequency of mutations among the six samples (4 patients and 2 controls) p=0.0002.

Conclusion: The results of this study indicates that the (-308) polymorphism in TNF-α gene is associated with the susceptibility to chronic periodontitis.

Key words: Chronic periodontitis, genetic factors, tumor necrosis factor-α, single nucleotide polymorphism.

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Introduction

Chronic periodontitis (CP) has been defined as "an infectious disease resulting in inflammation within the supporting tissues of the teeth, progressive attachment loss, and bone loss" [1]. CP is the most prevalent type of periodontitis in adults, but it can be observed in children and adolescents in response to chronic plaque and calculus accumulation [2]. It is likely to be a complex genetic disease; several lines of evidence suggest that there is a significant genetic component associated with the resistance to CP [3]. However; Genetic and environmental factors, in additon to systemic conditions and other risk factors that associated with development of periodontitis, affect the age of onset, severity and development of periodontal disease (PD) and play a role in the predisposition to and progression of PD [4, 5]. Host susceptibility thus may largely be defined in terms of the genetic makeup of an individual or ethnic group [6]. A number of genetic polymorphisms have been studied for their association with CP in various populations, including several interleukin genes; the vitamin D receptor; the tumor necrosis factor-α (TNF-α) gene; and several HLA variants [7][8].

The TNF-α is the most important pro-inflammatory cytokine released at the site of PD that plays a prominent role in the pathogenesis of periodontitis [9]. This cytokine is involved at an early stage in the inflammatory cascade, as it is secreted from a variety of cell types [10] in response to bacterial challenge. It is implicated in PD due to its effects on bone and soft tissue metabolism. It has a high potential for increasing bone resorption and is involved in the degradation of connective tissue [11]. TNF-α can be used as a marker of tissue destruction, bone resorption, and clinical severity during active periodontitis [12].

The gene for TNF is located on the long arm of chromosome 6 within the MHC class III region at the location 6p21.3 which is a highly polymorphic region [13]. The TNF synthesis may be influenced by the presence of certain gene polymorphisms and the effect of this polymorphism on the expression of TNF-α gene was studied [14] and it had an important role in genetic regulation of the inflammatory responses, resistance, and/or susceptibility to inflammatory diseases [15]. Numerous studies have investigated genetic polymorphism in TNF–α gene as putative risk factor for periodontitis [16]. Eight single nucleotide polymorphisms (SNPs) in the promoter region have been found [17], of which -308 locus G/A is one of the best described [18]. And also reported to be linked to chronic PD susceptibility. The rare allele such as the 308 A allele of TNF-α gene is associated with high promoter and transcriptional activity as compared with the -TNF-α -308G allele and with high TNF-α production [19]. The severity of periodontitis may differ in carriers of this allele [17]. Galbraith *et al.* (1999) found that patients with PD carrying the rare allele at position 308 of the TNF-α gene had higher TNF-α production than the non-carriers [18]. This study was performed to study the genotyping of TNF-α at position (-308) and to find out whether any associations exist between the severity of periodontitis and the gene polymorphisms.

Patients and Methods

This study was approved by the Ethics Committee of College of Dentistry / Baghdad University. This study enrolled 50 Iraqi subjects suffering from CP attending the Iraqi National Blood Bank and the laboratories of Al-Yarmouk Teaching Hospital in Baghdad from May 2015 to October 2015. Additionally, 20 race-matched healthy subjects were recruited as a control group. The age range of the patients was from 30 to

50 years and that of the controls was 25-45 years. Of the 50 patients (60 %) were males and 40 % were females. The patients were subjected to a questionnaire including the name, the age, ethnicity, medical history, past dental history, type and duration of treatment, medications and smoking or alcohol drinking.

The diagnosis was done by assessing and recording at least four sites with probing pocket depth (PPD) ≥ 4 mm and clinical attachment loss of (1-2 mm) or more. Patients were classified into three groups according to the severity of clinical attachment loss [1], as follows:- Group I: Fifteen patients with mild CP (clinical attachment loss of 1-2mm), Group II: Fifteen patients with moderate CP (clinical attachment loss of 3-4mm), and Group III: Twenty patients with severe CP (clinical attachment loss of ≥5mm). Clinical oral examination was performed for all patients by the same examiner. All periodontal variables were recorded considering four surfaces (buccal/labial, lingual/palatal, mesial and distal) for all teeth except the 3rd molars, the examination was done using Michigan O probe with William's markings at 1,2,3,5,7,8,9 and 10 mm. Periodontal parameters used in this study were plaque index (PLI), gingival index (GI), probing pocket depth (PPD), clinical attachment level (CAL) and bleeding on probing (BOP). A blunt periodontal probe inserted to the bottom of the periodontal pocket/sulcus for four surfaces of each tooth and is moved gently along the tooth (root) surface. If bleeding occurred within 30 seconds after probing, the site was given positive score (1), and a negative score (0) for the non-bleeding site [1].

Five ml of venous blood was withdrawn from each subject under aseptic technique and transferred into EDTA tube (1.5 mg / ml) and kept at –70 °C for the TNF-α analysis. DNA was extracted from blood samples by using DNA IQ™ Casework Pro Kit for Maxwell® 16 and Casework extraction kit (Promega /USA). The genomic DNA extraction was done in the laboratories of the Iraqi Medical Legal Institute/ Ministry of health. All DNA was stored at -20°C until tested. Genotyping procedures were conducted in the Laboratory of Musayyib Bridge Office. Fifty subjects were only selected for the TNF-α polymorphism study 35 with CP and 15 healthy individuals. The primers were designed by extracting the gene sequence for TNF-α from National Center Biotechnology (NCBI) www.ncbi.nlm.nih.gov and the program primer 3 plus http://www.bioinformatics.nl/cgi-bin was used to design primers that covered most parts of the genes to detect the possible mutations that present in the diseased cases to compare them with the normal sequences. DNA samples were amplified using GCCAAGACTGAAACCAGCAT (forward primer) and AACAAGCACCGCCTGGAG (reverse primer).

Polymerase chain reaction (PCR) was performed using Bioneer's AccuPower ® PCR PreMix kit. AccuPower ® PCR PreMix is a new, rapid and ready-to-use PCR reagent optimized for more accurate PCR amplification.It contains DNA Polymerase, dNTPs,a tracking dye and reaction buffer in premixed format, freeze-dried into a pellet. The patented chemical stabilizer of this product enables to maintain the activity of pre mixture for over a month even when stored at room temperature (25°C), and over 2 years in a -20°C freezer. The samples were put in MyGenie™ 96/384 Gradient Thermal Block in order to start the PCR amplification performance. Thirty five cycles of incubation at 95°C for 30 sec (denaturation), 59ºC for 45 sec (annealing) and 72°C for 1 min (extension) then 1cycle 72°C for 5 min (final extension) were the steps involved for DNA amplification. Then the samples were loaded

on agarose gel to perform electrophoresis using AgaroPower™ which is an agarose gel electrophoresis kit.

Following electrophoresis, the separated DNA were visualized under ultraviolet light and the images obtained were stored digitally for later analysis. After the emergence of the gene fragments in the PCR with the expected size, the results were sent to Macrogen Company for the detection of the nucleotide sequences of these fragments. The sequences analyzed by using Basic Local Alignment Search Tool Program (BLAST) which is available at the NCBI information site on this website http://blast.ncbi.nlm.nih.gov/Blast.cgi.

Statistical analysis

Collected data then submitted to both descriptive and inferential statistical analysis using SPSS v.21 program for windows. Comparing the frequency of mutations and the distribution of the mutations according to types compare among groups was done by chi-square test and Fisher's exact test. The significance of these differences was assessed by fisher's exact probability (P). In the statistical evaluation, the following levels of significance were used: $P > 0.05$ non-significant (NS), $0.05 \geq P > 0.01$ significant (S) and $P \leq 0.01$ highly significance (HS).

Results

The demographic variables and clinical parameters (PLI and GI) of the 70 subjects enrolled in this study are illustrated in table (1) which shown that there was non-significant difference between both study groups regarding age and gender. The table also shown that the mean value of PLI and GI were significantly higher ($P< 0.0001$) in patients group when compared to controls group.

Table (1): Demographic variables and clinical parameters (PLI and GI) in the study and control groups.

			Control No.=20	Chronic Periodontitis No.=50	P-value
Age	Range		25-45	30-50	0.1243^{NS}
	Mean±SD		34.05±6.228	36.08±4.323	
Gender	Male	No. (%)	10 (50%)	30 (60%)	0.5^{NS}
	Female	No. (%)	10 (50%)	20 (40%)	
PLI*	Mean±SD		0.444±0.429	1.819±0.366	$< 0.0001^{**}$
GI*	Mean±SD		0.372±0.358	1.634±0.383	$< 0.0001^{**}$

**: Highly statistical significant (<0.001). NS: None statistical significance (p>0.05). No= Number.
(PLI): plaque index, (GI): gingival index.

Regarding the CP subgroups, the number of sites examined for mild CP was (1524), for moderate CP was (1476) and for severe CP was (1832). The percentage of non-bleeding and bleeding sites in the mild CP subgroup were (51.71% and 48.29%) respectively, while in the moderate CP subgroup were (40.92% and 59.08%) respectively and in the severe CP subgroup were (19.71% and 80.30%) respectively. Chi-square test revealed a highly significant difference among the three subgroups as shown in (table-2).

Table (2): The number and percentage distribution of sites according to the presence or absence of BOP of the CP subgroups.

CP subgroups		BOP		Chi	df	p-value
		Score 0	Score 1			
Mild No. =1524	No. (%)	788 (51.71%)	736 (48.29%)	388.9	5	< 0.0001**
Moderate No. =1476	No. (%)	604 (40.92%)	872 (59.08 %)			
Severe No. = 1832	No. (%)	361 (19.71%)	1471 (80.30%)			

Considering the PIL, GI, PPD and CAL among the three subgroups of chronic periodontitis, a significant difference was observed p-value <0.0001, as seen in (table-3).

Table (3): The mean values and SD of the PLI, GI, PPD and CAL of the CP subgroups.

		Mild No. = 15	Moderate No. = 15	Severe No. = 20	F-test	p-value
PLI	Mean± SD	1.551±0.375	1.790±0.321	2.043±0.236	11.03	0.0001**
GI	Mean± SD	1.433±0.377	1.569±0.328	1.833±0.344	5.98	0.0049*
PPD	Mean± SD	1.519±0.317	3.313±0.283	4.744±0.390	387	<0.0001**
CAL	Mean± SD	1.8134±0.164	3.680±0.2	5.941±0.474	669.7	<0.0001**

**: Highly statistical significant (<0.001).
*: statistical significance (p<0.05).

The PCR products electrophoresis for the 50 subjects (35 with CP and 15 controls) using single round primers showed the absence of bands in only two (5.71%) samples of the patients group and one (6.67%) sample of the control group, (figure-1).

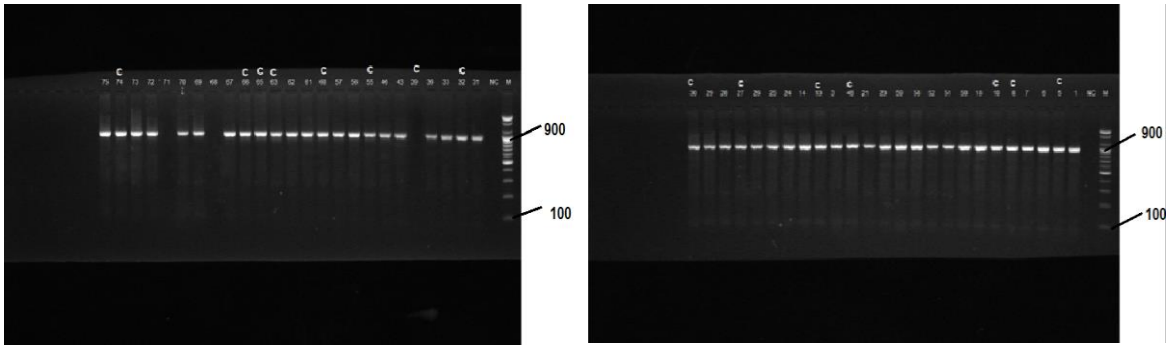

Figure (1): Electrophoresis of PCR products for TNF-α in both study group, M: marker size100 bp, C: control samples, the other numbers are patients samples.

Six samples (4 patients and 2 controls) were selected and sent to Macrogen Company for sequencing of TNF-α gene and locating the positions of possible mutations. The sequences of these samples were compared with the source sequence using Blast program. The results of the TNF-α gene sequencing demonstrated different mutations in the analyzed samples. These mutations were deletion, addition, and substitution mutations (transversion and transition). The mutations, their types, positions and their frequency are illustrated in (table-4).

Table (4): The mean, the numbers of mutations and their types for the TNF-α gene.

NO. of samples	Types of mutations	Mutations	Positions of mutations	Frequency of mutations	
Sample1	Substitution (Transversion)	A→C	31544973	1	11
		G→T	31545121-31545499	2	
		T→A	31545150-31545586	2	
		A→T	31545522	1	
		C→A	31545616	1	
	Substitution (Transition)	G→A	31545064-31545586	2	
		T→C	31545151	1	
	Deletion	C→×	31545626	1	
Sample 2	Substitution (Transversion)	C→A	31545193-31545632	2	15
		A→C	31545196	1	
		C→G	31545284-31545356	2	
		T→G	31545289-31545603	2	
		G→C	31545375	1	
		A→T	31545383-31545522	2	
		G→T	31545499	1	
	Substitution (Transition)	A→G	31545194	1	
		C→T	31545195	1	
		G→A	31545586	1	
	Deletion	C→×	31545626	1	
Sample 3	Substitution (Transversion)	T→A	31545512-31545598-31545603	3	16
		C→A	31545530	1	
		C→G	31545561	1	
		A→T	31545229	1	
		G→T	31545243	1	
	Substitution (Transition)	A→G	31545444-31545547	2	
		T→C	31545445	1	
		G→A	31545146-31545248-31545249	3	
		C→T	31545193-31545211	2	
	Addition	A×→	31545147	1	
Sample 4	Substitution (Transversion)	G→C	31545515-31545521-31545526-31545644	4	13
		C→A	31545540-31545614-31545570-31545600-31545618	5	
		G→T	31545499	1	
	Substitution(Transition)	C→T	31545584-31545588	2	
		G→A	31545619	1	
Sample 5 (control)	Substitution(Transversion)	A→C	31545363	1	3
		C→A	31545437	1	
	Substitution(Transition)	C→T	31545359	1	
Sample 6 (control)	Substitution(Transversion)	A→C	31545270	1	1
Total				59	

The distribution of mutations according to their types of the control and CP samples is illustrated in (table-6) which revealed a highly significant differences between the control and CP samples in substitution mutations P<0.0001 and non-significant differences in deletion or addition mutations (P=0.333 and 1.0000) respectively.

Table (5): The number and percentage distribution of mutations according to their types of the control and CP samples for the TNF-α gene.

		Chronic Periodontitis Samples		Control Samples		
Types of mutations	Frequency of mutations	No.	%	No.	%	P (Fisher's exact)
Substitution (Transversion)	38	35	92.11	3	8.57	<0.0001**
Substitution (Transition)	18	17	94.44	1	5.56	<0.0001**
Deletion	2	2	100	-	0	0.333 NS
Addition	1	1	100	-	0	1.0000 NS

Discussion

Genetic polymorphism in cytokine genes is regarded as a promising factor in inducing PD [20]. In periodontitis, the high variability of cytokine levels and the low frequency of this detection may contribute to the presence and/or severity of the disease [21]. Allelic variations in cytokine genes and factors regulating their expression results in phenotypic differences in cytokine response between individuals, which can be important in disease susceptibility and progression [22].

TNF-α -308 was chosen for gene polymorphisms analyzing because of their known biological significance in the pathogenesis of inflammatory and infectious diseases including periodontitis. This study is the first in Iraq to investigate the TNF-α genotype and sequencing for the detection of the nucleotide sequences of the gene fragments in Iraqi patients with CP.

Different types of mutations were observed such as deletion, addition, and substitution mutations (transversion and transition). The frequency of mutations for patients samples were 18.64, 25.42, 27.12, 22.03 respectively and for control samples were only 5.09 and 1.70 respectively. A Significant difference among the six samples with P=0.0002 were observed. The frequency of mutations in the samples according to their types showed high significant difference between control and CP for transversion and transition mutations, while non-significant differences between control and CP for both deletion and addition mutations. The deletion and addition mutations were observed only in CP samples.

According to these results, the TNF-α-308 gene polymorphism may associated with CP. These results was in agreement with other clinical studies considered the polymorphisms in TNF-α to be risk factors and associated with CP [23][24]. In one study conducted by Settin and colleagues (2006) on TNF-α (-308 A>G) polymorphism within an Egyptian population, greater frequency of GG homozygous genotype was observed in the patient group compared with the control group [25]. Furthermore, Ianni *et al.*, 2013 also showed that the GG genotype of TNF- α is a genetic risk factor for periodontitis (OR = 2.463) [26]. In study included 30 patients with CP, and 10 persons with healthy periodontium, the assessment of

TNF-α (G-308A) revealed that genotype GG was associated with CP in Bulgarian individuals and supposed that the G allele may play an important role in the development and progression of periodontal disease in this population [27].

At variance with the present results, Galbraith *et al.* (1998) determined TNF-α genotypes for three bi-allelic polymorphisms (-238, -308 or +252 gene polymorphisms) in Caucasian population and reported 19% frequency of A allele in TNF-α (-308) region in patients. They found no differences between patients and controls or between patients with different disease severity [28]. A subsequent study in mixed population (Caucasian 81% non-Caucasian 19%) to investigate four bi-allelic polymorphisms in the TNF- α gene which were all transitions from G to A, 3 in the promoter positions: – 376, – 308, – 238 and at position + 489, also found no significant associations between a different series of four TNF-α gene polymorphisms and periodontitis patients [29]. In addition, Moreira et al. (2009), also found no association of TNF-α gene polymorphisms with periodontitis in Brazilians population [15]. While other Brazilian showed that, the frequency of A allele in control groups was greater than periodontitis group. However, statistically no significant difference was observed between them [19]. In a meta-analysis by Nikolopoulos et al. in (2008) the great majority of studies were carried out in Caucasian population and no association of TNF-α (-308) was found with CP [30]. Another meta-analysis for CP showed lack of association of A allele with CP [31]. On the other hand, Flowaczny and colleagues (2004), showed that the frequency of allele between patient and normal subjects was similar, and there was no correlation between this polymorphism and periodontitis disease [32]. These inconsistent data may be partly explained by the different clinical methodological and statistical settings, as well as by variances in TNF-α genotype distribution within distinct races and/or populations [33].

In conclusion, the present study shows that the polymorphism in the locus-308 of TNF-α gene could be a risk factor for CP and might be associated with disease susceptibility in the Iraqi population. The identification of genetic markers for susceptibility to periodontitis will allow an early identification of individuals with high risk and could eventually help through individualized forms of therapy.

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