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New term (SNPs-genotypes combination principal) gives better understanding about effect of SNPs on the concentration of IL-1β and TNFα in clinical samples of periodontitis

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ABSTRACT

Aim: this study aim to evaluate the concentration of IL-1β in patients with periodontitis by using new term (SNPs-genotypes combination principal) in comparison with healthy individual in saliva and serum.

Materials and methods: A patients –healthy samples was conducted between February 2015- May 2015 and it included 100 Iraqi patients group with periodontitis and 30 number of healthy population group from Babylon Province and it was conducted in Faculty of Dentistry**.** Three types of samples have been taken from subjects in study pocket samples for bacterial identification, blood samples for DNA extraction and for serum isolation and saliva samples for evaluation of concentration of IL-1β. Identification of bacteria has been done by media and primers methods. PCR-RFLP and multiplex PCR have been done for all samples, genotype and allele frequency have been calculated, concentration of IL-1β in saliva and serum for both groups has been estimated according to SNPs-genotypes combination principal

Results: The most bacteria isolated in this study were *P. gingivalis* 65 in patient and 5 in healthy control, *P. intermedia* 41 in patient and 3 in healthy control, *T. forsythensis* 33 in patient and 1 in healthy control, *Peptostreptococcus spp.* 100 in patient and 30 in healthy control, *A. actinomycetemcomitans*, 47 in patient and 9 in healthy control *Streptococcus spp.* 44 in patient and 22 in healthy control, *Staphylococcus aureus* 3 in patient and 52 in healthy control. PCR-RFLP results of IL-1β +3953 gene polymorphism showed that patients with genotypes CC were more affected by periodontitis in 2.68 time than patients with genotypes TT while patients with genotypes CT were more affected by periodontitis in 1.05 time compare TT patients. While IL1-β -511 gene polymorphism showed that the patients with genotype CC and CT affected by periodontitis at 1.85 and 1.64 time respectively compare with TT genotype patients. Total concentration of IL-1β in saliva of patients and healthy was 46.238±40.013 pg/ml and 12.44±5.48 pg/ml respectively *P*= 0.145 this value was not significant because of the high value of stander deviation However this problem was solved by using SNPs-genotypes combination principal. We found that higher concentration of IL-1β in saliva was in patients with genotypes CT-CT (111.203±21.595 pg/ml) compare with healthy (20.593±3.78 pg/ml) P=0.000. While the lower concentration of IL-1β in saliva was in patients and healthy with genotypes CC-CC (0.00 pg/ml). While the higher concentration of IL-1β in serum of patients with genotypes CC-TT was (370.863±25.99 pg/ml) compare with healthy (91.763±3.9 pg/ml) P=0.000, and the lower concentration was (0.00 pg/ml) for genotypes TT-CC in patients and healthy control.

Conclusion: We concluded that SNPs-genotypes combination principal helped us to understand the changes in the concentration of IL-1β in periodontitis first conclusion. And the second conclusion in the present study we showed that the polymorphism in the loci +3954 and -511 of IL1B gene could be a risk factor for chronic periodontitis in Iraqi population.

KEY WORDS: Periodontitis, SNPs-genotypes combination principal, polymorphism, IL-1β gene.

1. INTRODUCTION

SNPs-genotypes combination principal is used to know concentration of gene product of gene that have more than single SNPs in their sequence in combination with genotypes **or** its used to know the effect of gene's SNPs on concentration of the gene product in clinical samples. For example when we test the effect of two SNPs on the concentration of IL-1β, it will give us better understanding than single SNP on the same gene. If the subject in study has or has no single SNP ,the expected forms of genotypes will be as following TT, TC and CC. we thought each form of genotype could give different concentration of gene product in clinical samples. While if the subject in study has two SNPs, the expected forms of genotypes will be as following TT-TT, TT-CT, TT-CC CT-TT, CT-CT, CT-CC, CC-TT, CC-CT, CC-CC (according to SNPs-genotypes combination principal).

2. MATERIALS AND METHODS

Study population: The study population included hundred consecutive patients with chronic periodontitis. All patients 25-65 years old showed clinical evidence of alveolar loss and periodontal pockets and they were diagnosed by radiograph. 30 healthy subjects 20-60 years old without clinical signs of periodontal disease were also selected. None of the periodontal patients or healthy subjects had received antibiotics for three months prior to sample collection.

Pocket swap specimens: Specimens were collected with complete aseptic method with the assistance of dentists. The site of specimen's collection was isolated with cotton rolls. For single sites, three sterile paper points (30-40#) were inserted to the bottom of the pocket for a 20-30 second period, the pocket depth is equal to or exceeding 3.5- 7

mm. and then transferred to Robertson's cooked meat medium. While the healthy subject's specimens were collected from sub gingival material with sterile paper points and transferred to Robertson's cooked meat media (Joshi and Vandana, 2007). The medium with specimens directly transported to the laboratory in 1-2 hours. The bacteria were diagnosed according to (Macfaddin, 2000) and by multiplex PCR.

Blood samples: Five ml of blood were obtained from each subject in study by vein puncture, 2 ml was put into EDTA tubes and the remaining 3 ml pushed slowly into disposable tubes containing separating gel. Blood in the EDTA tubes was stored in -40˚C in order to be used later in genetic part of the study, while blood in the gel containing tubes was allowed to clot at room temperature for 30 minutes and then centrifuged at 2000 ×g for approximately 15 minutes then the serum was obtained and stored at -20˚C until analysis.

Saliva specimens: Two common methods of saliva collection are: the passive drool technique, and the absorbent device technique (Vining, 1983). In this study we were used the passive drool technique, saliva from subjects were collected with complete aseptic precautions to sterile tube and it maintained in freeze (-20C°) until to period of need.

Extraction of DNA: DNA extracted from bacteria and WBCs was occurred by using DNA extraction kits (Favorgen).

Diagnosis of bacteria by specific primers: The extracted DNA from bacteria was subjected to multiplex PCR in two stages (PCR I and PCR II) using species specific primers (Table.1). The PCR conditions were comprised of initial denaturation of 95^0C for 5min, 35 cycles of 95^0C for 30 sec, 60^0C for 30sec (PCR I), 55^0C for 30 sec (PCR II), and 72^0C for 1 minute followed by a final extension of 72^0C for 7mins. The amplicons were visualized on 2% agarose incorporated with 1% ethidium bromide under UV illuminator (Tellapragada, 2014).

Table.1. Specific primers to diagnosis bacteria for multiplex PCR

Bacteria		Primers sequences	A.T (^{0}C)	Amplicon size (bp)
P.g*	F	5'-TGTAGATGACTGATGGTGAAAACC-3'	60	197
	R	5'-ACGTCATCCCCACCTTCCTC-3'		
P.i*	F	5'-TTTGTTGGGGAGTAAAGCGGG-3'	55	575
	R	5'-TCAACATCTCTGTATCCTGCGT-3'		
T.f*	F	5'-GCGTATGTAACCTGCCCGCA-3	60	641
	R	5'-TGCTTCAGTGTCAGTTATACCT-3		
A.a*	F	5'AGAGTTTGATCCTGGCTCAG3'	60	593
	R	5'CACTTAAAGGTCCGCCTACGTGCC3'		

*P.g, Pi, Tf and A.a: *Porphyromonas gingivalis, Prevotella intermedia, Tannerella forsythia, Aggregatibacter actinomycetemcomitans* respectively, A.T annealing temperature.

PCR Amplification for Interluekin-1beta (IL-1β +3953): For Interluekin-1beta (IL-1β +3953) genotyping, a set of primers as following forward primer: 5´-GTTGTCATCAGACTTTGACC-3´, Reverse primer: 5´-TTCAGTTCATATGGACCAGA -3´ (Santtila, 1998). PCR conditions were initial denaturation 97 for 2 min, followed by 32 cycles of following conditions DNA denaturation 97 for 1 min, primer annealing 55 for 1 min, and finally extension at 74 for 1 min, then followed by final extension 72 for 10 min. Taq1 (Biolabs) restriction enzyme has been used for digested the PCR product (Santtila, 1998). Incubate at the enzyme's optimum temperature (37C°) for 1hour, and then PCR product was loaded to 9% PAGE for 3 hours.

PCR Amplification for Interluekin-1beta (IL-1β -511): Conditions of the PCR and annealing temperature was determined as reported in Chaudhary (2008). A set of primers as following forward primer 5'-TGGCATTGATCTGGTTCATC-3', and Reverse primer 5'-GTTTAGGAATCTTCCCACTT-3'. PCR conditions were initial denaturation 95 for 2 min, followed by 35 cycles of following conditions DNA denaturation 95 for 1 min, primer annealing 54 for 1 min, and finally extension at 74 for 1 min, then followed by final extension 74 for 10 min and then PCR product was loaded to 1% agaros (0.5x) TBE buffer (40 min at 75v) and the bands visualized after staining with ethidium bromide under UV light. Ava1 (Eurex) restriction enzyme has been used for digested the PCR product. Incubate at the enzyme's optimum temperature (37^0C) for 1hour and then PCR product was loaded to 2% agarose for 2 hours.

Interluekin-1beta concentration in saliva and serum: Interluekin-1beta concentration and TNF-α were measured by Enzyme-Linked Immunosorbent Assay (ELISA) Kits (elabscience-china) for serum and saliva.

Statistical Analysis: All statistical analysis was performed by using SPSS 19 version. Data were expressed as (mean ± SD). The normality of the distribution of all variables was assessed by T independent test. Chi-square (χ^2) test have been used to determine the significant difference between the groups frequencies. Genetic analysis was performed using Hardy Weinberg. *P* values less than (0.05) is considered significant and less than (0.01) is considered highly significant.

3. RESULTS AND DISCUSSION

The types of bacteria that isolated in this study were listed in the table.2 by using two methods. High number of bacteria in patients may be belong to the suitable environment that produced in periodontal pocket ,that mean the destruction of periodontal ligament, and alveolar bone lead to bleeding of gingiva and formation of pocket and these condition makes suitable place for bacterial growth specially anaerobic bacteria, The leading cause of periodontitis is the deepening of gingival pockets due to inflammation brought on by the presence of undisturbed plaque (Van, 1996) while the number of bacteria in healthy people was low and this is predictable result because there is no suitable environment. Darveau (2010), showed that the number of bacteria in pocket in healthy people was 10^2-10^3 CFU , and most of organisms were gram positive , such as *Actinomyces* spp. and *Streptococci* spp., while other species such as *Porphromonas* spp. And *Provetella* spp. was in low levels (Ximenez-Fyvie, 2000).

Table.2. Isolation of microorganism from study groups by media method and multiplex PCR method.

Microorganisms	**Subjects N=130**			
	Healthy N=30		**Patients N=100**	
1. Anaerobic	**Media**	**Primers**	**Media**	**Primers**
P. gingivalis	5	24	65	87
P. intermedia	3	12	41	79
T. forsythensis	1	15	33	83
Peptostreptococcus spp.	30	Not use	100	Not use
2. Aerobic and facultative anaerobic				
Actinomycetam comitans	9	22	47	66
Streptococcus spp.	22	Not use	44	Not use
S. aureus	3	Not use	52	Not use
Candida albicans	non	Not use	22	Not use
3. Other species	yes	-	yes	-

From table.3 multiplex PCR method was more effective in isolation of some oral bacteria than media methods. In both methods the most bacteria isolated were *P. gingivalis*, *P. intermedia*, *T. forsythensis*, *Peptostreptococcus spp.*, *A. actinomycetemcomitans*, *Streptococcus spp*, and *Staphylococcus aureus* in addition to *Candida albicans*. Many studies were indicated that the presence of periodontopathogens, *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola* (called the red complex) and *Aggregatibacter actinomycetemcomitans*, considered the major etiologic factors in periodontitis (Feng and Weinberg**,** 2006).

Interluekin-1beta3953 (IL-1β +3953): PCR product of IL-1β +3953 gene amplification was 249 bp, figure.1.

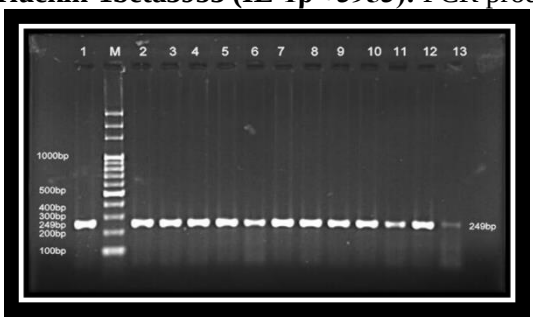

Figure.1. electrophoresis pattern of PCR product of IL-1β +3953 gene, the optimum annealing temperature was 55^{0}C

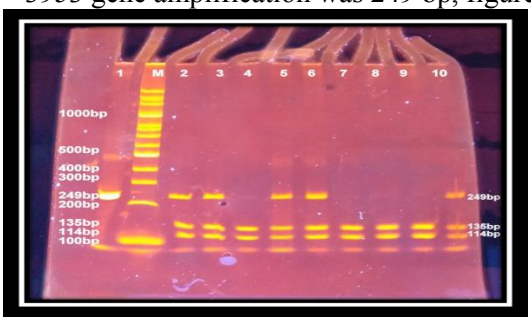

Figure.2. Electrophoresis pattern of IL-1β +3953 PCR-RFLP by 4% PAGE gel for PCR product (249bp) with restriction enzyme TaqI. Lane M DNA ladder Lane

(1): PCR product, Lane (2, 3, 5, 6, 10) heterozygote (CT) genotype, Lane (4, 7, 8, 9) homozygote (CC) genotype. Ladder. Lane (1): PCR product. Lane (2, 3, 5, 6, 10) heterozygote (CT) genotype, Lane (4, 7, 8, 9) homozygote (CC) genotype.

Genotype of IL-1β +3953 gene polymorphism with Allele frequency between the two groups healthy and patient were detected using PCR-RFLP technique.

Results from figure (2) show the genotype of IL-1β +3953 gene in the two study groups healthy and patients (the healthy were 30 samples while the patients were 100 samples), TT homozygote represented (249 bp), CT heterozygote represented (249bp, 114bp and 135bp) and CC homozygote represented (135bp and 114 bp).

Genotype frequencies of TT, CT, and CC of IL-1β +3953 gene polymorphism were 11 (36.67%), 14 (46.67%) and 5(16.66%) in the healthy group, while 17 (17%), 48 (48%) and 35 (35%) in the patient group.

Table.3.Genotype of IL-1β +3953 gene polymorphism with Allele frequency of periodontitis patients

Genotype IL-1β +3953	Healthy	Patients	χ2 (1,N=130)	P value	OR CI 95%
TT	11 (36.67%)	17 (17%)	6.673	0.036	0.35
CT	14 (46.67%)	48 (48%)	6.555	0.038	1.05
CC	5 (16.66)	35 (35%)	6.426	0.11	2.69
Total number	30	100			
Allele frequency					
Allele		Control		Patient	
T		0.6 (60%)		0.41 (41%)	
C		0.4 (40%)		0.59 (59%)	

Results were showed that the *P-value* of the genotypes frequency of IL-1β +3953 gene in the two study groups were (0.0036) which is less than 0.05. So it were significant ($p \leq 0.05$). Data of allele frequencies of point mutations on IL-1β +3953 gene in two study groups were presented in Table.3. For healthy group, allele frequency of (T) variant allele was 0.6 (60%), but (C) allele variant frequency was 0.4 (40%) according to Hardy- Weinberg equation. While for patient group , allele frequency of (T) variant allele was 0.41 (41%), but (C) allele variant frequency was 0.59 (59%) according to Hardy- Weinberg equation, while the OR of patients showed that the patients with CC genotypes were more effective by periodontitis than healthy people by 2.69 .

Interluekin-1beta -511 (IL_1β -511) genotyping PCR: PCR product of IL_1β -511 gene amplification was 304bp figure.3.

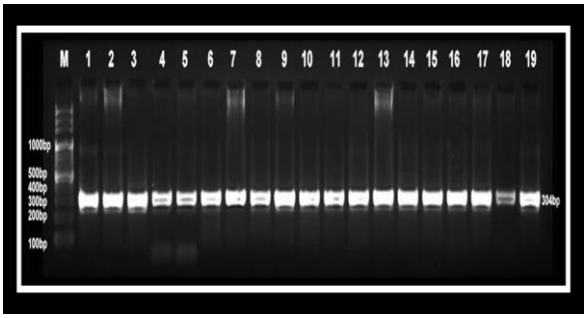

Figure.3. electrophoresis pattern of PCR product of IL_1β -511 gene, the optimum annealing temperature was 54^{0}C.

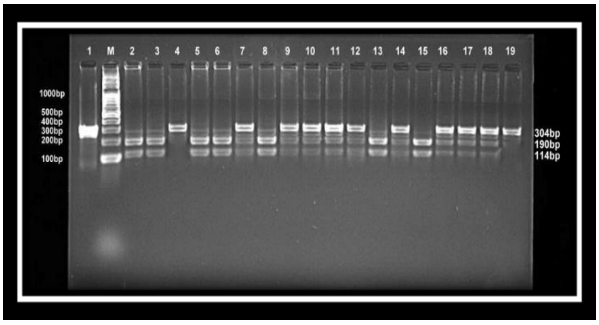

Figure.4. Electrophoresis pattern of PCR-RFLP by 2% agarose gel for PCR product (304bp) with restriction enzyme AvaI. Lane M DNA ladder.

Lane (1): PCR product. Lane (7, 9, 10, 11, 12, 14, 16, 17, 18) heterozygote (CT) genotype, Lane (2, 3, 5, 6, 8, 13, 15) homozygote (CC) genotype. Lane: (4, 19) homozygote (TT) genotype**.**

Genotypes of IL_1β -511 gene polymorphism with allele frequency between the study groups were detected using PCR-RFLP technique. Results from figure (4) showed that the genotypes of IL_1β -511 gene in the two study groups healthy and patients were TT homozygote which was represented (304bp), CT heterozygote was represented (304bp, 190bp and 114bp) and CC homozygote was represented (114bp and 190bp)**.**

Data of allele frequencies of point mutations on IL_1β -511 gene in two study groups were presented in table (4). For healthy groups the allele frequency of (T) variant allele was 0.6(60%), but (C) allele variant frequency was 0.4(40%) according to Hardy- Weinberg equation. While for patient groups the allele frequency of (T) variant allele was 0.43(43%), but (C) allele variant frequency was 0.57(57%) according to Hardy- Weinberg equation while the O.R. of each genotype were as showed in table.5**.** In this study, we were detected a polymorphism at locus +3954 and -511 of IL1B gene in a samples of the Iraqi population suffering or not from periodontal disease and we were found an association between the polymorphism of IL-1β and chronic periodontitis. These results were in accordance with the results that found by (Huang & Zhang, 2004). IL-1β has pro-inflammatory properties and it is found in increased levels in diseased periodontal tissues. Furthermore, the polymorphisms in the IL-1 genes have direct functional role by altering the gene transcription or the protein production and it has also been associated with other complex diseases (Loos, 2005; Shapira, 2005).

Table.4. Genotype of IL-1β - 511 gene polymorphism with Allele frequency of periodontitis patients

Genotype IL-1β -511	Healthy	Patients	χ2 (1,N=130)	P value	OR CI 95%
TT	11 (36.67%)	14 (14%)	7.791	0.02	0.28
CT	14 (46.67%)	59 (59%)	7.055	0.029	1.64
CC	5 (16.66%)	27 (27%)	5.725	0.17	1.85
Total number	30	100			
Allele frequency					
Allele		Control		Patient	
T		0.6 (60%)		0.43 (43%)	
C		0.4 (40%)		0.57 (57%)	

The strongest evidence for an association between IL-1 polymorphisms and periodontitis was provided by the study of Kornman (1997). However, clinical studies investigating and the functional role of the genotype have shown different results. The polymorphisms in the IL-1 genes have direct functional role by altering the gene transcription or the protein synthesis and it has also been associated with other complex diseases (Loos, 2005; Shapira, 2005). Some study indicated that the effect of SNPs could increase or decrease the level of gene product (protein synthesis). However there is no study interpreted the high or low level of gene product very well. We did not find any study about serum and saliva levels of IL-1β. Our results may be the first report on serum and saliva levels of IL-1β in patients with periodontitis. The relationship between the genotype and the IL-1β levels in periodontitis is not yet fully understood. But we here gave a small idea about this relationship by estimation the concentration of IL-1β in saliva and serum according to SNPs-genotypes combination principal. We were found that the concentration of IL-1β was changed according to the number of SNPs in their genes. The concentrations of IL-1β in saliva for all patients were risen in all genotypes compare with healthy people. Total concentration of IL-1β in saliva of patients and healthy was 46.238±40.013 pg/ml and 12.44±5.48 pg/ml respectively *P*= 0.145 this value was not significant because of the high value of stander deviation However this problem was solved by using SNPs-genotypes combination principal. We found that higher concentration of IL-1β in saliva was in patients with genotypes CT-CT (111.203±21.595 pg/ml) compare with healthy (20.593±3.78 pg/ml) *P*=0.000. While the lower concentration of IL-1β in saliva was in patients and healthy with genotypes CC-CC (0.00 pg/ml). While the higher concentration of IL-1β in serum of patients with genotypes CC-TT was (370.863±25.99 pg/ml) compare with healthy (91.763±3.9 pg/ml) *P*=0.000, and the lower concentration was (0.00 pg/ml) for genotypes TT-CC in patients and healthy control table 5 and 6.

Table.5. Concentration of IL-1β in saliva of study groups of periodontitis according to SNPs-genotype combination principal

Genotypes IL-1β -511 - +3953	Subjects N=130				P value
	Mean± SD Pg/ml				
	Healthy	n=30	Patients	n=100	
TT-TT	0.00	4	10.20±1.088	4	0.000
TT-CT	1.711±1.01	6	7.208±1.924	5	0.000
TT-CC	29.72*	1	31.944±1.391	5	0.218
CT-TT	0.000	4	98.49±21.76	12	0.000
CT-CT	20.593±3.78	8	111.203±21.595	32	0.000
CT-CC	19.55±0.565	2	27.847±14.874	15	0.454
CC-TT	28.313±5.533	3	28.313*	1	0.53
CC-CT	-	0	100.94±22.16	11	-
CC-CC	0.00	2	0.00	15	-
Total mean± SD	12.44±5.48	-	46.238±20.013	-	0.045
Total n		30		100	

*only one subject presents with these genotypes. (_) no subject with these genotypes.

Table.6. Concentration of IL-1β in serum of study groups of periodontitis according to SNPs-genotype combination principal

Genotypes IL-1β -511 - +3953	Subjects N=130				P value
	Mean± SD Pg/ml				
	Healthy	n=30	Patients	n=100	
TT-TT	2.15±0.37	4	36.94±9.22	4	0.000
TT-CT	2.52±0.68	6	84.66±23	5	0.004
TT-CC	0.00	1	0.00	5	-
CT-TT	30.51±43.14	4	64.341±184.77	12	0.81
CT-CT	99.257± 37.56	8	114.374±167.908	32	0.645
CT-CC	0.00	2	46.731±10.00	15	0.000
CC-TT	91.763±3.9	3	370.863±25.99	1	0.000
CC-CT	_	0	99.543±8.221	11	-
CC-CC	108.36±11.879	2	107.667±44.97	15	0.983
Total mean± SD	40.43±33.238	-	91.73±60.562	-	0.021
Total n		30		100	

*only one subject presents with these genotypes. (-) no subject with these genotypes.

4. CONCLUSION

The study concluded that SNPs-genotypes combination principal helped us to understand the changes in the concentration of IL-1β in periodontitis first conclusion. And the second conclusion in the present study we showed

that the polymorphism in the loci +3954 and -511 of IL-1β gene could be a risk factor for chronic periodontitis in Iraqi population.

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