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MOLECULAR STUDY OF INTERLEUKIN-6 (IL-6) AMONG PERIODONTITIS IN BABYLON PROVINCE

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ABSTRACT : Periodontitis considered as one of the main inflammatory disease that affect teeth supporting tissue. Interleukin-6 (IL-6) is biologically active minor protein molecules known as cytokines. Interleukin-6 (IL-6) is a multifunctional cytokine playing a central role in inflammation and tissue injury. The aim of studywas to investigate the association between the Interleukin-6 (IL-6) _174 gene polymorphism and susceptibility to periodontitis and body mass index (BMI) association with periodontitis in Babylon province. The total subjects of the present study is 100, divided into 2 groups; 60 subjects with periodontitis and 40 subjects healthy controls, Un-stimulated salivary sample was taken from each subject and was investigated for thepresence of Interleukin-6 (IL-6) by using Polymerase chain reaction (PCR) sequencing technique.Observed the difference is not important in the distribution of Interleukin-6 (IL-6) rs1800796 genotypes between periodontitis subjects and healthysubjects in this study also the BMI didn't association with periodontitis. The results suggests that Interleukin-6 (IL-6) rs1800796 gene polymorphism wasn't a putative risk factor for periodontitis and didn't associated with periodontitis

Key words : Saliva, interleukin-6(IL-6) rs1800796, BMI, periodontitis.

INTRODUCTION

Periodontal diseases are progressive and destructive inflammatory conditions that involve tooth supporting structures like gingiva, periodontal ligament, cementum, and alveolar bone. The term of periodontal disease refers to gingivitis and periodontitis. Gingivitis is a reversible inflammation of the gingiva, While periodontitis is defined as an inflammatory disease initiated by dental plaque biofilm and perpetuated by a deregulated immune response Via several biological mechanisms and it consider irreversible inflammation (Batool *et al*, 2018; Suvan *et al*, 2011). Periodontitis is destructive inflammatory disease of the tooth supporting tissues induced by an opportunistic mixed infection. Periodontitis affects of the adult population and represents the major cause of tooth loss in adults, Periodontitis is a multifactorial inflammatory illness and both environmental and genetic factors play a major role in the progression of the disease with consequent tissue destruction around the dental roots and alveolar bone is associated with systemic alterations (da Silva *et al*, 2017; Babel *et al*, 2006).

In recent years studies have demonstrated that periodontitis is associated with genetic variants of some cytokines confer susceptibility to periodontitis , the genetic factors and the genes herefore that play a necessary role within the immune pathology of periodontitis like protein genes are the most candidates for the analysis of polymorphisms and may be possibly associated with periodontal disease. 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 deterministic in disease outcome (Laine *et al*, 2010; Gabriela Teixeira *et al*, 2014; Shao *et al*, 2009). Cytokines are soluble proteins that bind to specific receptors on target cells and initiate intra cellular signaling cascades resulting in phenotypic changes in the cell via altered gene regulation. Cytokines play a major role in various biological activities such as differentiation proliferation, regeneration, development, repair inflammation and homeostasis they play a fundamental role in inflammation including periodontal disease (Preshaw *et al*, 2011). Pro-inflammatory cytokines that are generated by immune system cells and mediate many kinds of immune responses are kinds of endogenous

polypeptides, they are also the effectors of the autoimmune system, interleukin-6(IL-6) becomes a protagonist among them since it predominately induces pro-inflammatory signaling and regulates massive cellular processes. It has been ascertained that interleukin-6 (IL-6) is associated with a large number of diseases with inflammatory background, such as (Luo *et al*, 2016). Interleukin-6 (IL-6) is an important mediator of host response to tissue injury and infection. It plays a major role in differentiation of B cell and also enhances proliferation of T cell and bone resorption. The correlation between tissue levels of Interleukin-6 (IL-6) and the severity of the coincident inflammation is significant. In mononuclear cells isolated from inflamed gingival tissues of patients with periodontitis spontaneous production of Interleukin-6 (IL-6) has been reported. The levels of Interleukin-6 (IL-6) may correlate with the severity of periodontal disease the Interleukin-6 (IL-6) is increasedin sites of refractory periodontitis compared to stable sites, which indicates that IL-6 could be a diagnostic marker for sites of active periodontal disease (Alwan *et al*, 2018).

MATERIALS AND METHODS

Sampling

The subjects enrolled in the present study were (100) subjects of both genders. The age range was from (30-65) years. Most of the subjects were from attendants to the Department of Periodontics; College of Dentistry, University of Babylon, Hilla city, Iraq, between November 2018 to February 2019. A questionnaire was designed to include: name, age, dental history, family history of periodontal disease and history of systemic disease etc. were recorded according to the participant testimony, while the participant's weight and height were measured by electronic balance and measuring tape, respectively.

Body mass index was calculated according to WHO (2004) by the equation $\frac{Weight(kg)}{Height(m^2)}$

The participants of this study were 100 subjects where they were divided into two categories:

1. Control group (C): included (40) subjects who had healthy periodontium

2. Periodontitis group (P): included (60) subjects 30% of teeth with clinical attachment level ≥ 5mm were considered severe (Kornman *et al*, 1997). The determination of disease severity was based on criteria established in 2017 at World Workshop on the Classification of Periodontal and Peri Implant Diseases and Conditions (Tonetti *et al*, 2018).

Exclusion criteria

All individuals with less than 20 natural teeth, history of any systemic disease such as: diabetes mellitus, rheumatoid arthritis, cardiovascular diseases, hepatic diseases, kidney diseases etc. Smoker. pregnant or lactating woman .alcohol drinking. Systemic antibiotic or anti-inflammatory therapy within the last three months

Collection of salivary samples

Unstimulating entire salivation accumulation as per alteration of the strategy depicted by Navazesh (1993). Saliva test gathered between 9 am to 12 pm. All subjects washed mouth with distil water (10mL) for 30 s–1 moment to evacuation any flotsam and jetsam. Subjects of all gatherings gather five milliliters of an animate entire spit forward into a plastic holder. put all examples in the ice box. All examples were taken to the research facility, put in test tubes and centrifuged at (3000 rpm for 10 minutes). The cleared up supernatant isolated by micropipette and disseminated in excess of one Eppendorf tubes and test were quickly solidified and capacity at (-20ºC) in the microbiology research centers to the point that time of test accumulation finish before investigation.

DNA Extraction from sample of saliva

Under sterile condition Salivawere collected from mouthwash the DNA Extraction from sample of Saliva in manual Procedure by Quinque *et al* (2006).

PCR amplification of Interleukin-6 (IL-6)

One PCR fragment was selected for amplification, which supposed to cover 311 bp of Interleukin-6 (IL-6) gene (Table 1).

Single strand conformation polymorphism SSCP

A. Procedureof SSCP

1. The glass plates were cleaned thoroughly with warm tap water, and then rinsed with tap water, deionized water, and finally ethanol. They were dried either by wiping or by air.
2. The long plate was put down first on a clean surface, and then the left and right spacers were placed along the sides of the long plate. The short plate was put on top of the spacers so that it was even with the bottom edge of the long plate.
3. The single screw of each sandwich clamp was loosened and each clamp was placed by the appropriate side of the gel sandwich with the locating arrows facing up and toward the glass sandwich.
4. The gel sandwich was held firmly and fit it into the left and right clamps. The screws were tightened

Table 1 : show optimized reaction mixture for PCR.

	Composition	Concentration	Volume
1	Nucleases free water		11 µl
2	DNA sample	10-20 ng/µl	2 µl
3	$Mgcl_2$	25mm	1 µl
4	Forward primer	10 PM	0.5 µl
5	Revers primer	10 PM	0.5
6	Master mix	2.5X	10 µl
	Total volume		25 µl

Table 2 : show optimized PCR Condition of Interleukin-6 (IL-6).

Stage	Time	Temperature	Steps	Cycles
1	Initial denaturation	94	5min	1
2	DNA denaturation	94	30sec	35
	Primer annealing	66	30sec	
	Extension	72	30 Sec	
3	Final extension	72	5min	1

Table 3 : Primers for amplification of Interleukin 6 (IL-6).

Primers		Sequence (52 -32)	Amplicon size bp
Interleukin -6(IL-6)	F	GCAGCCAACCTCCTCTAAGT	311bp
	R	CAGGCTAGAATTTAGCGTTCCAG	

enough to hold the plates in place. It was checked whether the plates and spacers were even at the bottom. If this was not the case, the plates and spacers were realigned to obtain a good seal. Failure to do so could have resulted in a gel leakage when casting, as well as buffer leakage during the run.

5. The gray sponge was placed onto the front casting slot. The sandwich assembly was placed on the sponge with the short glass plate facing forward. The sandwich was pressed down, and the handles of the camshaft were held down as well to lock the sandwich in place.
6. A gel solution was made with the desired polyacrylamide percentage volume of reagents used to cast polyacrylamide gel 10% including H_2O (3.4 ml), TBE (1.5 ml), 10%APS (100 µl), TEMED (5 µl), 30% Acrylamide (2.4 ml).
7. The gel solution was poured into the sandwich.
8. A comb was inserted into the top of the sandwich to form the sample wells and was left to polymerize at room temperature for at least 1 h.
9. After polymerization, the comb was removed by pulling it straight up slowly and gently. The gel sandwich was released from the casting stand and attached to the core with the short glass plate facing the core. The core was turned to its other side and the second gel sandwich was attached.
10. The upper and lower chambers were filled with 0.6 X TBE buffer and the wells were rinsed out thoroughly with running buffer using a syringe and needle
11. The core and the attached gel sandwiches were placed into the electrophoresis tank; allowing the core to lock in place. The lid was put on and the system was connected to an external water chiller. The temperature was set and pre-run for 20 min to reach the desired temperature.

B. Sample preparation

Five µL PCR product was mixed with 5 µL SSCP gel loading buffer and heated at 90ºC for 5 min, and was then immediately placed on ice.

C. Electrophoresis

1. When the running buffer had reached the desired temperature, the pre-run was stopped, the wells were rinsed with running buffer again, and 10 µL of the samples was loaded into the wells using "long" tips.
2. The gel was run at a constant power of 100 v for 6–12 h at 5–15ºC.
3. After the electrophoresis were completed, the power supply and water chiller system off were turned off, the electrodes were disconnected, and the core out of the electrophoresis tank was pulled out carefully.

D. Silver Staining

1. The core and gel sandwich were laid on a padded surface to absorb buffer spills. The gel sandwich was removed from the core. The gel was removed carefully from the plates, and it was rinsed briefly in deionized water.
2. The gel was immersed in a tray containing solution 1 (45 ml deionized water, 5ml ethanol, 0.1 gram silver nitrate and 250 µl acetic acid) and the tray was placed on top of a shaker to be mixed for at least 30 min.
3. Solution 1 was poured off and the gel was briefly rinsed with deionized water.
4. Solution 2 was put in a water bath at 55 C. As soon as solution 1 was removed, solution 2 (1.5 g NaOH, 75 µL formaldehyde, 50 ml deionized water) was added for 20 min.
5. When the bands were clearly visible, the second solution was thrown and the third solution (5 ml ethanol, 45 ml deionized water and 250 µl acetic acid) was added for 5 min.
6. The gel was placed on top of the LED light, then the

bands were read and the results on the gel were recorded and a photo was taken afterwards.

RESULTS

General sample parameters

In this study, the total number of subjects in this study are 100 persons devided in two groups, the first group consist of population have periodontitis (case) consist 24 were female and 36 were male, while the the other group healthy population (control) consist 27 were female and 13 were male.

The age of the participant in periodontitis infected group range from 30 years old to 65 years old , the mean of population have periodontitis are 44.15, while the mean in the healthy control group are 38.03.

Interleukin IL-6 genotyping polymerase chain reaction (PCR)

The Polymerase chain reaction PCR product of the IL-6 gene (311 bp) was amplified in all samples, the PCR products were electrophoresed on 2% agarose gel as shown in the Fig. 1.

Single strand conformation polymorphism SSCP

Four patterns were detected by using Single strand conformation polymorphism (SSCP). The SSCP Technique reveals a different single strand pattern. The Fig. 2 shows Single strand conformation polymorphism SSCP electrophoresis pattern.

DNA Sequence of Single strand conformation polymorphism SSCP pattern

The resolved PCR amplicons were commercially sequenced from both (forward and reverse) termini according to instruction manuals of the sequencing company (Macrogen Inc. Geumchen, Seoul, South Korea). Only clear Chromatographs obtained from ABI sequence files were further analyzed, ensuring that the annotation and variations are not because of PCR or sequencing artifacts. By comparing the observed DNA sequences of this study samples with the retrieved neighboring DNA sequences of the NCBI Blast engine, the virtual positions and other details of the retrieved PCR fragments were identified.

The observe variants of the DNA sequence alignment with its corresponding reference sequences of the 311

Table 4 : show Allele association of Interleukin 6 rs1800796 gene polymorphism with Periodontitis.

Allele	Control		Case		OR (95% CI)	P-value
	Count	Proportion	Count	Proportion		
G	50	0.62	65	0.56	0.765(0.42-1.36)	0.366
C	30	0.38	51	0.44	1.30(0.73-2.34)	

Table 5 : The association of each genotype with periodontal disease.

Model	Genotype	Control	Case	OR (95% CI)	P-value
Codominant	G/G	22 (55%)	30 (51.7%)	1.00	0.47
	G/C	6 (15%)	5 (8.6%)	0.61 (0.17-2.26)	
	C/C	12 (30%)	23 (39.7%)	1.41 (0.58-3.42)	
Dominant	G/G	22 (55%)	30 (51.7%)	1.00	0.75
	G/C-C/C	18 (45%)	28 (48.3%)	1.14 (0.51-2.56)	
Recessive	G/G-G/C	28 (70%)	35 (60.3%)	1.00	0.32
	C/C	12 (30%)	23 (39.7%)	1.53 (0.65-3.61)	
Over dominant	G/G-C/C	34 (85%)	53 (91.4%)	1.00	0.33
	G/C	6 (15%)	5 (8.6%)	0.53 (0.15-1.89)	

* P value > 0.05 *Two tailed p value of chi square.

Table 6 : Descriptive statistic of body mass index (BMI) with Interleukin-6 (IL-6) rs1800796 polymorphism.

Genotype	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
C/C	31	231.13	67.5383	12.130	206.3616	255.9081	117.83	428.99
G/C	11	217.47	44.2838	13.352	187.7224	247.2230	129.74	301.40
G/G	48	234.48	62.2116	8.9794	216.4227	252.5515	110.90	359.80
Total	90	231.25	61.8941	6.5242	218.2894	244.2164	110.90	428.99

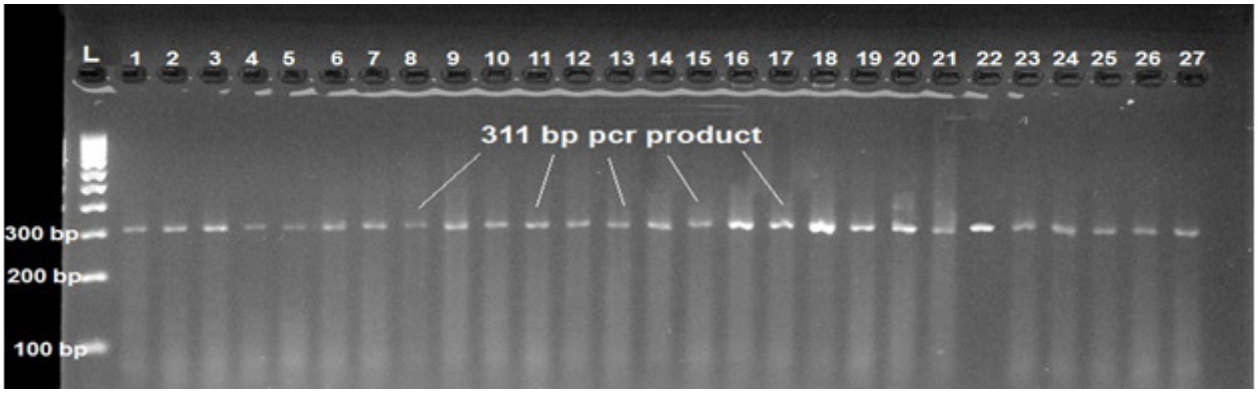

Fig. 1 : Electrophoresis pattern of IL-6 gene amplicon (311pb).

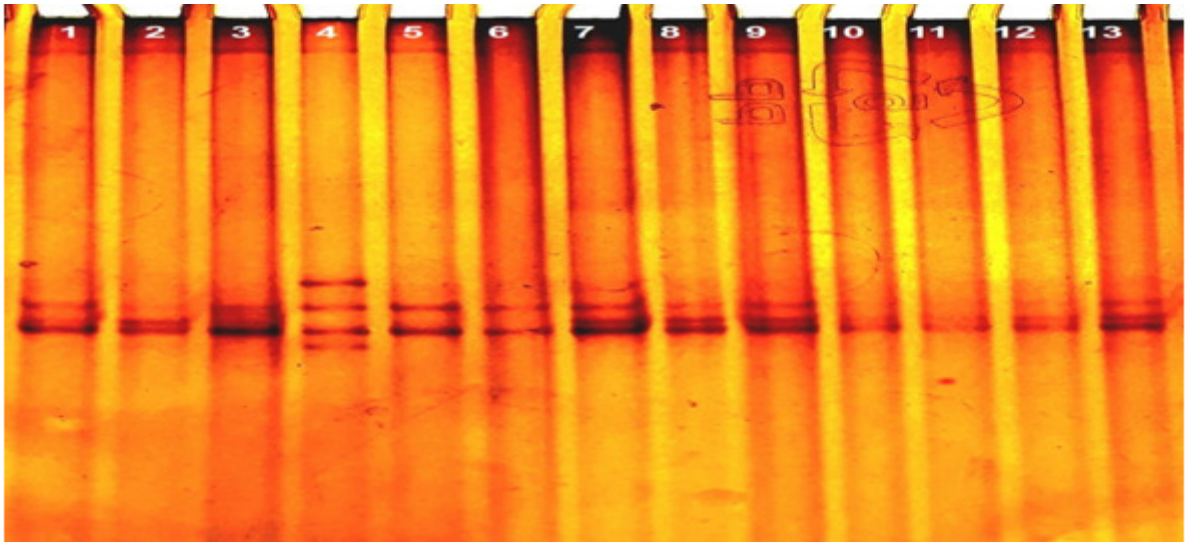

Fig. 2 : shows the SSCP pattren of IL-6 amplicon,the corresponding genotype of each pattern as the folowing (lanes 3.4.5 and 6 GG genotype : lanes 2.10.11and 12 CC genotype : lanes 1.7.8.9 and 13 GC genotype).

bp amplicon of the IL-6 sequences (1 – 311 nucleotides range). After positioning the 311 bp amplicons' sequences within the chromosome no. 7, the details of its sequences were highlighted, in terms of the positioning of both forward and reverse primers of the 311 bp amplified amplicon.

The Alignment results of all sequencing samples

The alignment results of all sequencing samples revealed the presence of only one SNPs, in which one substitution mutations were observed. Only the cite 148th in our amplicons show polymorphism (G>C). The sequencing chromatogram of the observed SNP was documented in Fig. 3.

Therefore, the observed variant had exhibited three different distributions in the analyzed samples in terms of the targeted rs1800796; in which S2 and S4 exerted G allele homozygous status (G/G) in the 148th position of the analyzed 311 bp amplicon, while S3 exhibited heterozygous status (G/C) in the same analyzed position, whereas S1 had shown a C allele homozygous status (C/C) in the same position within the PCR amplicon. Thus, the currently observed three sequencing patterns had confirmed the observed four different SSCP patterns by showing an obvious form of Zygosity status distributed in three groups of investigating samples.

The Association of Interleukin-6 (IL-6)rs1800796 Gene polymorphismwith Periodontitis.

An Allelic Association of Interleukin-6 (IL-6) rs1800796 gene Polymorphism : An Allelic associationof Interleukin-6 (IL-6) rs1800796 gene polymorphism for case and control groups is listed in Table 4. The results show that there is no significant allelic frequency difference between case and control groups.

The association of each genotype with periodontal disease was further tested under different models of inheritance. The result shows that there was no significant association of any genotype with periodontal. Under any tested inheritance model, as in Table 5.

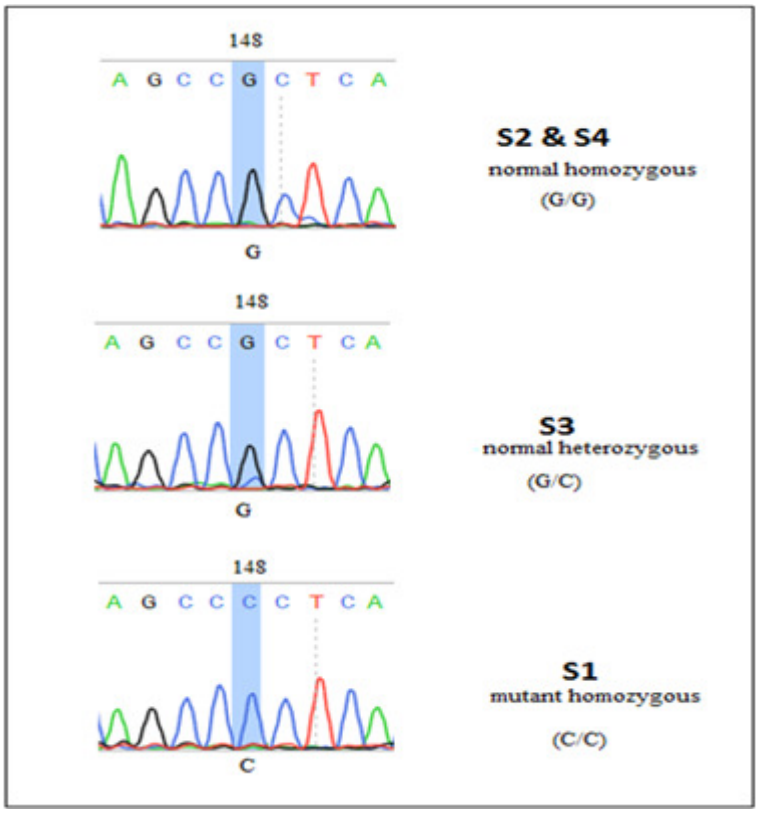

Fig. 3 : The pattern of the observed substitution mutation within the DNA chromatogram of the targeted 311 bp amplicons within the IL-6 gene. The observed substitution mutation is highlighted according to its position in the PCR products. S1 – S4 refer to the studied no. 1 to no. 4 samples.

Table 7 : Association of body mass index (BMI) with Interleukin-6 (IL-6) rs1800796 polymorphism

(I) group	(J) group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
C/C	G/C	13.66211	21.88646	0.534	-29.8396	57.1638
	G/G	-3.35224	14.36945	0.816	-31.9131	25.2086
G/C	C/C	-13.66211	21.88646	0.534	-57.1638	29.8396
	G/G	-17.01436	20.84669	0.417	-58.4494	24.4207
G/G	C/C	3.35224	14.36945	0.816	-25.2086	31.9131
	G/C	17.01436	20.84669	0.417	-24.4207	58.4494

Association of body mass index (BMI) with Interleukin-6 (IL-6) rs1800796 polymorphism

Table 6 shows the association of body mass index (BMI)with Interleukin-6 rs1800796gene polymorphism .The result shows that there was no significant difference between body mass index (BMI) and Interleukin-6 (IL-6) rs1800796 gene polymorphism in the Table 6.

DISCUSSION

The etiological factors that play a potential role as a risk factors associated with periodontal disease, periodontitis is a destructive, nonreversible condition resulting in loss of tooth connective-tissue attachment to bone, which ultimately leads to loss of the involved teeth (Dumitrescu, 2010).

Michalowicz *et al* (2000) suggested that the individual genetic background and environmental factors effected by pathogenesis of periodontitis and that the half variance of periodontal disease attributed to genetic factors.

Sequencing technique is a very important technique in genetic study because it discover novel SNPs and a large number of single nucleotide polymorphisms (SNPs) in studyingthe genetic polymorphisms. Sequencing technique is to investigate of the single nucleotide polymorphisms (SNPs) in the target area of the gene and study association of (SNPs) with the disease and the target area of this study is part promoter and also part of

Interleukin 6 gene that related with periodontitis.

In this study, there was no relationship between Interleukin-6 IL-6-174 (G/C) gene polymorphism and periodontitis and the frequency distribution of genotypes and alleles showed no significant difference between subjects with periodontitis and healthy subjects.

The results of DNA sequencing are only one single nucleotide polymorphisms (SNPs) mutation in this part of the gene, but this (SNPs) mutation was not gave any significant difference between case and control group.

The association of Interleukin-6 (IL-6) rs1800796 Gene polymorphism with Periodontitis

The association of Interleukin-6 (IL-6) rs1800796 gene polymorphism with periodontitis was statistically no significant allelic frequency difference between case and control groups. This result was in accordance with previous studies by Sanchooli *et al* (2012) in Iranian population samples which consisted of (100) healthy individuals, and (100) patients with periodontitis, they found a statistically no significant association of Interleukin-6 (IL-6)-174 (G/C) gene polymorphism with periodontitis between these two groups.

In addition, this result was also accorded with the result of previous study among Brno, Czech Republic population sample by Holla *et al* (2005), which studied the association of Interleukin-6 IL-6 gene polymorphism with periodontitis, the sample of this study was consisted of (255) subjects (148 patient sample of periodontitis and 107 control samplese and showed that there was no significant association between patient and control group.

At the same time, the result of our study was disagreed with the results showed by Line *et al* (2003), which studied the association of Interleukin-6 IL-6 gene polymorphism of with periodontitis; the sample was collected froma Caucasian Brazilian population (48 patients and 36 healthy). They found that there was statistically significant between patients and healthy group.

Association of body mass index (BMI) with Interleukin-6 (IL-6) rs1800796 gene polymorphism

Body Mass Index (BMI) is a commonly used index to categorize the individuals for obese, normal and underweight , it is simply calculated as the ratio of weight (kg) per square the height (m). WHO put a cut off Body Mass Index (BMI) values for four main classes ,which are underweight, normal weight ,overweight and obese as listed in Table 8 (WHO, 2004).

The associationof body mass index (BMI)with Interleukin-6 rs1800796gene polymorphism that there was no significant difference between body mass index (BMI) and Interleukin-6 (IL-6) rs1800796 gene polymorphism . further research needs to be conducted to investigate the myriad cellular and molecular pathways affected by the IL-6 polymorphisms.

Table 8 : BMI cut-off values for the four main classes.

Class	Cut-off value
Underweight	<18.50
Normal range	18.50 - 24.99
Overweight	25.00-29.99
Obese	≥30.00

This result was agreed with the result of previous study by Boeta *et al* (2018) revealed that minor allele frequencies (MAFs) for SNPs was compared between subjects with and without obesity phenotypes categorized by BMI and waist circumference. The samples collected from Mexican-American population the participants consisted of 437 subjects .the result showed there were no associations found between SNPs and BMI (Boeta-Lopez *et al*, 2018).

Oana *et al* (2014) disagree with the results presented in this study in which studied associationof body mass index (BMI) with Interleukin-6 rs1800796 gene polymorphism in children's.the sample of this study was consisted of (222) of childrencollected in a pediatric (Caucasian) population in Romania (southeastern Europe). The result showed obesity is most frequently associated in children with IL-6 174 C allele carriers.

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