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Association of TNFα and IL1β genotypes with the risk of *Staphylococcus aureus* causing recurrent tonsillitis in a sample of Iraqi patients

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Abstract

Background and aim: Tonsillitis is an inflammation of the tonsils, usually caused by an infection by viruses or bacteria; recurrent tonsillitis occurs when tonsillitis occurs several times a year. The current study aimed to investigate the association of TNFα and IL1β genotypes with the increased risk of recurrent Staphylococcus aureus tonsillitis in a sample of Iraqi patients. Methods: A total of 50 blood samples were collected from patients with recurrent tonsillitis of S. auerus from three hospitals in Najaf city, and 50 samples were collected from apparently healthy volunteers with a free medical history of recurrent tonsillitis as control group. Genomic DNA was extracted from peripheral blood and genotyped for TNF-α 308G/A and IL1β C/T SNP using Nested tetra-primer amplification refractory mutation system PCR (T-ARMS-PCR). Results: The results of the distribution IL β –rs1143627 C/T alleles in S. aureus tonsillitis patients showed CC wild type genotype was in 11 patients (22%), CT genotype was in 30 patients (60%) and the TT genotype was in 9 patients (18 %) also C allele frequency was (0.42), whilst T allele frequency was (0.58) with Odds Ratio (1.507) at significant difference (P≤ 0.01), while prevalent of TNF-α –rs1800629 G/A alleles genotype in S. aureus tonsillitis patients revealed that GG wild type genotype was 37 patients (74%), GA genotype was 13 patients (26%) and AA genotype was 0(0%) also, G allele frequency was (0.87) and A allele frequency was (0.13) with Odds Ratio (1.507) at no significant differences in both the patient and control. Conclusion: The incidence of IL1 β -31 genotypes, whether in heterozygous (CT) or homozygous (TT) plus the existence (C) allele in S. aureus patients, could be related to the development of tonsillitis, while there is no association between the occurrence of TNF α -308 genotypes with the development of recurrent tonsillitis in this Iraqi population.

Keywords: *IL1β, Iraqi patients, Staphylococcus aureus, TNFα , Tonsillitis*

Introduction

Tonsillitis, a common type of upper respiratory tract infection characterized by an inflamed condition of palatine tonsils, pharyngeal tonsils, tubal tonsils, and lingual tonsil. Viral or bacterial infection and immunologic factors lead to tonsillitis and its complications, overcrowded conditions, and malnutrition are also contributing factors. Tonsillitis is the most commonly encountered health-related problem in the general population (1). Good hydration and the use of analgesics in conjunction with antibiotics are important for treatment. Penicillin was recommended as the first-line treatment for bacterial tonsillitis, but now has been replaced mostly by newer antibiotics (2). The pathogenesis of tonsillitis is most likely to be based on its anatomic location and its inherent function as an organ of immunity, processing infectious material and other antigens, and then becoming a focus of infection/inflammation. Tonsillitis has been associated with a wide variety of infectious agents. Staphylococcus aureus is one of the most pathological factors. In the development of tonsillitis, the bacterial factor plays an important role, but it may not be the only one, because certain genetic backgrounds may be related to greater inflammation (3). The most important elements of tonsillitis are cytokines. Cytokines are intercellular messengers in the immune system, where they integrate the function of several cell types in various body compartments into a coherent immune response. Interleukin-1 β (IL-1 β) and tumor necrosis factor alpha (TNFα) are the most inflammatory cytokines that play an important role in a number of chronic and acute inflammatory diseases (4). The mRNA of IL-1 beta and TNF α were expressed in the whole tonsillar tissue of all the subjects tested with recurrent tonsillitis. Genetic variants of cytokines may alter the inflammatory response and several genes encode pro-inflammatory cytokines (5). Among the well-studied single nucleotide polymorphisms (SNPs) that can affect the inflamma-

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tory response is TNF α -308G/A, besides, the ±31C/T SNP at the promoter of the IL1 β gene has been associated with greater inflammation in multiple populations(6). Genetic polymorphisms have been reported as risk factors for the development of recurrent tonsillitis, but the evidence is not strong enough (7). In view of the importance of this health issue, it was decided to conduct this study, which investigates the relative contribution of immunogenetic factors such as TNFα and IL1β in the development of recurrent S. auerus tonsillitis in the Iraqi population.

Materials and Methods:

Blood samples were obtained from 50 patients with recurrent tonsillitis under the approval ethics committee Institute of Genetic Engineering and Biotechnology for Postgraduate Studies of the University of Baghdad for the use of human samples of number (2550). The age of the patients ranged from (2-60) years with a mean age (31) years for both sexes and (50) samples of children of healthy volunteers of the same age and gender who came to the hospital for routine tests to check the level of calcium in the blood as a control group. Staphylococcus aureus was diagnosed by bacterial cultures, biochemical tests, and the Vitek system. Two ml of blood was taken from each subject, DNA was extracted using genomic DNA purification kit (Geneaid, Taiwan), DNA concentration was estimated by Nano drop spectrophotometer and measured in(ng\ µl) as well as the purity was estimated by reading the absorbance at a wavelength of between 260/280 nm. Genotyping for TNFα-308G/A and IL1 β C/T SNP was done by using Nested T-ARMS PCR method. In brief, it consisted of Taq polymerase and four primers with two outer nested primers. Two pairs (instead of one pair) of PCR primers are used to amplify an 8 fragment(outer forward (OF), outer reverse (OR), inner forward (IF), and inner reverse (IR) primers. The primers for Nested T-ARMS PCR and their sequences are listed in table (1and 2).

Table.1 Primers of SNP of TNF-α gene (308 G/A) rs1800629

Target gene		Sequence 5'-3'	Tm (°C)	Product size	References
Nested	F	AGTCTCCGGGTCAGAATGAAAGAAG	60	bp 622	Designed in this study
	R	GAAAGAATCATTCAACCAGCGGA			
Outer	F	TCCAACCCCGTTTTCTCTCC	60	647bp	
	R	CTCACACTCCCCATCCTCCCT			
Allele A	F	CAATAGGTTTTGAGGGGCAGGA	60	117bp	
Allele G	R	GGAGGCTGAACCCCGTACC	60	176bp 161	

Table 2 SNP primers for the IL-1 β gene (±31 C/T) rs1143627

Target gene		Sequence (5'-3')	Tm (°C)	Product size	References
Nested	F	CATGTATAAATCTGTGTGTCTTCCAC	60	bp 847	Designed in this study
	R	GTGTGATTTCTCTCAGCATCCAG			
Outer	F	CTACTCCTTGCCCTTCCATGAAC	60	bp 260	
	R	CAATGAAGATTGGCTGAAGAGAA			
Allele C	F	CTCCTACTTCTGCTTTTGAAATCC	60	bp 121	
Allele T	R	GTTTCTCCCTCGCTGTTTTTCTA	60	bp 185	

The ratio of the inner primer to the genotype-specific primer is 1:10 in a final reaction volume of 50 µl. The components of the mixture to process Nested T-ARMS PCR are mentioned in Table (3).

Table 3: Components of the Mixture to Process Nested T-ARMS PCR.

Components	Concentration	Volume (µl)
2X PCR *Taq* Master Mix	1X	25
Forward primer	µM/µl 10	4
Reverse primer	µM/µl 10	4
ddH_2O	-	13
DNA	ng 40	4
Total		50

The Nested T-ARMS PCR Cycling program DNA to detect TNFα-308G/A and IL1β C/T gene polymorphism was done as illustrate in Table (4).

Table 4: Nested T-ARMS PCR Cycling Program for TNFα-308G/A and IL1β C/T genes.

Phase	Tm (°C)	Time	Cycles
Initial denaturation	94	5 min	1X
Denaturation	94	30 sec.	35X
Annealing	60	30 sec.	
Extension	72	1 min	
Final extension	72	5 min	1X

Agarose gel electrophoresis for the Nested T-ARMS PCR product was performed to visualize the size of the PCR product after finishing the PCR program using 2% agarose gel and DNA Red safe dye, at voltage (70) for (30) minutes. The genotypes were differentiated by checking the amplicon sizes in reference to molecular size markers.

Statistical analysis

The Statistical Analysis System- SAS (8) program was used to detect the effect of difference factors in study parameters. The least significant difference –LSD test (Analysis of variance -ANOVA) was used to compare between means and the Chi-square test, which was used to compare between percentages (0.05, 0.01 probabilities) in this study.

Results:

The result revealed that the highest number of recurrent tonsillitis was located in age group (31-40) year, which represented 16 (32%) of the total, followed by year (11-20) that showed 11 (22%) and almost a similar number of 10 (20%) was gained from each age group (1-10) and (21-30) years, while the lowest number of tonsillitis was in age groups older than 50 years and (41-50) years which showed 2 (4%) and 1 (2%) respectively with a high significant difference ($P \leq 0.01$) as illustrated in table (5).

Table 5: Distribution of S. aureus recurrent tonsillitis according to age groups.

Age groups	No.	(%)
1-10	10	20
11-20	11	22
21-30	10	20
31-40	16	32
41-50	1	2
50<	2	4
Total	50	100%
(Chi-Square-χ^2 (P-value	---	(0.0073) ** 8.923
.(P≤0.01) **		

The distribution of patients with recurrent tonsillitis of S. aureus according to sex was 27(54%) males and 23 (46%) females, with no significant difference, as shown in Table (6).

Table 6: Distribution of patients with recurrent tonsillitis according to sex.

sex	No.	%
Male	27	54
Female	23	46
Total	50	100
Chi-Square-χ^2 (P-value)	---	NS 0.320 (0.571)
NS: Non-significant.		

The results of the distribution of S. aureus tonsillitis according to the patient's family history revealed that 26 (52%) of cases with family history (Present) and 24 (48%) without family history (absent) without significant differences as illustrated in Table (7).

Table7: Distribution of patients with recurrent tonsillitis according to family history.

Family history	Patients N = 50 (%)	Chi-Square-χ^2	P- Value
Present	26 (52%)	NS 0.081	0.777
Absent	24 (48%)		
NS: Non-significant.			

Total genomic DNA was extracted from whole frozen blood using a genomic DNA purification kit, the concentration and purity of DNA were carried out using Nanodrop. The result showed that the purity was good and ranged from (1.8-1.85) and the concentration ranged from (10-12ng/µl). Figure (1) showed sharp bands of chromosomal DNA when analyzed by gel electrophoresis using (1%) agarose gel and DNA Red safe dye, at voltage (70) for (30) minutes.

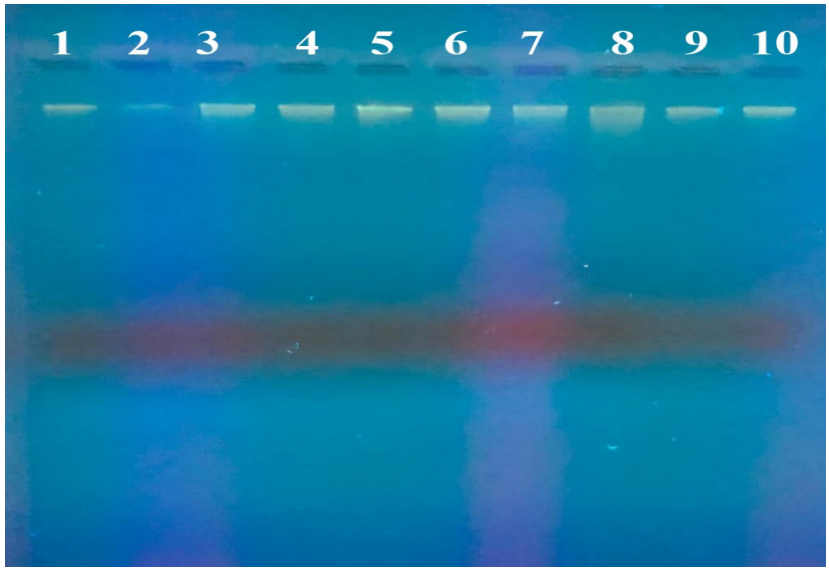

Figure1: Genomic DNA gel electrophoresis in (1%) agarose gel at (70) volt/cm2 for (30) min, stained with DNA dye is RedSafe and visualized under ultraviolet light.

A modified T-ARMS PCR technique was implemented in which a nested PCR product was introduced prior to T-ARMS PCR for the human gene IL-1β rs1143627 SNP. The T-ARMS-PCR products of these samples were divided into four bands: (847 bp) represented the nested outer PCR that serves as a DNA template for the inner PCR (260 bp) and (185 bp) for the allele (T) and (121 bp) for the allele (C). The wild-type genotype (CC) would have only three bands (847, 260 and 121 bp). The heterogenotype (CT) would have four bands of (847, 260, 185 and 121 bp), and homogenotybe (TT) would have three bands (847, 260 and 185 bp). T-ARMS- PCR results for eleven patient and control samples shown in figures (2) and (3).

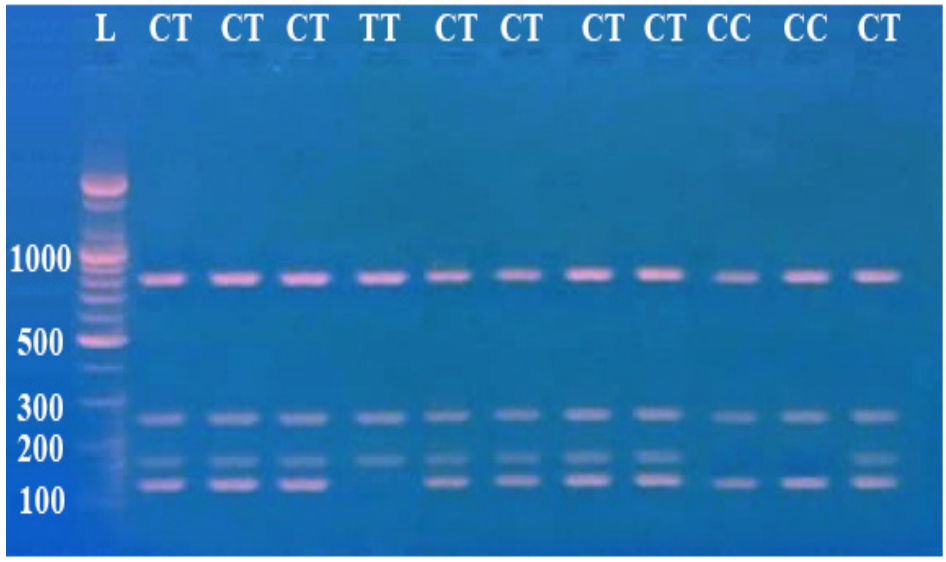

Figure 2: T-ARMS-PCR bands of the human gene IL-1β rs1143627 SNP. (patient) (2%) agarose, DNA dye is red safe, V: 70, Time: 90 minutes, L: DNA ladder.

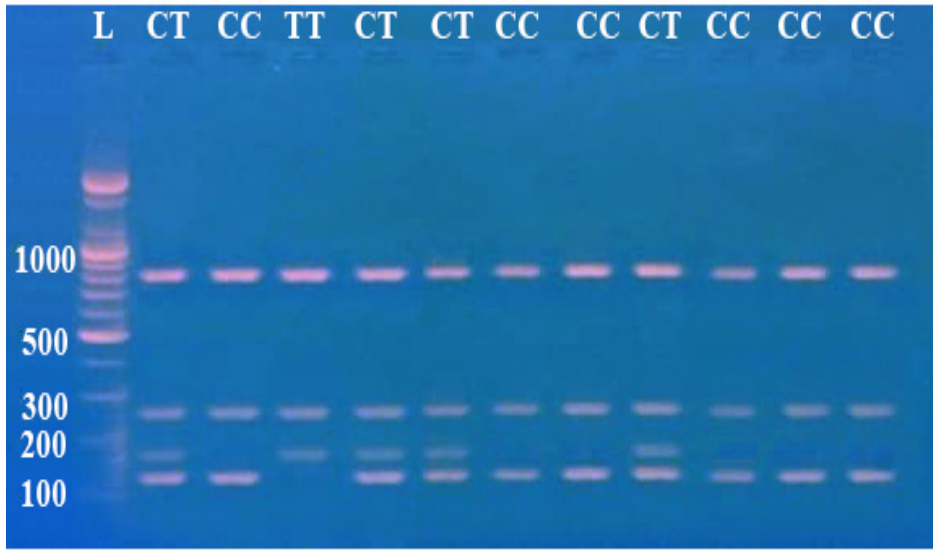

Figure 3: T-ARMS-PCR bands of the human gene IL-1β rs1143627 SNP. (control) (2%) agarose, DNA dye is RedSafe, V: 70, Time: 90 minutes, L: DNA ladder.

In the current study, T-ARMS PCR was performed for the human gene TNF -α was done to detect the rs1800629 SNP, the T-ARMS-PCR product of this sample was divided into four bands: (622 bp) represents the nested outer PCR which serves as a DNA template for the inner PCR (253 bp) and (176 bp) for allele G and (117 bp) for allele A. The wild-type genotype (GG) would have only three bands (622, 253 and 176 bp). The heterogenotype (GA) would have four bands of (622, 253, 176 and 117 bp). The homogenotype (AA) would have three bands (622, 253 and 117 bp). T-ARMS- PCR results for eleven patient and control samples shown in Figures (4) and (5).

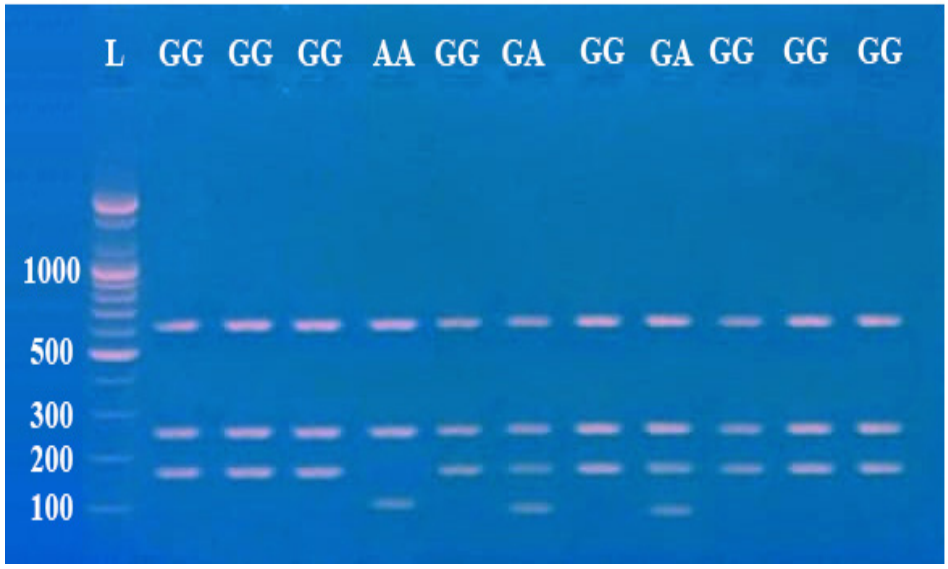

Figure 4: T-ARMS-PCR bands of the human gene TNF-α rs1800629 SNP (control) (2%) agarose, DNA dye is red safe, V: 70, Time: 90 minutes, L: DNA ladder.

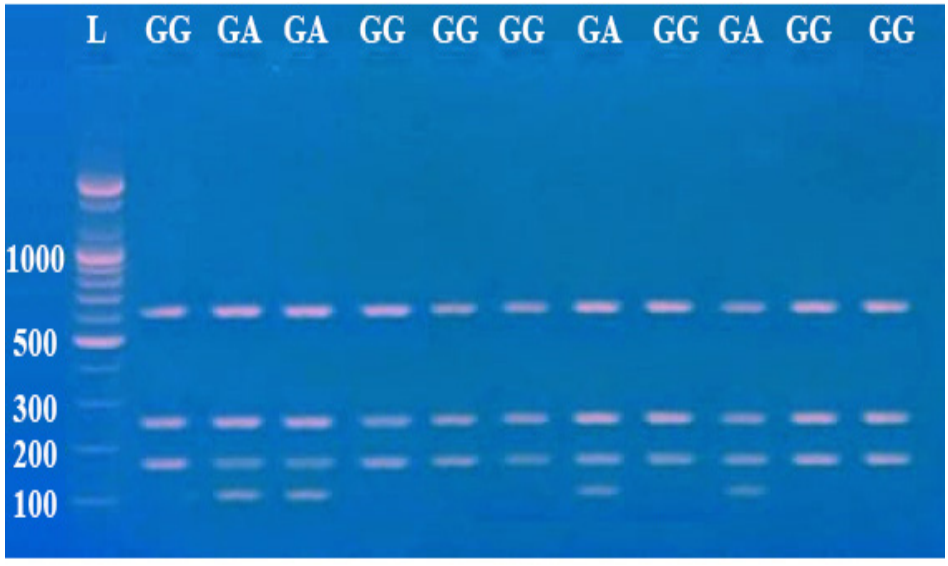

Figure 5: T-ARMS-PCR bands of the human gene TNF-α rs1800629 SNP (patient) (2%) agarose, DNA dye is Red Safe, V: 70, Time: 90 minutes, L: DNA ladder.

The results of the relationship between ILβ and TNF-α polymorphisms and the patient with s. aurous tonsillitis showed that the distribution of the ILB –rs1143627 C/T alleles among the tonsillitis patient was 11 (22%) of the wild type CC genotype, while the heterogeneous CT genotype was 30 (60%) and the homogeneous genotype of the TT mutant was 9 (18 %), also the frequency of C alleles was (0.42), while the frequency of the T alleles was (0.58), while the distribution of ILβ genotype between the control individuals was 32 (64%) of the wild type CC genotype, the heterogeneous CT genotype was 12 (24% and the homogeneous TT mutant homogeneous genotype was 6(12%), also the frequency of C allele was (0.76) and the frequency of T allele was (0.24) with odds ratio (1.507) at significant difference (P≤ 0.01), as shown in Table (6).

Table 6: Distribution of ILβ –rs1143627 C/T genotypes in tonsillitis patients and control.

Case CC		IL1 β			*Allele frequency*		*P.* Value	*Odds ratio (CI)*
		CT	TT	C	T			
Patient	Count	11	30	9	0.42	0.58	0.0001 **	(0.86-1.77) 1.507
	within % the case	22 %	60%	18 %				
Control	Count	32	12	6	0.76	0.24		
	within % the case	64 %	24 %	12 %				
** (P≤0.01).								

Furthermore, the prevalence of TNF-α –rs1800629 G/A alleles genotype in patients with S. aurous tonsillitis was 27(74%) of the wild-type GG genotype, while the heterogeneous genotype of GA was 13(26%) and the homogeneous genotype of the AA mutant was 0(0%) also, the frequency of G alleles was (0.87) and the frequency of A alleles was (0.13). Although the distribution of TNF-α genotype in control was 39 (78%) of the wild type GG genotype, the GA genotype was 9 (18%) and the AA genotype was 2(4%) also the frequency of the G allele was (0.86) and the frequency of the A allele was (0.14), with the odds ratio (1.507) without significant differences in both the patient and control as described in Table (7).

Table7: Distribution of TNF-α –rs1800629 G/A genotypes in patients with tonsillitis and control.

Case GG		TNF-α			*Allele frequency*		*P.* Value	*Odds ratio (CI)*
		GA	AA	G	A			
Patient	Count	37	13	0	0.87	0.13	0.853 .N.S	1.14-) 1.966 (2.52
	within % the case	74.0%	26.0%	0.0%				
Control	Count	39	9	2	0.86	0.14		
	within % the case	78.0%	18.0%	4.0%				
No significant differences								

Discussion:

The result of the current study revealed that the highest number of recurrent tonsillitis was located in the age group (31-40) year, the reason for this may be due to the fact that this age group is the most exposed to environmental factors due to its continuous mixing with different segments of society, as it is the productive age group, this result was agreed with the study by Katkowska et al. (9), who found high incidence in young percents also Katkowska et al .(10) found S. aureus the most common pathogen in patients with recurrent tonsillitis affected patients between 21 and 30 years of age, the outcome of the present study showed the distribution of the S. aureus patients with respect to gender found that men infected more than females , this may be due to that probably because the number of patients admitted was more than female patients, The prevalence of tonsillitis in the current study agreed with the study of Klug (11) when he noticed the frequency of S. aureus tonsillitis was more spread in male patients compared with female ones. In recurrent tonsillitis, genetic factors may be related to sever inflammation, as well as polymorphisms in cytokine genes may alter the inflammatory response, especially genes encoded in pro-inflammatory cytokines such as IL-1β and TNFα. The result showed that patients with at least one copy of the IL1 β -31(C) allele had

a higher risk of recurrent tonsillitis, as well as there was an association between the existence of the IL1 β -31 (T) allele, whether in heterozygous (CT) or homozygous (TT) genotypes, with the development of recurrent tonsillitis. Interleukin 1 β is a potent pro-inflammatory cytokine involved in host defense against infections and regulates the innate and acquired immune is the product of some activated regulating inflammatory response. These findings support the key role of inflammation in the response to recurrent tonsillitis. Although no association between the appearance of TNFα-308 locus with the development of recurrent tonsillitis, single amino acid gain of function mutations results in high amounts of actively secreted IL-1β elevated IL-1 secretion of IL-1β is related to inflammation in patients with these mutations. The primary role of TNF is in the regulation of immune cells. Dysregulation of TNFα production has been implicated in a variety of human diseases. The genetically regulated immune response appears to play a crucial role in determining the intensity of tonsil damage (12). study by González-Andrade et al. (13) one of the few studies that has analyzed the genetic immune response and the development of recurrent tonsillitis that showed the presence association between the presence of the IL1β-31 (T) mutant allele of IL1-31 (T) and the development of recurrent tonsillitis.

Conclusions

In conclusion, the results suggest that the incidence of IL1β -31m (T) allele may be represented as a risk factor for tonsillitis in the case of S. aureus infection. If this result is confirmed in populations other than Iraqi, it may be used as a diagnostic tool at increased risk for recurrent tonsillitis.

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ethical approval

The ethical approval was by the committee of the Institute of Genetic Engineering and Biotechnology for Postgraduate Studies of the University of Baghdad for the use of human samples of number (2550).

Authors' Contribution

Howraa Fadhil carried out the experiment. Bushra Jasim Mohammed wrote the manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

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