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ORIGINAL RESEARCH

Effect of TNF-α −308G/A (rs1800629) Promoter Polymorphism on the Serum Level of TNF-α Among Iraqi Patients with Generalized Vitiligo

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Background: Vitiligo is a chronic acquired pigmentary disorder of the skin; it results from immunological distruction of functioning melanocytes. The cytokine TNF-α plays a central role in the initiation of melanocyte apoptosis in vitiligo. Single nucleotide polymorphism (SNP) in the promoter region of the gene coding for serum TNF-α may affect its production.

Objective: The aim of this study is to assess serum TNF-α as a risk factor for generalized vitiligo among Iraqi patients and to rule out that polymorphism at the −308 position affects serum TNF-α.

Materials and Methods: This case–control study was conducted at Sulaymaniyah Dermatology Teaching Center (SDTC), Iraq. Serum concentration of TNF-α was measured using enzyme linked immunosorbent assay (ELISA) technique in 80 patients with generalized vitiligo and 40 clinically healthy controls. The amplification refractory mutation system polymerase chain reaction (ARMS–PCR) technique was used for detection of TNF-*308G/A* gene polymorphism. TNF-α level correlated with TNF-*308G/A* gene polymorphism. Serum concentration and TNF -*308G/A* gene polymorphism have been analyzed in correlation with demographic features and clinical characteristics of patients with generalized vitiligo.

Results: Statistically significant elevation of serum TNF-α seen in patients compared to a control group (*p*-value 0.01). Significantly higher TNF-α level (*p*-value 0.01) found among patients with active generalized vitiligo. Elevated serum levels of TNF-α were significantly associated with both TNFA1 (TNF-*308G*) allele (*p*-value 0.04) and TNFA2 (TNF-*308*A) allele (*p*-value 0.03). TNF-α −*308GA* polymorphism was not affected by demographic features and clinical characteristics of patients with generalized vitiligo.

Conclusion: TNF-α in the serum is a risk factor for generalized vitiligo among Iraqi patients. Patients with active vitiligo have a higher serum TNF-α level. No difference was found between serum level of TNF-α with TNF-α polymorphism at position −308 (TNF −308). This involves substituting G allele for the A allele.

Keywords: generalized vitiligo, serum TNF-α −308G/A promoter polymorphism, Iraq

Introduction

Vitiligo is a common acquired chronic depigmenting disorder of the skin leading to development of white macules or patches due to selective destruction of functioning melanocyte in skin, hair, or both.[1] The global prevalence of vitiligo varies (0.2–1.8%) and is seen in both sexes and in all ethnicities. The disease may develop at any age although it is most common in children and young adults.[1,2] In Iraq, vitiligo is the most common type of hypopigmented skin disorder.[3] Although exact prevalence is not

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determined; in a hospital-based study of 1000 participants, 25% had vitiligo including both localized and generalized variants with their subtypes.[4]

Vitiligo is classified into generalized vitiligo and localized vitiligo. The generalized variant is the most common pigmentation disorder, occurring at a frequency of approximately 0.2–1.0% in different populations around the world[5] and includes subtypes of vitiligo vulgaris, acrofacial vitiligo, vitiligo universalis, and mixed vitiligo. Localized vitiligo includes variants of focal, segmental, and mucosal vitiligo.[6]

Generalized vitiligo is characterized by well-defined, depigmented amelanotic (milk- or chalk-white) macules or patches surrounded by normal skin, However, at their onset or when actively spreading, lesions of vitiligo may be more ill-defined and hypo- rather than depigmented. Lesions are asymptomatically symmetrically distributed, round, oval, irregular, or linear in shape and ranging from millimeters to centimeters in diameter. In the vulgaris subtype, scattered patches are widely distributed on the face, dorsal aspect of the hands, nipples, axillae, umbilicus, sacral, inguinal and anogenital regions. While on the extremities, it favors the elbows, knees, digits, flexor wrists, dorsal ankles and shins. In the acrofacial variant, distal extremities and the face are involved, typically, facial vitiligo occurs around the eyes and mouth (periorificial). Mixed type of vitiligo presents as a combination of segmental subtype, unilateral and band shaped white macules with acrofacial and/or vulgaris types and, in universal vitiligo, complete or nearly complete depigmentation of the skin.[7,8]

The diagnosis of vitiligo is generally made through the appearance of reduced or lost skin pigmentation in a typical distribution pattern (including periorificial, segmental, lips and tips of the fingers, toes and/or penis, flexural surfaces, and frictional areas)[1,9]

The etiology of vitiligo is not fully understood: Most studies suggest an autoimmune mechanism is responsible for the pathogenesis of vitiligo, especially in the generalized variant. Autoimmune destruction of melanocytes mediated by innate immunity, cell-mediated, and humeral immunity as well as the action of cytokines.[10]

Tumor necrosis factor alpha (TNF-α) is extensively studied in vitiligo.[11] TNF-α or cachectin, the prototypic member of the TNF superfamily with diverse functions in cell differentiation, inflammation, immunity, and apoptosis,[12] is primarily secreted from activated macrophages although it may also be secreted by other cell types (including monocytes,

T-cells, mast cells, NK cells, keratinocytes, melanocytes, fibroblasts, and neurons). TNF-α is synthesized as a transmembrane precursor protein (mTNF-α) with a molecular mass of 26 kDa.[11,13] mTNF-α is cleaved by the action of the matrix metalloproteinase known as TNF-α converting enzyme (TACE) and released as soluble form (sTNF-α) that circulates throughout the body and confers TNF-α with its potent endocrine function far away from the site of its synthesis.[14,15] The gene encoding for TNF-α is located in the short arm of chromosome number 6 in the major histocompatibility (HLA) complex class III region.[16] Various polymorphisms have been identified inside the TNF-α promoter region, positioned relative to the transcription start site: −1031 (T→C), −863 (C→A), −857 (C→A), −851 (C→T), −419 (G→C), −376 (G→A), −308 (G→A), −238 (G→A), −162 (G→A), and − 49 (G → A).[17] One polymorphism that directly affects TNF-α expression is located at nucleotide position −308. Here, a guanine is defined as the common allele (TNFA1); guanine substituted by adenosine forms the rarer allele (TNFA2).[18] In vitiligo, TNF-α initiates melanocyte apoptosis, decreases melanogenesis, inhibits melanocyte stem cell differentiation, and increases melanocyte cytotoxicity[19,20] Evidence from various studies showed that epidermal TNF-α plays a central role in melanocyte disappearance in vitiligo, while few studies determined role of serum TNF-α in vitiligo pathogenesis. These illustrate the differences in the distribution of HLA alleles and how they lead to variations in associations between TNF-α promoter gene polymorphisms in different geographical areas.[21] Accordingly, we were encouraged to study the serum TNF-α level among Iraqi patients with generalized vitiligo and the association between TNF-α −308 GA promoter polymorphisms with serum levels of TNF-α.

Materials and Methods
Study Design and Setting
A case–control study was conducted in Sulaymaniyah Dermatology Teaching Center in the city of Sulaymaniyah, Iraqi Kurdistan from April 2018 to December 2019. The study group comprised 80 consecutive patients presented with subtypes of generalized vitiligo. Patients with localized vitiligo including focal, segmental, and mucosal vitiligo have been excluded in this study. In addition, all patients with generalized vitiligo associated with hypo- or hyperthyroidism, autoimmune thyroiditis, diabetes mellitus, pernicious anemia, and Addison's disease; based on clinical

examination and laboratory investigations have been excluded as well.

This study was approved by the (Research Ethics Committee) of the College of Medicine, University of Sulaimani, in accordance with the Helsinki Declaration. Both, lingual and informed written consent have been taken from every participant. Demographic and biometric information included (eg, age, gender, time of onset of vitiligo, duration of presentation, family history of vitiligo, history of associated diseases, etc.). Forty age and sex-matched healthy volunteers were also enrolled as a control. Among the control group, neither themselves nor their first-degree relatives had any evidence of vitiligo, hypo- or hyperthyroidism, or other autoimmune disease including autoimmune thyroiditis, diabetes mellitus, pernicious anemia, and Addison's disease.

Venous blood samples (10 mL) were collected from patients and the control group under strict laboratory sterile condition, then divided into 2 (5 mL) tubes, Serum separated from one of the 5 mL was stored at −80 °C for further cytokine estimation. The remaining 5 mL blood samples were immediately stored in ethylenediamine tetraacetic acid (EDTA) tubes and used for deoxyribonucleic acid (DNA) extraction then subsequent ARMS-PCR and Sangers sequencing.

Analysis of Serum Level of TNF-α

Serum levels of TNF-α in patients with generalized vitiligo and healthy controls were measured using the ELISA technique (DRG Instruments GmbH, Germany). The DRG TNF-ELISA is a solid phase assay performed on a microtiter plate. The assay uses monoclonal antibodies (MAbs) directed against distinct epitope of TNF. Standards and samples react both with capture monoclonal antibody (MAb1) coated on microtiter wells and with a monoclonal antibody (MAb2) labelled with horseradish peroxidase (HRP). After an incubation period that allowed the formation of sandwich coated MAb1/human TNF/MAb2/HRP, the microtiter plates are washed to remove unbound enzyme-labelled antibodies. Bound enzyme-labelled antibodies were measured via a chromogenic reaction. The chromogenic solution tetramethylbenzidine (TMB) in dimethylformamide was added and incubated. The reaction then stopped upon addition of stop solution and the microtiter plate was then read at the appropriate wavelength. The amount of substrate turnover was determined via a color change (via absorbance), and this is proportional to the TNF concentration. A calibration curve was then plotted and the

TNF-α concentration in samples was determined via interpolation from the calibration curve and expressed as picograms per milliliter (pg/mL).

Detection of TNF-α −308G/A Polymorphism

TNF-α *−308G/A* gene polymorphism was detected on serum samples both in patients with generalized vitiligo and the healthy control, using ELISA technique (DRG Instruments GmbH, Germany). The DRG TNF-ELISA is a solid phase assay performed on microtiter plate. The assay uses monoclonal antibodies (MAbs) directed against distinct epitopes of TNF. Standards and samples both react with capture monoclonal antibody (MAb1) coated on microtiter wells and with monoclonal antibody (MAb2) labelled with horseradish peroxidase (HRP). After an incubation period allowing the formation of a sandwich coated MAb1/human TNF/MAb2/HRP, the microtiter plates are washed to remove unbound enzyme-labelled antibody. Bound enzyme-labelled Ab was measured via a chromogenic reaction. The chromogenic solution tetramethylbenzidine (TMB) in dimethylformamide was added and incubated. The reaction was stopped upon addition of stop solution and the microtiter plate was then read at the appropriate wavelength. The amount of substrate turnover was determined via a color change (absorbance). This is proportional to the TNF concentration. Calibration curve plotted and TNF-α concentration in samples determined via interpolation from the calibration curve and expressed as pictograms/milliliter (pg/mL).

Detection of TNF-α −308G/A Polymorphism

TNF-α *−308G/A* gene polymorphism was detected via ARMS–PCR. A genomic DNA extraction kit (RIBO-prep; AmpliSens, Moscow 111,123 Russia) used to extract DNA from a fresh whole blood sample according to the manufacturer's protocol. ARMS–PCR is a variant of PCR based on the principle that under optimized conditions, the primers with 3′ end mismatch with the complementary template DNA will not result in amplification of a targeted DNA fragment. For each DNA template, two complementary reactions were established in each set of primers: one forward primer to screen for the G allele and the A allele as well as a common reverse primer.[22]

Forward G allele primer: 5`-ATAGGTTTTGAGGGGC ATCG-3`

Common Reverse Primer: 5`-AAGAATCATTCAACC AGCGG-3`

Forward A allele primer: 5`-ATAGGTTTTGAGGGGC ATCA-3

Common Reverse Primer: 5`-AAGAATCATTCAACC AGCGG-3`

Each PCR reaction contained 10 microliters master mix (MyTaq^TM HS Mix-Bioline, USA), 10 pmol primer, and 20 ng of template DNA. The PCR was performed using three-step cycling protocol with initial denaturation for polymerase activation (95 °C/5 min) 1 cycle followed by denaturation (95 °C/30 sec), annealing (56 °C/30 sec), and extension (72 °C/30 sec) 40 cycle. Final extension was 72 °C/5 sec for 1 cycle (Bio-Rad C1000 Thermal Cycler, USA). Known positive and negative controls were included in the run with every batch of the amplification.

PCR products were analyzed in 1% agarose gel (Canvax Biotech): 1.0 g of agarose was dissolved in 100 mL (1X TAE buffer TAE: Tris-acetate EDTA) stained with SYBR Safe Dye (GENETBIO). Gels were placed horizontally in plastic tanks (BioRad) and run at 110 volts for the appropriate time. The size of the target DNA bands compared with 100 bp DNA ladders (GENETBIO). Later, gels were documented with images taken under ultraviolet light (Ultraviolet Transilluminator, BioDoc-It). A random sample of 10 patients has been selected for Sanger sequencing.

Statistical Analysis

Continuous variables were presented as mean, standard deviation, and median. Chi-squared tests were used to compare the categorical data. The results were analyzed statistically using independent *t*-test and analysis of variance (ANOVA) test; $p \leq 0.5$ was assumed significant. All the calculations were performed using Statistical Package for the Social Sciences from IBM (SPSS), version 24.

Results
Demographic and Clinical Characteristics of Patients with Generalized Vitiligo

The mean age of vitiligo patients and controls were (28.8 ±12.4) years and (33.0±12.7) years, respectively. Age varied from 7–50 years in patients and from 9–49 years in the healthy control. Mean age of onset of patients with generalized vitiligo (21.53 ±12.77) years. Females with vitiligo were 57 (71.2%) compared to 23 males (28.8%). The control group consisted of 18 females (45%) and 22 males (55%).

Fitzpatrick's skin phototype IV (light brown skin) constituted the most common skin phototype of the patients (53.0%). Vitiligo vulgaris was the most frequent variant of generalized vitiligo (n = 71; 88.8%) followed by acrofacial vitiligo (n = 5; 6.35%). Cosmetic appearance recorded to be the most frequent chief complaint in patients and accounted for 67 patients (83.7%). The majority of the patients (70%) had vitiligo for more than 2 years. Positive family history among first-degree relatives was 22.5%. Grade +2 vitiligo disease activity (VIDA) score was the most common grade of vitiligo activity reported in 35 patients (43.8%). Vitiligo extent score was between 2–10 and found in 30 patients (37.5%). Table 1 shows the demographic and clinical characteristics of patients with generalized vitiligo.

Serum Level of TNF-α in Patients and Controls

In the current study, serum concentration was measured both in patients with generalized vitiligo and the clinically healthy control using ELISA. The TNF-α concentration was significantly higher among the patient group versus the age- and sex-matched healthy control group (*p*-value 0.01) (Table 2, Figure 1).

Odds ratio for high level TNF-α (more than cutoff point which is found by ROC curve as 5.7) is 2.81 with 95% CI (1.285 to 6.143), *p*-value (0.01), that indicates a significant elevation of TNF-α among patients with generalized vitiligo compared to healthy controls.

Correlation of Serum Level of TNF-α with Patient Demographic and Clinical Characteristics

The elevated serum level of TNF-α was analyzed in relation to age, gender, disease duration, family history of vitiligo, and activity of vitiligo (based on VIDA score and VES scores, both used to determine extent of skin surface involvement). There was no significant correlation of elevated serum level of TNF-α with the mean age of the patients (*p*-value 0.31), age of onset (*p*-value 0.33), gender (*p*-value 0.55), positive family history of vitiligo (*p*-value 0.78) and duration of generalized vitiligo (*p*-value 0.27). Using the VIDA score, the correlation of serum TNF-α concentration with the activity of generalized in patients with active generalized vitiligo was statistically significantly (*p*-value 0.01), higher serum level in more active disease. Using VES score,

Table 1 Demographic and Clinical Characteristics of Patients with Generalized Vitiligo

Variables	Frequency	Percentage
Gender		
Female	57	71.2%
Male	23	28.8%
Fitzpatrick's skin phototypes		
II (Fair skin blue eyes)	11	1.3
III (Darker white skin)	20	25.0
IV (light brown skin)	43	53.0
V (Brown skin)	16	20.0
Clinical variants of vitiligo		
Vitiligo vulgaris	71	88.75
Acrofacial vitiligo	5	6.3
Vitiligo universalis	3	3.8
Mixed vitiligo	1	1.25
Chief complaint		
Cosmetic appearance	67	83.8
Cosmetic appearance and pruritus	10	12.5
Pruritus	2	2.5
Sun burn	1	1.3
Duration of presentation		
Vitiligo > 2 years	56	70.0
Vitiligo ≤ 2 years	24	30.0
Family history of vitiligo		
Positive for first degree relative	18	22.5
Positive for second degree relative	16	20.0
VIDA score		
+4 Activity of 6 weeks or less	7	8.8
+3 Activity of 6 weeks to 3 months	11	13.8
+2 Activity of 3–6 months	35	43.8
+1 Activity of 6–12 months	13	16.3
0 Stable for 1 year or more	4	5.0
−1 Stable with spontaneous re-pigmentation for ≥1 year	9	11.3
VES score		
< 2	31	38.8
2–10	30	37.5
10–20	11	13.7
>20	8	10.0
Associated leukotrichia	24	30
Premature hair greying	15	18.8
Positive Koebner phenomenon	22	27.5

Notes: Mean age ± SD (years): (28.8 ± 12.4). Mean age of onset ± SD (years): (21.53 ± 12.77).

correlation of skin surface involvement with serum level of TNF-α was statistically not significant among the four groups of VES score (p-value 0.98) (Table 3).

Table 2 Serum Concentration of (TNF-α) of Patients and Control Groups

TNF-α Concentration (pg/mL)		
	Range Values	Mean ±SD
Patients (n = 80)	3.06–80.07	12.92 ± 14.40
Controls (n = 40)	2.64–22.51	6.43 ± 4.24

Note: p-value< 0.05; significant.
Abbreviations: SD; standard deviation; pg/mL, picogram/milliliter (concentration of TNF-α in the serum).

TNF-α −308 G/A Promoter Polymorphism

Single nucleotide polymorphism in the promoter region of TNF-α (−308G/A) in patients with generalized vitiligo and clinically healthy control subjects was detected using ARMS-PCR. The distribution of genotypes and alleles of TNF-α (−308G/A) is shown in Table 4. No statistically significant difference between the allele and genotype frequencies for TNF-α (−308G/A) was seen, both in patients and the control (p-value 0.21). Odds ratio for genotype G/A compared to G/G genotype was 1.12 with 95% CI (0.469 to 2.676) which means it was not significant (p-value 0.798). The result of Sanger sequencing on PCR product for 10 random samples were comparable to that of ARMS–PCR.

Correlation of TNF-α −308 G/A Promoter Polymorphism with Demographic and Clinical Characteristics of Patients with Generalized Vitiligo

TNF-α −308GA polymorphism has been analyzed in correlation with mean age, onset of the disease, gender, family history, VIDA score and VES score. A statistically non-significant correlation was found between TNF-α −308G/A polymorphism with age (p-value 0.80), age of onset (p-value 0.74), gender (p-value 0.82), positive family history of vitiligo (p-value 0.23), VES score (p-value 0.76) and VIDA score (p-value 0.66) (Table 5, Figure 2).

Correlation of Serum Level of TNF-α with −308 G/A Promoter Polymorphism

Elevated serum level of TNF-α was significantly associated with both TNFA1 (TNF-308G) wild alleles (p-value 0.04) and TNFA2 (TNF-308 A) rare allele (p-value 0.03) (Table 6).
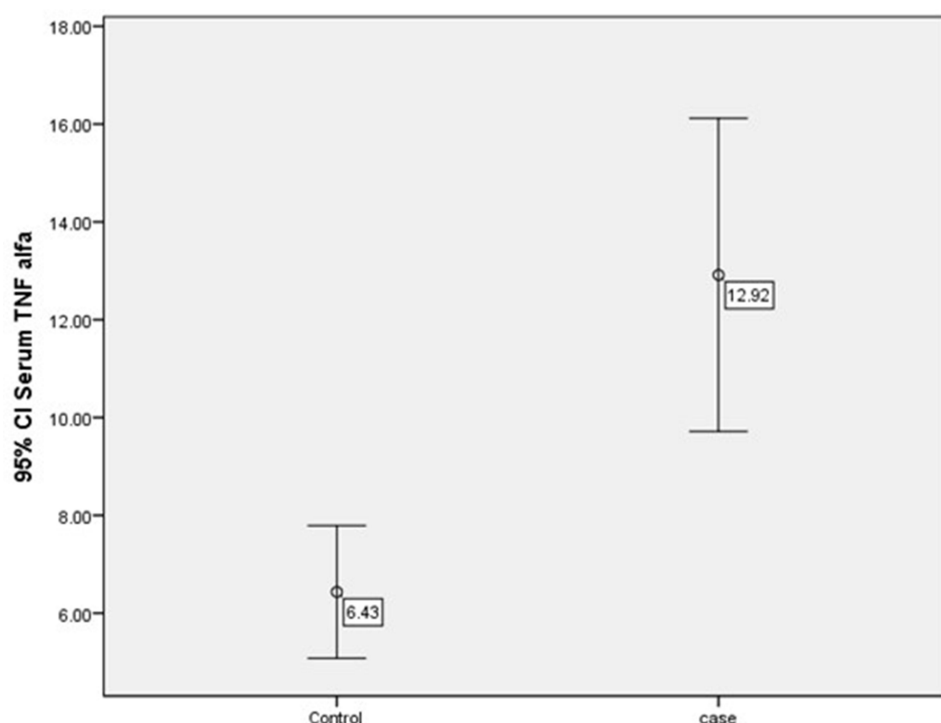
Figure 1 Error-bar: differences in serum TNF-α level in patient and control groups.

Discussion

Vitiligo is a polymorphous, multifactorial, and polygenic disease characterized by loss of melanocytes from the epidermis and hair follicle reservoirs.[23] Many studies indicated that vitiligo is mostly acquired early in life, and the majority of patients present in the second or third decade of life.[24]

In our study, 22 patients (27.5%) were younger than 20 years old, these results were comparable to that of Al-Mutairi and Al-Sharma,[25] Sonja et al[26] and Oguz Topal et al.[27]

Mean age of onset (±SD) was 21.53±12.77, this is consistent with a study in India by Mahajan et al[28] that reported the mean age of onset of vitiligo as 20.5 years for 945 patients, and a study by Oguz Topal et al in Turkey 30.2±16.9 years.

Although vitiligo is found in both sexes, some studies reported it to be more common in women.[29,30] In the current study, 57 (71.2%) patients were female compared to only 23 (28.8%) males and male to female ratio (0.4:1). Study by Akay et al[31] in Turkey also reported 80 patients with vitiligo consisted of only 30 males (37.5%) with the rest 62.5% female. In two other studies one in Turkey by Gönül et al[32] and the other in China by Lu et al,[33] male to female ratio were more similar. Difference in the sample size and female patients seeking treatment more than males may explain the difference in the result between these studies.[34]

In our study, vitiligo vulgaris was the most frequent variant of generalized vitiligo, accounting for 71 (88.8%), followed by acrofacial vitiligo 5 (6.35%), vitiligo universalis

Table 3 Correlation of Serum TNF-α Level with Clinical Characteristics

Variables	Serum Level of TNF-α (Mean ±SD) pg/mL	p-value
Family history of vitiligo		0.78
Positive	13.39 ± 13.49	
Negative	12.49 ± 15.32	
Duration of the disease		0.27
≤ 2 years	15.62 ± 18.99	
> 2 years (n= 56)	11.76 ± 11.93	
VIDA score		0.02
−1	6.10 ± 4.63	
0	22.98 ± 16.91	
1	8.27 ±6.22	
2	16.78 ±8.11	
3	12.51 ±4.99	
4	13.20 ±10.89	
Vitiligo extent score		0.98
< 2	12.37±16.90	
2–10	13.77±14.20	
10.1.20	12.79 ± 10.97	
> 20	12.02 ± 10.12	

Table 4 Genotype and Allele Distribution of TNF-α (−308G/A) in Patient and Control

Genotype and Alleles	Patients (No.)	Controls (No.)	Total	p-value*
Genotype				
Homozygous G/G	46	27	73	0.21
Heterozygous G/A	21	11	32	
Homozygous A/A	13	2	15	
Total	80	40	120	
Alleles				
G (Wild)	67	38	105	0.31
A (Rare)	34	13	47	
Total	101	51	152	

Note: *p-value< 0.05; significant.

Table 5 Correlation of Serum Level of TNF-α with −308 GA Promoter Polymorphism

Serum TNF-α/-308 GA Polymorphism	TNF-α Concentration (Mean±SD) in pg/mL		p-value
	Patients	Controls	
Homozygous G/G	13.51 ± 16.56	6.85 ± 4.79	0.046
Heterozygous G/A	11.50 ± 7.81	5.84 ± 2.93	0.03
Homozygous A/A	12.96 ± 14.53	4.06 ± 0.62	0.42

Note: p-value < 0.05; significant.
**Abbreviations:** SD, standard deviation; pg/mL, picogram/milliliter concentration of TNF-α in the serum.

3 (3.8%), and mixed vitiligo was recorded in only one (1.3%) patient, similar results were also found in earlier studies by Oguz Topal et al[27] in Turkey, Mahajan et al[28] and Shah et al[35] in India. Cosmetic appearance and/or disability was the most frequent complaint(s) (67 patients; 83.7%) to enroll more females into this study. Cosmetic appearance associated with pruritus was recorded in 10 (12.5%) patients while sunburn was the chief complaint for only one patient (1.3%). A study done by Kanwar et al[36] found the majority of the participants experienced significant photosensitivity as a second most common complaint to itching in addition to cosmetic concern.

A positive family history for vitiligo among first-degree relatives of patients with vitiligo was 22.5%, while 20% of patients had a positive family history of vitiligo among their second-degree relatives. A study by Mahajan et al[28] showed that among 945 participants, a positive family history of vitiligo was found in 150 (15.9%) patients with first-degree relatives being 9.5% and second/third-degree relatives in 6.3% of patients.

Shah et al[35] reports positive family history of vitiligo in half of the enrolled cases.

TNF-α is a major cytokine initiating apoptosis of melanocytes in vitiligo[37] Adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) are overexpressed in melanocytes from vitiligo lesions, and cytokines such as TNF-α can induce their expression on the surface of epidermal melanocytes[38,39] This pathway could influence melanocyte target recognition by T-cells, and mediate immunologic cytotoxic damage. TNF-α can inhibit melanogenesis by decreasing the intracellular levels of tyrosinase and tyrosinase-related protein 1 an abundant melanosomal glycoprotein involved in both melanogenesis and prevention of melanocyte death.[40,41] In our study, there was a significant elevation of serum TNF-α among patients with generalized vitiligo. Similar results were found in earlier studies by Laddha et al,[42] Sushama et al[43] and Singh et al,[44] although the result was not statistically significant in the later study. Statistically no significant difference was found between serum level of TNF-a with each of age, age of onset, and gender. A study by Laddha et al,[42] showed significantly higher transcript and protein levels of TNF-a in female patients compared to males. Mean (±SD) of TNF-α serum concentration for 24 patients with duration of vitiligo ≤2 years was 15.62 ± 18.99 (pg/mL) and for 56 patients with duration of vitiligo >2 years was 11.76 ± 11.93 (pg/mL). A negative correlation was found between serum level of TNF-α and duration of generalized vitiligo (p-value 0.27). Sushama et al[43] was the first to find a negative correlation of serum level of TNF-α with the duration of vitiligo, like our study. In the current study, in 56 patients (70%) there were appearances of new white macule of vitiligo while in the remaining 24 patients (30%) there were enlargement of preexisting vitiligo lesion (as a marker of disease activity over variable periods of time using VIDA score). We found that patients with active generalized vitiligo have a significantly higher TNF-α level, a similar result was reported by Laddha et al[42] and Aydingoz et al.[45] A negative correlation of serum level of TNF-α and extent of skin involvement is found in our study, similar to a study by and Laddha et al[42] but contrary to a study by Sushama et al[43] that found elevated serum level of TNF-α among localized and generalized skin surface involvement groups. The underlying mechanism that results in the increase in the serum level of TNF-α is unclear. There are several SNPs in the promoter region of TNF-α gene that affected the expression and thus elevation of the serum level
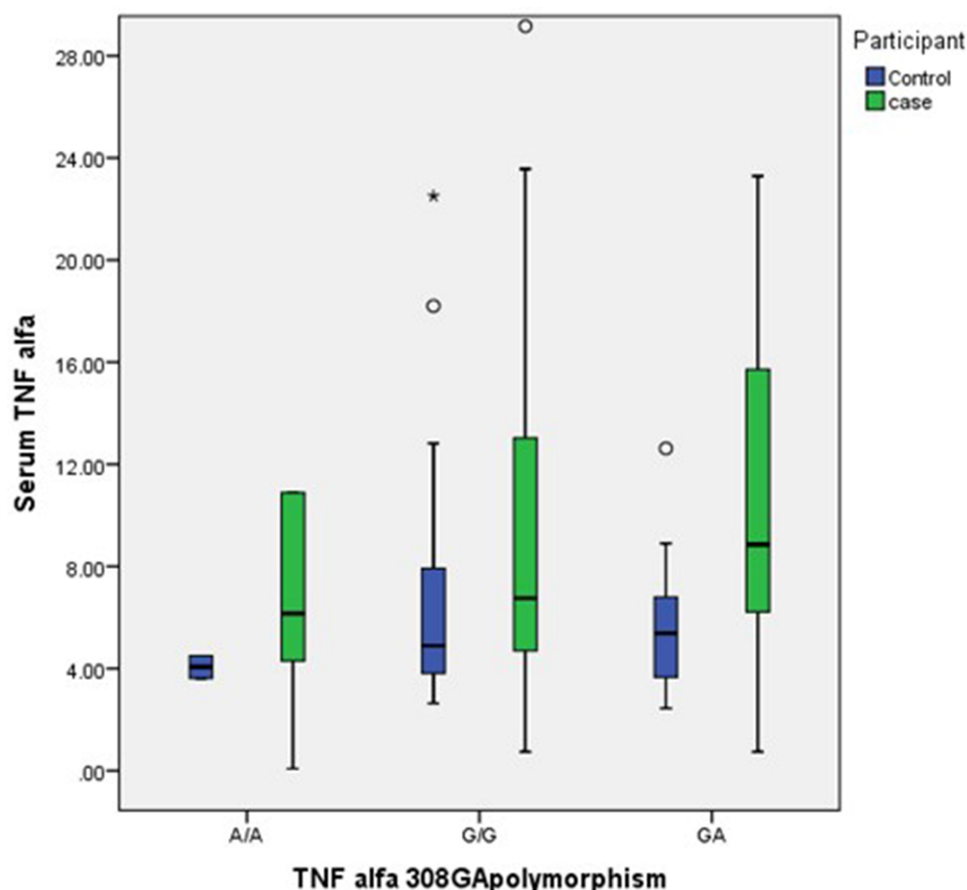
Figure 2 Box plot showing correlation of serum TNF-α level with genotypes and allele frequencies in patient and control groups.

of TNF-α[46,47] These include the association of SNPs at the promoter region of TNF-α (*−308 GA*); its association with vitiligo has been studied in different populations. To our knowledge, there are no reported or published works studying the Iraqi population, our study will be the first on this topic.

TNF-α *−308G/A* polymorphism displays increased gene transcription. Allele A (TNF-α 2 allele) lies on the extended haplotype *HLA-A1-B8-DR3-DQ2* and has been demonstrated to be a much stronger transcriptional activator that produces levels of TNF-α transcription 6–7 fold greater, this is associated with high TNF-α production. Moreover, TNF-α (*−308G/A*) promoter polymorphism has a direct effect on the TNF-α gene regulation (increased TNF-α expression) and may be responsible for association of allele A with the high TNF-α phenotype and more severe diseases.[48] A single-base polymorphism within the promoter region of TNF-α gene results in 2 allelic forms, one in which guanine defines the common allele (TNFA1) (homozygous G/G) and the other in which guanine is substituted by adenosine forms the rarer allele (TNFA2)

(heterozygous G/A). Serum level of TNF-α is elevated among patients with homozygous G/G allele compared to controls (*p*-value 0.046). Similarly, an elevated serum level of TNF-α among patients with heterozygous G/A was found compared to the control group (*p*-value 0.03). Comparative statistical analysis of serum level of TNF-α with different genotypes done with the *p*-value of 0.21, and with different alleles *p*-value of 0.31 was found. This result is similar to Yazici et al[49] in Turkey who reported a lack of difference in G/G and G/A alleles distribution between study group (*p*-value > 0.05) Our study is also comparable with that done by Odeh et al that reported similar results in the Jordanian population.[50]

Our study is in contradiction with two earlier studies by Aydıngöz et al[51] in Turkey and Al-Harthi et al[51] in Saudi Arabia, both confirmed increased TNF-α *−308G/A* polymorphism with increased levels of this cytokine is patients with vitiligo. These conflicting results can be explained by differences in distribution of HLA alleles among different populations and within subgroups of the same population. The two studies in Turkey were between two different

Table 6 Correlation of TNF-α Promoter Polymorphism with Patient Clinical Characteristics

Variables	TNF-α Promoter Polymorphism			p-value
	G/G	G/A	A/A	
Age (years)				
Mean ±SD	34.5± 7.0	24.5 ± 8.0	19.0±9	0.8
Onset of the disease	22.22 ± 12.98	21.29 ± 12.80	19.46 ± 12.69	0.79
Gender				
Males	13	6	4	0.97
Female	34	14	9	
Family history of vitiligo				
Positive	20 (43.5%)	9 (42.9%)	9 (69.2%)	0.23
Negative	26 (56.5%)	12 (57.1%)	4 (30.8%)	
VIDA score				
−1	4 (8.9%)	1 (4.8%)	2 (15.4%)	0.66
0	5 (11.1%)	4 (19%)	2 (15.4%)	
1	20 (44.4%)	11 (52.4%)	4 (30.8%)	
2	9 (20%)	2 (9.5%)	2 (15.4%)	
3	2 (4.4%)	0 (0%)	2 (15.4%)	
4	5 (11.1%)	3 (14.3%)	1 (7.7%)	
VES score				
< 2	5 (11.1%)	3 (14.3%)	1 (7.7%)	0.76
2–10	15 (32.6%)	8 (38.1%)	7 (53.8%)	
10–20	6 (13%)	3 (14.3%)	2 (15.4%)	
> 20	4 (8.7%)	3 (14.3%)	1 (7.7%)	

subgroups with different results. Differences in the sample size between the various studies may also explain the different results as well.

Conclusion

Serum TNF-α is significantly elevated among Iraqi patients with generalized vitiligo. No difference was found between serum levels of TNF-α with TNF-α polymorphism at position *−308* (TNF *−308*). This involves substituting the G allele for the A allele.

Abbreviations

TNF-α, tumor necrosis factor alpha; SNP, single nucleotide polymorphism; ELISA, enzyme linked immunosorbent assay; ARMS–PCR, amplification refractory mutation system polymerase chain reaction; VIDA score, vitiligo disease activity score; VES score, vitiligo extent score; SD, standard deviation; ANOVA, analysis of variance; pg/mL, picogram/milliliter.

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