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Article

The Association of TNFα -308 Polymorphism and TNFα serum level in a sample of Multiple Sclerosis Iraqi patients

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ABSTRACT
Tumor necrosis factor-alpha TNF-α) is a pro-inflammatory cytokine that is involved in the pathogenesis of Multiple Sclerosis. The current study was designed to examine the association between TNF-α level and TNF-α gene polymorphisms in Multiple Sclerosis of Iraqi patients. Blood samples were collected from fifty Iraqi patients who suffered from Multiple Sclerosis (20 men and 30 women) with ages ranging between 23-54 years, and 50 healthy volunteers as a control group. The serum level of TNF-α was detected by using an Enzyme-Linked Immuno-sorbent assay (ELISA), and TNF-α-308 gene polymorphism was assessed by TaqMan Real-time Polymerase Chain Reaction (Taq-RT-PCR). The results of the estimation of TNF-α level showed high elevation in the patients' group (4.88 ± 0.17 pg/ml) with a high significance difference (P≤0.01) as compared with the control group (2.96 ± 0.09 pg/ml). While detection of TNF-α-308 polymorphism in MS patients revealed that the wild genotype G/G was 3 (6.00%), heterogeneous genotype GA was 15 (30.00%). Homogeneous genotype AA was 32 (46.00%), while G allele frequency was 0.21 and A allele was 0.79 with significant difference (P≤ 0.005) and even as in control G/G genotype was 47 (94 %), GA genotype was 3 (6.00%), AA genotype was 0 (0.00%), G allele frequency was 0.97. A allele was 0.03 with significant difference (P≤ 0.01). The result revealed a significant difference between TNF-α-308 genotype and TNFα serum level in MS patients and control.

Keywords: TNF-α-308, MS, ELISA, Taq-PCR.

INTRODUCTION

Multiple Sclerosis (MS) is a chronic inflammatory demyelinating and neurodegenerative disease. It is a heterogenic and multifactorial disease. Like most other autoimmune disorders, MS is associated with the factors involved in immune responses, such as cytokines[1]. Cytokines are one of the most important factors in the regulation of inflammatory immune response; therefore, they can affect the pathogenesis of autoimmune diseases such as

MS. Tumor necrosis factor alpha (TNFα) is a potent inflammatory cytokine, which stimulates cytokine and has a crucial effect of on the immune responses. Because TNFα increases inflammation, they can influence the pathogenes of the MS[2]. Some single nucleotide polymorphisms (SNPs) were found in the promoter of the TNFα gene. Among these SNPs, TNFα-308 G/A polymorphism has been studied in several diseases. Substitution of Guanine (G) nucleotide in -308 positions creates G allele, which is the common allele, while substation of Adenosine (A) nucleotide in this position makes the rare allele. The rare allele with unknown exact mechanisms was found in higher expression of TNFα. But in contrast to these studies, some research reported the other effect of TNFα-308 A allele on the expression or production of TNFα gene[3]. Among different studies on the TNFα-308 position, only a few studies demonstrated a significant association of this position with MS, whereas their results were inconsistent. Given the importance of the topic, this study aimed to investigate the association between TNFα-308 polymorphism and serum TNF levels in multiple sclerosis Iraqi patients.

MATERIALS AND METHODS

The study was carried out under the Ethics Committee on Human Research. Four ml of venous blood was taken from 50 patients suffering from Multiple Sclerosis (20 men and 30 women) with mean age (23-54 years), and 50 healthy volunteers as a control group. Each sample was divided into two parts; first, 2 ml was put in a gel tube, then left the tube for 10- 30 min to allow for clotting of serum then centrifuged for 10 min at 3000 rpm and kept in the (-20 °C) until use for evaluation of TNF-α serum level by ELISA; the rest 2ml was put into EDTA anticoagulant tubes, mix gently and stored at (-20 ° C) till subjected to DNA extraction for detection TNF-α 308 (rs1800629)by Taq Man-Polymerase Chain Reaction (Taq-PCR) method.

Estimation of TNF-α serum level

Estimation of TNF-α serum level was done by using the ELISA technique and TNF-α ELISA kit (Changsheng/China), according to Mohammed[4].

Genomic DNA extraction

Genomic DNA was extracted from frozen blood using a DNA purification kit (Promega/USA). According to Mohammed [5], DNA purity in ng/µl and concentration within the accepted ratio 260/280 nm were determined by Quantus Fluorometer (Promega/USA).

Detection of TNF-α -308gene polymorphisms

Detection of TNF-α- 308 (rs1800629) gene polymorphisms was done by TaqMan-Polymerase Chain Reaction (Taq-PCR) using a specific primer supplied by Genomic deoxyribonucleic acid (gDNA) Miniprep System (Promega/ USA), depending on NCBI as illustrated in Table (1).

Primer	Size bp	Forward
F	37	5-GGACCCTGGAGGCTGAAC-3
R		5-TCCTGCATCCTGTCTGGAA-3

Table 1: The TNF-α- 308 (rs1800629) primer sequences

Component of PCR mixture reactions of 10µl volume including GoTaq Green Master mix (Promega/USA) as shown in Table (2)

Master-mix components	Volume µl
Master Mix	5
Forward primer	0.5
Reverse primer	0.5
Probe 1	0.5
Probe 2	0.5
Nuclease Free Water	2
DNA	1

Table 2: Reaction component for PCR reactions

The PCR amplification was done using the Taq-PCR method by RT-PCR device with Mic Tube (BioMolecular System / Australia)according to the program shown in Table(3).

Steps	°C	M:S	Cycle
Initial Denaturation	95	05:00	1
Denaturation	95	00:30	40
Annealing	55	00:30	
Extension	72	00:30	

Table 3: Amplification fragment 37 bp PCR program

Statistical analysis

Results analysis was done by using the program Statistical Analysis System-SAS[6] to estimate the effect of different factors in work parameters. T-test was used to significantly compare between means, and the Chi-square test was used to significantly compare between percentages.

RESULTS

The result of distribution of MS patients according to age revealed that the highest incidences rate appeared in (30-39) year age group which reached to 28 (56%), followed by(40-49) year age group with 10 (20%), while (20-29) year age group represented 9 (18%), and 50≥ year age group had lowest percentage 3 (6%), with high significant difference (P≤0.01) as clarified in Table (4)

Age group (years)	Patients. (%)
20-29	9 (18%)
30-39	28 (56 %)

40-49	**10 (20%)**
≥50	**3 (6%)**
P-value	**0.0001 ****
**** (P≤0.01).**	

Table 4: Distributions of MS patients according to age

The finding of distribution of MS patients group according to MS family history revealed that (80%) nonexistent MS family history and (20%) existent MS family history with a high significant difference (P≤0.01) as shown in Table (5)

Family history	No	Percentage (%)
Yes	**10**	**20%**
No	**40**	**80%**
Total	**50**	**100%**
P-value	**---**	**0.0001 ****
**** (P≤0.01).**		

Table 5: Distribution of patients according to MS Family history in the patient group

The outcome of distribution of MS patients according to treatment showed Interferon-ß medicine with trade name Beta feron had the largest percentage in treatment of study sample 20 (40%), followed by Fingolimod with trade name Gilenya 15 (30%), Retuxan with trade name Rituximab 12 (24%), and Natalizumab with trade name Tysabri drug had the lowest percentage 3 (6%) with high significant (P≤0.01) as illustrated in Table (6).

Treatment	No	Percentage (%)
Interferon-ß (Beta Feron)	**20**	**40.00 %**
Retuxan (Rituximab)	**12**	**24.00 %**
Fingolimod (Gilenya)	**15**	**30.00 %**
Natalizumab (Tysabri)	**3**	**6.00 %**
Total	**50**	**100%**
P-value	**---**	**0.0027 ****
**** (P≤0.01).**		

Table 6: Distribution of MS patients according to treatments

The result of the distribution of TNFα -308 (rs1800629) Polymorphism and allele frequency in patients and control groups revealed that the wild genotype G/G was 3(6%), heterogeneous genotype GA was 15 (30%), and homogeneous genotype AA was 32(64%), while G allele frequency was 0.21 and A allele was 0.79 with significant difference (P≤ 0.005) and even as in control G/G genotype was 47 (94%), GA genotype was 3 (6%), AA genotype was 0 (0.00%), G allele frequency was 0.97. Afrequency allele was 0.03 with significant difference (P≤ 0.01) as shown in Table (7).

Genotype G/A	Patients No. (%)	Control No. (%)	Chi-Square (χ^2)	P-value	OR (CI.)
Wild: GG	3 (6%)	47 (94%)	38.72 **	0.0001	Ref. =1
Hetero : GA	15 (30%)	3 (6%)	8.00 **	0.0047	1.06(0.71-1.83)
Mutant: AA	32 (64%)	0 (0.00%)	31.96 **	0.0001	1.89(0.96-3.67)
Total	50 (100%)	50 (100%)			
Allele	Frequency				
G	0.21	0.97	Ref. =1		
A	0.79	0.03	OR (CI) = 1.77 (0.91-3.72)		
** (P≤0.01).					

Table 7: Genotype distribution and allele frequency of TNFα308 (rs1800629) in patients and control groups

The results of the comparison between serum TNFα levels in MS patients and control groups revealed a noteworthy increase in the patient's serum level (4.88 ± 0.17pg/ml) as compared to the control group (2.96 ± 0.09pg/ml) with highly significant differences (P ≤ 0.01), as illustrated in Table (8) and Figure (1).

Group	Mean ± SEofserum TNFα (pg/ml)
Patients	4.88 ± 0.17
Control	2.96 ± 0.09
P-value	0.0001
T-test	0.432 **
** (P≤0.01)	

Table 8: Comparison between serum TNFα levels in MS patients and control groups

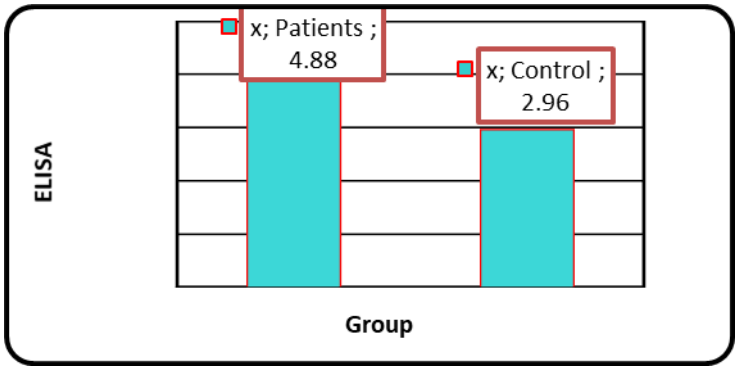

Figure 1: Comparison between serum TNFα levels in MS patients and control groups

The results of the relationship between TNFα serum level and TNFα-308 genotype in MS patients revealed that a substantial increase at AA genotype patients (P ≤ 0.01) in TNFα serum level (5.08 ±0.22pg/ml) followed by GA patients genotype (4.32 ±0.31pg/ml) and finally GG genotype patients (3.17 ±0.11pg/ml). Also, patients with at least one copy of the (A) allele had a higher risk of MS, as there was an association between the existence of the

mutant (A) allele heterozygous (GA) and homozygous (AA) with the serum TNFαin MS patients (Table 9).

Genotype	Mean ± SE
	TNFα serum (pg/ml)
GG	3.17 ±0.11
GA	4.32 ±0.31
AA	5.08 ±0.22
LSD value	0.569 **
P-value	0.0001
**(P≤0.01).	

Table 9: Relationship between TNFα serum level and TNFα-308 genotype in MS patients

DISCUSSION

This study found that the highest incidence rate of MS appeared in (30-39) years of age. It can be noticed that MS can attack individuals at any age. it also appeared that people over 50 were not considered the best candidate for this disease, but the preferred age group was (30-39) years. Also, the younger ones were rarely affected because they had perfect immune systems. However, studies have indicated infected younger age groups as[7] when reported the occurrence of the MS disease in pediatrics under 18 years of age. The reasons for that may be due to the modern lifestyle and nutrition based on fast food without paying attention to the basic elements of food and sitting long in front of the TV, computer and phone without going out to fresh air and exercising. The same results have been recorded in other studies, such as [8], which suggested that the incidence of MS occurred in 30 persons. The present result agreed with the study of 9, which explained that the prevalence of MS occurred at thirty years of age. However, some of the previous results contradicted the current study because of the interaction of many factors with the pathogenesis of the disease, as a study of [10] did not observe statistically significant changes between the ages of 35 and 32 years. The distribution of MS patients according to other autoimmune disease family history showed a highly significant (P≤0.01) increase in patients' number with no family history of another autoimmune disease, in contrast to the patients with a family history of autoimmune diseases. The explanation for this result could be that MS disease is considered hereditary with 20 %. This led to the study of another immune disease to prove this part of the study, so in comparison with others [11,] the probability of autoimmune diseases in patients with MS could be higher with several causes (older patients, longer duration of disease, and also in patients with higher age at the time of MS diagnosis). Other studies are needed to confirm results and for the main cause of the difference in the prevalence and type of autoimmune diseases and MS varying, but it could be due to the similarity of genetic factors, immune pathways, and environmental factors, which support the idea that it seems this area from the world could differ from other areas[12]. The present outcome was approved with a research of [13] who suggested no sign of familial autoimmunity when 265 families from the Multiple Autoimmune Disease Genetics Consortium (MADGC) examined

the prevalence of autoimmune diseases (ADs) among relatives of MS families. The study sample of 50 patients who suffered from MS had taken many treatments, some of them from a long time since the beginning of their onset of the disease, others had not; some of them used treatment and changed to another within time and according to the degree of the disease and benefit from the treatment to them. According to the results obtained in the current study, the drugs listed in Table (6) were the most frequently used in the study sample. In support of this result, many previous studies have been received that agreed with this result, as [14] reported that Beta-Feron, Rituximab and Gilenya showed a response to treatment of the patients compared to other treatments, which affects the autoimmunity of patients. Interferon-ß (Beta-Feron)was shown to have a higher response than in Fingolimod (Gilenya) treatment, depending on the duration of treatment and the EDSS. Fifteen suggested that Interferon-ß (Beta-Feron) has immune-modulatory and anti-proliferative properties, considered the most affected drug in MS patients. It also discussed that Fingolimod (Gilenya) effects, as mediated by modulation of sphingosine1-phosphate receptors, inhibit the egress of lymphocytes from lymph nodes and may have direct effects on the central nervous system. So, [17] had the same dialogue on the importance of Retuxan (Rituximab)as the first oral treatment used for MS, the next treatment. Also, one of the treatments used in MS cases is a chimeric monoclonal antibody directed at CD20, demonstrated to reduce inflammatory activity in patients with relapsing–remitting MS (RRMS). The current result showed the last but not the least treatment was Natalizumab (Tysabri). the importance of using this medicine agreed with the opinion of [18] who reported that this medicine is a highly specific α4-integrin antagonist used for severe relapsing-remitting MS. Failure to respond to other treatments can affect brain MRI or a significant increase of T2 burden, but it is not preferred to be used because the risks of inadequately treated MS or progression of the disease, and the relative benefit–risk profiles of alternative treatments. From the above results, it can be concluded that patients with at least one copy of the (A) allele had a higher risk of MS. Single-nucleotide polymorphisms are the most commonly studied genetic variations for disease susceptibility, and functional SNPs located in a gene's promoter region regulate that gene's transcriptional expression. The TNF-α is an important cytokine of the inflammatory response involved in the pathogenesis of MS. The TNF-α −308 Polymorphisms were implicated in MS risk[19]. Present results were consistent with preceding studies as [20] showed that the TNF-α −308 G/A polymorphism was observed as statistically significant when investigating the association between the polymorphism of IL-10 gene and susceptibility to MS. Furthermore, [21] found significant association between -308G/A polymorphism of TNF-α and MS in both allele model GA and dominant model AA in Asia population when compared with Europe population. Also, research of [22] cleared that the A allele appeared to be linked to increased risk of MS and suggested that -308G/A of the TNF-α gene was a risk factor for MS. While current results disagreed with the study of Wang et al· [21] who discussed in demographical study association between -308G/A polymorphism of TNF-α and found decreased risk of MS in Europe population, that may be referred to allele of this area is differ from other areas of the world. The above results showed a significant increase in the serum level of TNFα protein, which confirmed the role of this cytokine in inflammatory processes and resistance to infections

in the normal state and in interfering with autoimmune pathogenesis in abnormal cases. Such a result came almost compatible with a result of [23] that clarified elevated TNFα serum levels of MS patients and showed a significant variation between pre- and post-medicated patients with EDSS**.** Almost this result agreed with the previous study of [24] when it observed that serum TNF-α concentration of hospitalized MS patients was significantly increased than in convalescent once. From the results of Table (9), it has been demonstrated that the G/A polymorphism of TNF-a 308 directly affects TNF-a gene regulation, and the A allele is associated with a high level of TNF-a production. That's agreed with several clinical studies as [25] who suggested that diminished TNF-a activity was associated with the onset of central nervous system (CNS) inflammatory lesions and the TNF-a pathway is implicated in MS susceptibility, anti-TNF-a therapy in subjects with other diseases was associated with inflammatory demyelinating affecting the CNS and MS aggravation. TNF-α gene plays an important role in the pathogenesis of autoimmune diseases. the -308 G/A TNF-α promoter polymorphism appears to be closely associated with the development of these diseases; however, some conflicting results have been documented [26]. Also, a study of [27] was partially consonant with the current result when investigating the association of TNFA-308G > A with impaired cognitive functions in RRMS patients. It showed that the AG genotype was associated with higher serum TNF-a than the GG genotype. Also, the study of [28] somewhat agreed with this result and showed that the level of TNF-α in the serum of patients with RRMS was a significant association with the −308G/A TNF-α polymorphism and gender dependency when they explored the association between the promoter polymorphism−308G/A in the TNF-α gene (rs1800629) with genetic susceptibility to RRMS.

CONCLUSION

It can be concluded that TNF-α played a critical role in immune responses and, subsequently, the MS susceptibility. Patients with at least one copy of the (A) allele had a higher risk of MS, as there was an association between the existence of the mutant (A) allele heterozygous (GA) and homozygous (GG) with the serum TNF-α status in Iraqi MS patients.

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Conflicts of interest

The authors declare no conflicts of interest.

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