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Association of Tumor Necrosis Factor Receptor Two Single Nucleotide Polymorphism with the Response to Etanercept in Iraqi Rheumatoid Arthritis Patients.

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A B S T R A C T

Rheumatoid arthritis (RA) is an autoimmune chronic inflammatory disease which characterized by many features like autoantibodies presence, inflammation of joints and destruction that lead to damage of articular cartilage. However, the prognosis of RA patients in the last decades has improved due to knowledge extension of the disease biology and etiology, which led to the creation of numerous active medications. Etanercept therapy has been widely used to treat Iraqi rheumatoid arthritis patients, but some of these patients did not respond to the treatment, which led to financial and moral losses. Given the importance of tumor necrosis factor alpha (TNF-α) RA disease, single nucleotide polymorphisms(SNPs) in tumor necrosis factor receptor 2 (TNFR2) was studied to find any possible relationship among genetic variation with RA disease susceptibility and response to treatment. The study included 60 RA patients and 25 healthy unrelated controls. Blood samples were taken and DNA extraction was done. The patients were followed up for (3-6) months then distributed for two groups, responders and non-responders to etanercept. The rs1061622 flanked region was amplified by polymerase chain reaction (PCR). The products of PCR were sent for sequencing. The results may indicate the protective role of TT genotype at rs1061622 in decreasing the susceptibility to RA. The TT wild type at rs1061622 was higher in non-responders group which may correlates with failure to response to etanercept.

Keywords: Rheumatoid arthritis, TNF-α, PCR, SNPs, rs1061622

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune chronic inflammatory disease which characterized by many features like autoantibodies presence, inflammation of joints and destruction that lead to damage of articular cartilage (Firestein and McInnes, 2017). However the prognosis of RA patients in the last decades has been enhanced due to knowledge expansion of the disease pathophysiology and etiology which lead to development of many active drugs (Smolen *et al*., 2018). The disease onset can arise at any age, the age of 50 years represents the peak of disease occurrence (Gibofsky, 2014). The smokers with a positive rheumatoid factor (RF) are up to three times more affected by RA than non-smokers (Sugiyama *et al*., 2010). The important breakthroughs in the understanding of RA is the recognition of lung as a main site of the initial pathogenic events of the disease and the strong correlation of RA with numerous airborne noxious. Smoking represent one of the most important of such exposures which can be the main risk factors for RA development (Malmström *et al*., 2017). Pathological features of RA involve inflammation and the swelling of synovial membrane. Innate and adaptive immunity contribute to the disease pathogenesis by cellular interaction between the two systems. The exact factors that triggers the inflammatory response at the affected joint are unclear. The activated synovial macrophages (SM) which are located at the cartilage–pannus junction secrete the proinflammatory cytokines, for example interleukin -1, interleukin-6 and TNF-α. Synovial macrophages are suggested to be the key regulator of inflammation in RA (Kinne *et al*., 2007). In RA, extra-articular manifestations are characterized by rheumatoid nodules, systemic comorbidities like weight loss, vasculitis, pulmonary affectations and cardiovascular diseases (Kim *et al*., 2020). Evidence has demonstrated the higher risk of RA patients to have depression and anxiety, which lead to worsens the disease state and reduce the conventional treatment responsiveness (Soósová *et al*., 2017). In inflammatory and autoimmune disorders, TNF plays different roles by inducing different cellular pathways (Sedger and McDermott, 2014). Macrophages and immune cells are the main TNF cellular source which produce TNF in response to tissue damage or infection (Fischer and Maier, 2015). TNF-α exists as a trans membrane and a soluble molecule which targets the natural two receptors, TNFR1 and TNFR2 (Brenner *et al*., 2015). The different effects of TNF-α is depend on its two receptors and their downstream distinct signaling pathways (Wajant and Siegmund, 2019). The activation of TNFR2 has many role such as promotes cell activation, proliferation and migration, which plays protective role in the cells and affects the function and amplification of Treg, also it mediates apoptosis by in cooperation with TNFR1 as shown in figure (1-5) (Medler and Wajant, 2019). the using of TNF-α inhibitors in autoimmune diseases have revolutionized the strategy of treatment used in these diseases,

indicating improved efficacy of therapy when compared to another treatments (Jang *et al*., 2021) . Therefore, the application of anti-TNF therapy to combat such diseases have been applied successfully even though it can have a number of o combat some of these diseases even though it can also have some important adverse effects (Cho *et al*., 2019). Etanercept is a biologic inhibitor of TNF-α which is commonly used in controlling plaque psoriasis, psoriatic, ankylosing spondylitis, arthritis, juvenile idiopathic arthritis and RA. Etanercept, induce inhibition of the inflammatory response in skin and joints which are distinctive features of these autoimmune diseases. The administration of drug can be as monotherapy or can be taken along with additional immune suppressants drug, such as methotrexate (Davis *et al*., 2003). Although there is a strict of drug pre-marketing trials, the medicines safety is not understood completely because the achieved results of pre-authorization clinical trial are obtained in controlled settings which are different from real-world settings (Gagliardi *et al*., 2022). The presence of polymorphism in some loci in the TNF-α receptors has shown a positive relationship with the response to treatment, which makes pre-screening of polymorphism of these loci is very useful in choosing the appropriate drug depending on the genetic profile of the patients (Pallio *et al*, 2020).

MATERIAL AND METHODS

Sixty Iraqi patients with RA were chosen according to European League against Rheumatism (EULAR)/ American College of Rheumatology (ACR) Classification Criteria for RA to conduct an observational cross-sectional study. The blood samples were collected from the patients whom aged between 20 and 69 years old. The patients were diagnosed and treated for at least one year in the clinics of RA, Teaching Hospital in Medical City of Baghdad. Twenty five healthy unrelated individuals were also enrolled in this study through the period from September 2022 to February 2023. The patients sex were 48 Females and 12 males who chosen at baseline and followed up for 3-6 months. The RA patients were distributed into two groups, non-responders (primary failure) and responders after the follow up. This distribution was done according to laboratory results and clinical examination. Twenty four had a primary response failure to drug, while 36 patients achieved remission. The genomic DNA of the RA patient and healthy control have been extracted from blood samples by using DNA extraction kit supplied by Favorgen/ Taiwan. The PCR technique was used to amplify the flanked region of rs1061622 by using a pair of primers shown in table (1).

Table (1): primers used in the amplification of rs1061622 region

Primer		**Sequence (5'- 3')**	**Tm (C°)**	**Product Size(bp)**	**Location**
For rs1061622	Forward **(18b)**	GCACACATCGTCACTCTC	55.5	379	Chr 1 TNFRSF1B
	Reverse **(20b)**	AAGGAGTGAATGAATGAGAC	52.6		

The reaction mixture and conditions used in the amplification was listed in table (2) and (3) respectively.

Table (2): Components of reaction mixture

Component	**Volume (µl)**
Master Mix: *Taq*DNA Polymerase, dNTPs, MgCl2, and reaction buffer	12.5
Forward Primer	1
Reverse Primer	1
DNA Template	4
D.W.	6.5
Total	**25**

Table (3): PCR amplification program.

Steps	**Temperature (C°)**	**Time**	**No. of Cycles**
Initial Denaturation	94	5 min.	1
Denaturation	94	30 sec.	27
Annealing	58	30 sec.	
Extension	72	30 sec	
Final Extension	72	10 min.	1

The PCR products were sent to Macrogen Company (Korea) for sequncing. The (Geneious Prime) software was used to analyzed and compared the sequence results with the National Center for Biotechnology Information (NCBI) gene bank information. Hardy-Weinberg equation (HWE) and Chi-square ($\chi 2$) were calculated to investigate the distribution of our samples and whether it follows the normal distribution or not. The risk was determined according to the ODDS ratio, which was calculated by online software: "https://www.medcalc.org/calc/odds_ratio.php" to check the risk or productivity property for alleles and genotypes in interval confidence (CI estimate at 95%). The standard error of the mean (SEM) was calculated for continuous variables. The T-test was used to explore the presence of variation between parametric groups. Statistically significant values were considered when they had a p-value lower than 0.05.

RESULTS

Sixty cases of RA patients (12 Males: 48 Females) treated by etanercept were enrolled in this study. The patients were classified after (3-6 months) of follow up into two groups (responders and primary failure non-responders to etanercept). The responders group consisted of 36 (10 Males : 26 Females) patients, while the non-responders group was 24 (2 Males: 22 Females) patient. The patients age was in the rang 20-69 year with average (48 ± 1.35 SEM). The 24 non-responders age average was (50 ± 1.37 SEM), while the 36 responders age average was (47 ± 2.08 SEM). The BMI of the patients was around 30 which is higher than the reference population. The ESR level was higher in responders (53.2 ± 5.55 SEM) than non-responders group (42.3 ± 4.94 SEM). The purity of extracted DNA was ranged 1.8-2 with concentration ranged from 150-80 µg/ml. The region of rs1061622 was amplified by using specific primers and optimum conditions. The illustrated results in figure (1) showed a clear bands with (379 base pair) after electrophoresis of PCR products on agarose gel (2%). These fragments represent the target region in TNFR2 gene which contains rs1061622.

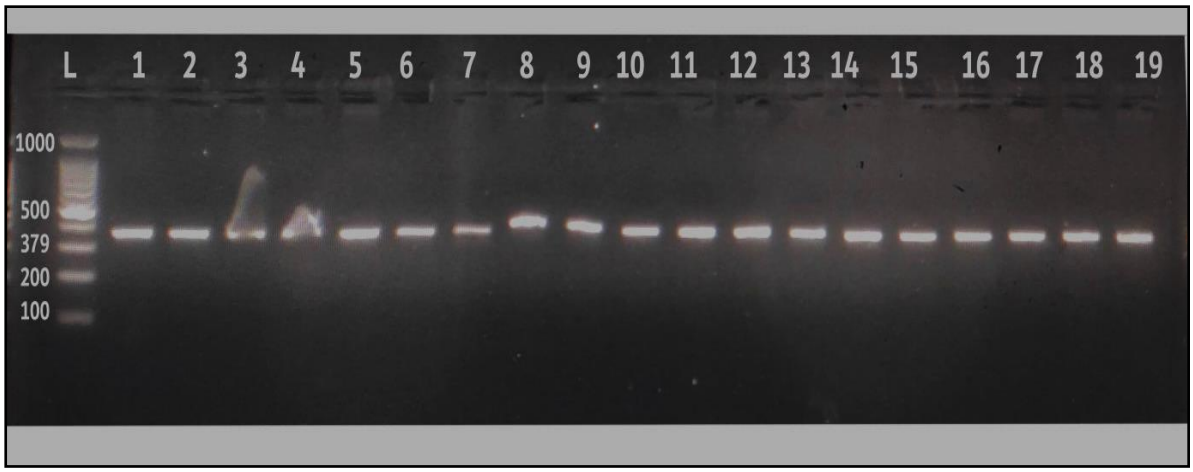

Figure (1): Gel electrophoresis of PCR products of the specific TNFR2 region that contain rs1061622 SNP on agarose gel (2%) by using 70v and 400 mA for 45 minutes with the presence ladder marker of 100bp.

Sanger sequencing method was used to achieve the sequence of amplified PCR products . The sequencing of the amplification products of the flanked regions of study SNPs by Macrogen/ USA. Then, the sequences of these products were compared with the reference data in the NCBI's GenBank for the TNFR2 genes. The results showed the RA patients have no significant difference within HWE equilibrium, but the controls group was significantly out of HWE normal distribution as shown in Table (4).

Table (4): Results of the HWE for the TNFR2 polymorphism rs1061622 T/G between RA patients and controls.

Groups	NO.	TT (%)	TG(%)	GG (%)	χ^2	P-value
RA patients	Observed	29 (48%)	27 (45%)	4(7%)	0.419	0. 81
	Expected	(50%)	(42%)	(8%)		
Control	Observed	24(96%)	1(4%)	0	20.488	0. 00004
	Expected	(77%)	(20%)	(3%)		
Total Observed		53	28	4		

The genotyping and allele frequency for the TNFR2 polymorphism rs1061622 T/G among RA patients and controls is illustrated in Table (5).

Table(5): Genotypes and allele frequency results for the TNFR2 polymorphism rs1061622 T/G between RA patients and controls.

rs1061622	Genotype (%)		P-value	Odd ratio (95% CI)
	Control (NO.= 25)	Ra patients (No. =60)		
TT (Wild)	24 (96%)	29 (48 %)	---	1.00 (Reference)
TG	1(4%)	27(45%)	0.0032	22.34(2.82 to 176.71)
GG	0	4(7%)	0.1844	7.47(0.38 to 145.76)
Allele frequency				
T	49 (98%)	89 (74%)	---	1.00 (Reference)
G	1 (2%)	31 (26%)	0.0059	17.06(2.26 to 128.87)

The results shown in Table (6) revealed the comparison between responders and non-responders group regarding genotyping and allele frequency for the TNFR2 polymorphism at rs1061622.

Table(6): Genotypes and allele frequency results for the TNFR2 polymorphism rs1061622 T/G between responders and non-responders group.

rs1061622	Frequencies (%)		P-value	Odd ratio (95% CI)
	Responders (NO.= 36)	Non-responders (No. =24)		
TT(Wild)	16(44.4%)	13 (54 %)	---	1.00 (Reference)
TG	16(44.4%)	11 (46%)	0.75	0.84(0.29 to 2.44)
GG	4(11.2%)	0	0.1934	0.13(0.006 to 2.75)
Allele frequency				
T	48 (67%)	37 (77%)	---	1.00 (Reference)
G	24 (33%)	11 (23%)	0.221	0.6(0.25 to 1.36)

DISCUSSION

Sixty cases of RA patients (12 Males: 48 Females) treated by etanercept were enrolled in this study. This result indicate that the females represents the major percentage of patients which was reported by other studies (Al -Yasiri *et al*., 2013, Law *et al*., 2021, Aljubran *et al*., 2022). Sex has a role in many epidemiologic and pathogenic aspects of RA, resulting in significant differences among affected females and males as it mentioned by Smolen *et al*.,2023. Our results agreed with other results which reported that the females response rate to anti TNF was significantly less than that seen in males among RA treated patients (Hyrich *et al*.,2006, Jayakumar *et al*.,2012,Souto *et al*.,2016). On the other hand the results of the current study disagreed with several studies in RA which revealed that a better response to etanercept was seen in females more than males (Anderson *et al*.,2000, Jassim, 2015). The BMI of the patients was around 30 which is higher than the reference population. This results similar to other studies (Albrecht *et al*.,2016, Vallejo *et al*.,2021). There is no significant correlation of the BMI and the response to etanercept which agreed with Mariette *et al*.,2017 and Vallejo *et al*.,2024. While the study results disagree with other studies which have shown that obesity is related to the negative response to the TNF-α inhibitors therapy (Singh *et al*.,2018, Shan and Zhang, 2019). Some studies suggest that the obesity may affect the response to biological anti TNFα because obesity represents a systemic low-grade inflammatory condition that may share a pathological pathway which is commonly related to immune-mediated diseases (Versini *et al*.,2014). Also, the body weight of an individual can affect the distribution of drug's volume (Berends *et al*.,2019). The ESR level was higher in responders (53.2 ± 5.55 SEM) than non-responders group (42.3 ± 4.94 SEM), which may affect the response to etanercept. Although the results of ESR,WBC and other parameters at baseline showed no significant impact on the response to drug. This result agreed with other study that showed non-significant difference in ESR baseline between responders and non-responders (Zhang and Jiang, 2019). While other study

concluded that the high level of ESR and WBC was associated to the response failure to etanercept (Mohammed *et al*.,2022). The results showed in table (5) the TT wild type at rs1061622 was predominant (96%) in controls group, while it was (48%) in RA patients. The TG genotype in RA patients was significantly higher than controls group, while the GG was present only in RA patients. This results may indicates the protective role of TT genotype at rs1061622 in decreasing the susceptibility to RA, while the TG genotype is correlates with the susceptibility to RA which agreed with a meta-analysis study that demonstrated the functional rs1061622 is associated with RA susceptibility. Also Jumaah *et al*, 2023 reported the association of TG and GG genotype at rs1061622 with susceptibility to RA. On the other hand two studies revealed that polymorphism at rs1061622 was not correlates with the genetic susceptibility of RA (Canet *et al*, 2015; Xie *et al*, 2016). The G allele had significantly higher in RA patients which may correlates with the risk of RA. The rs1061622 T/G lead to change amino acid from methionine to arginine which results in affecting functional properties of TNFR2. The susceptibility Various autoimmune diseases have been linked to the G allele, including RA (Ticha *et al*, 2018).

The results shown in Table (6) revealed the comparison between responders and non-responders group regarding genotyping and allele frequency for the TNFR2 polymorphism at rs1061622. The TT wild type was higher in non-responders group which may correlates with failure to response to etanercept. This result agreed with Chen *et al*., 2015 who reported that RA patients with TNFAR2 rs1061622 T allele had a worst response to anti-TNF-α therapy compared to patients with the TNFAR2 rs1061622 G allele. While other studies showed poor response to anti-TNFα is associated with patients having the G allele at rs1061622 (Fabris *et al*, 2002; Vasilopoulos *et al*, 2011; Jumaah *et al*, 2023). Allele frequency of allele T in responders and non-responders group was predominant in both groups and it was higher in non-responders group, but there is no significant difference.

CONCLUSION

In the TNFR2 gene, GG genotype at rs1061622 appear only in responders group which may correlates with the response to etanercept**.**

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CONFLICT OF INTEREST

No conflict of interest indicated in this report.

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