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Genetic Identifications for Sample of Multiple Sclerosis Iraqi Patients

Fadel S. Hammood, Bushra J. Mohammed

Institute of Genetic Engineering and Biotechnology for Post Graduate Studies, University of Baghdad,

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Abstract: Multiple sclerosis (MS) is an inflammatory condition that affects central nervous system (CNS) causing neurological dysfunction; T cell immunoglobulin and mucin domain -3(*TIM-3*) is a transmembrane protein which widely expressed on the surface of many immune cells including macrophages and T helper cell (Th)1 that regulates T cell responses. This study was planned to examine the relationship between multiple sclerosis and genetic and demographic aspects, using statistical analysis and genetic techniques. Blood samples were collected from fifty of multiple sclerosis patients (Men and women) with age ranged between 20-57 years, in comparison with fifty of apparently healthy volunteers as a control group. Information for demographic study was taken from patients according to a questionnaire that included, name, gender, age treatments, family history for MS and other autoimmune disease. Through the molecular study, DNA was extracted by using the genomic isolation kit, then subjected to Taq-Polymerase Chain Reaction (Taq-PCR). PCR analysis by using pair of primers and pair of probes. The results of the demographic study revealed that the highest number of MS patients located at the age group (30-39) represented 26(52%) of the total number with significant difference (P≤ 0.05). The females constituted 30(60%) more than males 20 (40%) with the high significant (P≤ 0.01). The distribution of MS according to MS family history revealed that 7(14%) of patients had MS family history, whilst 43(86%) hadn't with a high significant (P≤ 0.01). Moreover, the distribution MS patients regarding to other autoimmune diseases family history showed 2(4%) of patients had autoimmune diseases family history, while 48(96%) hadn't. Also, distribution of MS patients according to treatment, showed that Betaferon medication had the highest percentage 18(36%), followed by Retuximab 15(30%), Jelinya 12(24%), and Tysabri 5(10%) patients. The results of genetic study for *TIM-3* (rs10515746) genotyping clarified that wild genotype AA was 4(8%),heterogeneous genotype AC was 15(30%), homogeneous genotype CC was 31(62%) frequency of A allele was (0.23) and C allele was (0.77) with OR (C.I.: 1.729) and frequency of C allele was (0.77) with OR (C.I.: 0.578) in MS patients, while wild genotype AA was 25(50%),heterogeneous genotype AC was 10 (20%),and homogeneous genotype CC was 15(30%), frequency of A allele was (0.60) and C allele was (0.40) in control with Significant differences (P≤0.05). Conclusion: *TIM-3* plays a suppressive role in immune responses and genetic variants: CC and AC SNPs may influence an individual's vulnerability to MS.

Keywords: *TIM-3*, MS, Taqman –PCR, Polymorphism.

Corresponding author: (Email: fadelshaker6@gmail.com).

Introduction

Multiple sclerosis (MS) is a chronic autoimmune inflammatory disease of the central nervous system characterized by demyelination and axonal loss (1), believed to be mediated by autoreactive T cells directed against myelin antigens (2). Multiple sclerosis is conceder as the commonest cause of serious physical disability in adults of working age (3). Although usually it not life-shortening, MS is often interfering with life and career plans of an individual (4). The prevalence of MS has increased progressively over time, in 2019, MS accounts over 2.5 million affected

individuals worldwide with an estimated cost of $2–3 US billion annually (5). Multiple sclerosis incidence has been reported from different regions, suggesting that environmental factors, as well as geographic and genetic ones, play an important role in MS (6). The T cell immunoglobulin mucin domain 3 (*TIM-3*) is a transmembrane protein which widely expressed on the surface of many immune cells including macrophages and T helper cell (Th)1 that regulates Th1 responses (7,8). It acts as an immune checkpoint inhibitor, contributes to immune tolerance by inducing T cell apoptosis or by suppressing the activation of innate immune cells (9). Deregulated or upregulation of *TIM-3* has been associated with many tumors and chronic infectious diseases, while dysregulated, downregulation or dysfunction of *TIM-3* leads to many kinds of autoimmune diseases such as multiple sclerosis (1). At Recent years, showed that *TIM-3* may control T cell response indirectly via regulating the function of innate immune cells (10). However, the mechanism by which *TIM-3* expression mediates immune tolerance, especially innate immune tolerance and function in human T cells, remains largely unclear (11). Several single nucleotide polymorphisms (SNP) have been shown in promoter and coding region of *TIM-3* gene have been shown in previous studies that associations with susceptibility in different autoimmune diseases (8). Multiple sclerosis is not rare in Iraq; however, there are insufficient data to determine the spread of the disease, despite serious studies by many Iraqi researchers who concluded their results by MS demographicand clinical data as Al-Hamadani *et al*. (12); Falah and Al-Araji (13). However, up to available knowledge, it has not yet been investigated between the relationship between *TIM-3* gene polymorphism and susceptibility to MS, that's what encouraged us to conduct such a study, which examined the relationship between multiple sclerosis and genetic and demographic aspects

Materials and methods

Fifty of MS Iraqi patients, and fifty apparently healthy Iraqi volunteers as control were employed in the study, with age ranged between (20-57) years and from both genders. A questionnaire form was filled for each subjects. The Questionnaire included information index about the patients as age and sex, treatments, family history for MS and other autoimmune disease. Under ethics committee on human research, two ml of venous blood was taken from all volunteers, was put in EDTA anticoagulant tube, mix gently and kept in the (-20 °C) until subjected to DNA extraction as mentioned by Mohammed *et al* (14), using genomic DNA purification kit, concentration and purity of the DNA were carried out according to Mohammed (15), by using Nanodrop (BioNeer /Korea). *TIM-3* A/C (rs10515746) single nucleotide polymorphism (SNP) was done by by taq man RT-PCR using specific primers and probes pairs which supplied by Macrogen company/ Korea (Table 1)). Analysis of data and determined sequence variation between *TIM-3* gene using allelic discrimination by real time PCR technique using the ability of specific probe to determine the specific genotype, the reaction setup and thermal cycling protocol used in this study are given in table (2).

Table (1): Sequences of primers and probes of *TIM-3* gene

Primers name	Sequence 5`- 3`	Annealing Temp. (ºC)	Reference
rs10515746-F	CAGTGAATGGCATGTTCCTTATCC	55	This study
rs10515746-R	GGAAACTGAGACTCAGCAAGGTTA		
rs10515746-PA	FAM-TTACAGACCATAGCAACTC		
rs10515746-PC	HEX-CAGACCATCGCAACTC		

Table (2): The Reaction setup and thermal cycling of real time PCR

Steps	°C	m: s	Cycle
Initial Denaturation	95	5:00	1
Denaturation	95	00:30	40
Annealing	95	00:30	
Extension	72	00:30	

Results analysis was done by using the program of Statistical Analysis System-SAS (16) to estimate the effect of difference factors in work parameters. T-test was used to significant compare between means and Chi-square test was used to significant compare between percentages.

Results

The results of distribution of MS patients according to gender revealed that female to male ratio was 1.5:1 was female 30(60%) and male 20(40%) with highly significant difference (P≤0.01) as shown in table (3).

Table (3): Distribution of MS patients according to gender

Gender	Patients No. (%)
Male	20 (40%)
Female	30(60%)
Total	50
P-value	**0.0001
** (P≤0.01).	

According to age, the results of distribution of MS patients showed that the group age (30-39) had the greatest frequency in the research sample 26(52%), followed by10 (20%) in both of (22-29) and (40-49) age groups, while the age group of >50 had lowers percent 4(8%) with high significant difference (P≤0.01) as illustrated in table (4).

Table (4): Distribution of MS patients according to age

Age group (years)	Patients No. (%)
20-29	10 (20%)
30-39	26 (52%)
40-49	10 (20%)
50≥	4 (8%)
P-value	0.0001 **
** (P≤0.01).	

The result of distribution patients according to MS Family history showed that 7(14%) of patients with MS family history of disease, while 43(86%) without MS family history of disease at

high significant (P≤0.01) as clarified in table (5).

Table (5): Distribution of patients according to MS family history in patients group

Family history	No	Percentage (%)
Yes	7	14%
No	43	86 %
Total	50	100%
P-value	---	0.0001 **
**** (P≤0.01).**		

The result of distribution MS patients according to other autoimmune disease family history showed high significant (P≤0.01) increase 48(96%) in patients number with no family history of other autoimmune disease, in contrast 2(4%) of the patients have a family history of autoimmune diseases.

Table (6): Distribution of MS patients according to other autoimmune disease family history

Family history	No	Percentage (%)
Yes	2	4.00 %
No	48	96.00 %
Total	50	100%
P-value	---	0.0001 **
**** (P≤0.01).**		

In this study, the distribution of patients according to therapy showed that Beta feron medicine had the greatest frequency in study sample 18(36%), followed by Rituximab 15(30%), Jelinya 12(24%), and Tysabri drug had the lowest percentage 5(10%) with high significant (P≤0.01) as illustrated in table (7).

Table (7): Distribution of MS patients according to treatments

Treatment	No	Percentage (%)
Beta feron	18	36
Rituximab	15	30
Jilenya	12	24
Tysabri	5	10
Total	50	100%
P-value	---	0.0001 **
**** (P≤0.01).**		

The findings of genotype *TIM-3* (rs10515746) suggested that a significantly greater incidence (P≤0.05) of homogeneous genotype CC which was 31 (62%) while occurrence of heterogeneous genotype AC was 15 (30%) and wild genotype AA was 4 (8%), frequency of A allele was (0.23) with OR (C.I.: 0.578) and frequency of C allele was (0.77) with OR (C.I.: 1.729) in MS patients. Whereas in control group the greater incidence for AA genotype which was 25 (50%) while occurrence of AC genotype was 15 (30%), CC genotype was 15 (30%), frequency of A allele was (0.40) and frequency of C allele was (0.60) as shown in table (8) and figure (1).

Table (8): Genotype distribution and allele frequency of *TIM-3* gene in patients and control groups

Genotype	Patients No. (%)	Control No. (%)	Chi-Square (χ^2)	P-value	O.R.(C.I.)
Wild: AA	4 (8%)	25 (50%)	6.368 *	0.016	0.106 C.I (95%)
Heterozygous: AC	15 (30%)	10 (20%)	5.002 *	0.0317	9.375 C.I (95%)
Mutant: CC	31 (62%)	15 (30%)	7.642 *	0.0622	12.9 C.I (95%)
Total	50 (100%)	50 (100%)			
Allele	**Frequency**				
A	0.23	0.40		OR: 0.578 (C.I.)95% OR: 1.729 (C.I.)95%	
C	0.77	0.60			
*** (P≤0.05). Significant.**					

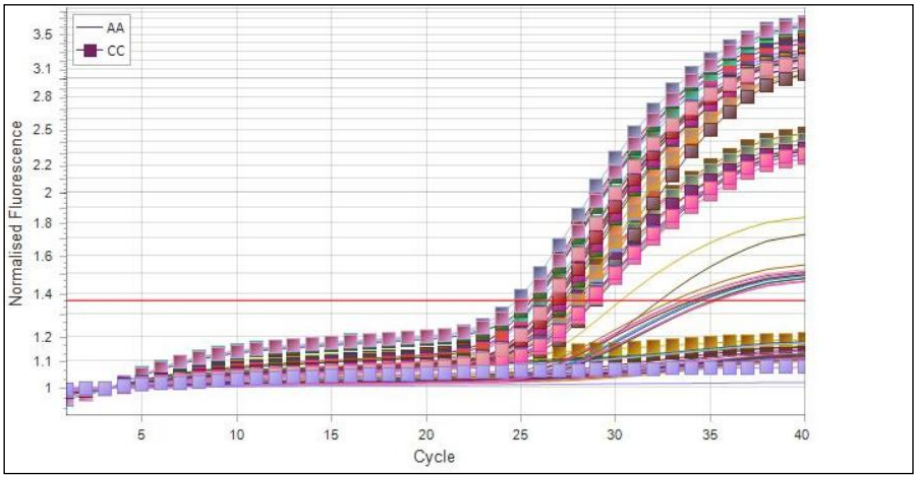

Figure (1): Allelic discrimination results (Taq man RT-PCR showing)

Discussion

Distribution of MS patients revealed that female more than male with highly significant difference. The X chromosome may possibly play a significant effect in autoimmunity, according to Selmi (17). The existence of two X chromosomes enhances vulnerability to encephalomyelitis in the experimental animals of MS experimentally autoimmune encephalomyelitis irrespective of hormones (18). Moreover, in women, X chromosome deactivation (the randomized deactivation of one X chromosome within every cell during embryogenesis) may be unbalanced, leading to an increased prevalence of MS genetic variants. The data gathered was comparable, with women accounting for more than half of the population in virtually all research, for instance, study of Al-Hamadai (19) agreed with current results who reported that females outnumbered males in cases with illness beginning as teens. Moreover, Hannikaine (20) revealed that females are highly represented amongst MS individuals, particularly in the relapsing-remitting (RRMS) phase, and may possess a better prognosis than men. While study of Khademi (21) disagreed with this result when not observe significant differences detected in sex incidence (78% male against 88 % female), or in illness severity. The

present sample of MS patients reveals that there was an increasing in females to males' ratio and that came in agreement with previous studies as Alonso(22); Holmberg*(*23); Westerlind (24)., also it is worth to mention that the same sex disparity seen in MS and many autoimmune disorders, Whitacre (25) reported that in patient population the percentage reached to over than 80%; and about 80% of autoimmune disease affect women, as well as Banwell (26) explained that may by female sex hormone that stimulate inflammatory response, which cause autoimmune diseases consequently. It can be explained that the increase get MS disease in (30-39 years) age group due to the fact that the immune system at this age is completely complete and more effective compared to the average of other ages. The other reason can be explained by the fact that people who lived this age period, whose date of birth ranged from 1982-1993, had lived through periods of war and siege, which negatively affected their psychological state and environmental conditions, as well as the reason can be attributed to the epigenetics factor it is well known that epigenetics means is the study of how behaviors and environment can cause changes that affect the way of genes work, and this definition is commensurate with the existing factors associated with Iraqi patients, including environmental factors and behavioral factors, including the frequent use of prohibited drugs, as well as the absence of drug control over most drugs that adversely affect the body. This interpretation is consistent with several sources, including by a progressive grade of neurodegeneration. Matching of these results have been recorded in other studies as Cheraghmakani *et al.* (27) who reported that the incidence of MS found in thirty age individuals, also study of Alroughani *et al*. (28) who explained that the prevalence of MS accord at thirty age. However, and because the disease is heterogeneous and has multiple influence factors, some studies contradict the current result, such as study of Khademi *et al*. (29) that did not observe statistically significant changes between the ages of 35 and 32 years, that may be due to the time of complaint beginning in MS is often determined by a patient's ability to remember, date, and describe occurrences and may be impacted by variables such as the kind and form of the first symptom(s), sex (or gender), and the first MS clinical profile. The results of this study showed that the prevalence of the disease in individuals without MS family history Thus, current results were coming in consistent with study of Barcellos *et al*. (30) who noted that determining the causation of MS is hampered by the issue of heterogeneity. Therefore, for the purpose of defining the multiple sclerosis phenotype, careful characterization of concomitant autoimmune illnesses in instances of multiple sclerosis and clustering of certain diseases in family members becomes essential. For disease-gene identification and genotypic–phenotypic associations in MS, large family-based or case-control studies that combine this clinical information will be particularly useful. The current study showed distribution MS patients with no autoimmune disease family history The familial clustering of autoimmune illnesses may be explained by common genes, shared environmental exposures, or a mix of the two. Independent genome-wide linkage searches of numerous autoimmune illnesses, in addition to MS, have been published, showing more complicated patterns than

conventional linkage studies of monogenic diseases. Clinical or phenotypic variability has likely also contributed to the discrepancy seen across linkage screenings in multiple sclerosis, where various loci may contribute to certain disease manifestations. Although the findings of the biggest linkage screen in families with numerous occurrences of multiple sclerosis show that only very minor genetic effects are acting in multiple sclerosis, it may not be desirable to regard multiple sclerosis as a single phenotype (31). This result agreed with study of Criswell *et al*. (32) who reported that there was no indication of familial autoimmunity when used 265 families from the Multiple Autoimmune Disease Genetics Consortium (MADGC) to examine the prevalence of autoimmune diseases (ADs) among siblings of multiple families However, there was a selection bias since families chosen for admission were not acquired in the same way. Therefore, the necessity for family studies in genomic research on MS s bolstered by the fascinating scientific discussion over the best effective technique for identifying hereditary variations of the illness. After a genome-wide association study (GWAS) failed to identify a link between MS and a mutation discovered in two families with MS, family studies were compared to case-control studies. The results Beta feron medicine had the greatest frequency in study sample were agreed with study of Hoer *et al*. (33), who stated that IFN beta-1a and IFN beta-1b (Beta feron) were the most commonly prescribed medications in 2009, accounting for more than two-thirds of all medications, whereas GA meds grew to almost one-quarter of the overall. To date, several disease-modifying therapies (DMTs) have been indicated for the treatment of multiple sclerosis MS in the European Union (EU). These therapies have varying mode of action, paths and intensities of administration, levels of efficacy, and security profiles. These therapies include subcutaneous interferon-β pegIFNβ-1a, (IFNβ)-1a, and IFNβ-1b, small-molecule oral agents (dimethyl fumarate, teriflunomide, ozanimod, fingolimod, cladribine), subcutaneous glatiramer acetate, intravenous monoclonal antibodies (mAbs) (natalizumab, ocrelizumab, alemtuzumab), and intravenous mitoxantrone. Efficient therapies for advancing types of MS are more restricted, with just a few numbers of beneficial medicinal drugs obtainable (34). Also the result of Rituximab medicine had the second greatest frequency in study sample came in accordance with search of Zecca *et al*. (35) a large multicenter, retrospective Italian-Swiss study analysing data from over 350 progressive MS patients treated with rituximab. Ultimately, the research contributes to the body of knowledge by proving that rituximab is successful and reasonably safe in the treatment of multiple sclerosis. The findings of genotype *TIM-3* suggested that a significantly greater incidence of homogeneous genotype CC and heterogeneous genotype AC *TIM-3* is one of the checkpoint receptors that is expressed on helper T cells, regulatory T cells, and innate immune cells. Numerous researches have established an association among the *TIM-3* polymorphism and ADs including MS (36). Chae *et al*. (12) research had discovered a variety of SNPs in the regulator and coding *TIM-3* gene, also, Yaghoobi *et al*. (37) described the correlations of *TIM-3* SNPs with illness vulnerability in ADs. Mazrouei

et al. (38) reported that the findings of prior research linking *TIM-3* -1541C>T polymorphism to RA is comparable to their findings indicate that in the Isfahan community with MS, since the aetiology of this illness and MS are similar. The findings indicated that the frequency of the CT genotype *TIM-3* -1541C>T was much greater in the MS sample than in the reference sample, and that there is a strong correlation between CT genotype and MS prevalence. Recently, Pouladian *et al*. (39) indicate that the +4259 A>C polymorphism in the *TIM-3* gene may be a significant genetic determinant influencing MS vulnerability in Iranian people. Present results were consistent with previous studies as Chae *et al*. (40) and Yaghoobi *et al*.(37) that identified several SNPs in the promoter and coding area of the *TIM-3* gene, which may regulate the protein's expression level. They revealed an association between *TIM-3* single nucleotide polymorphisms and vulnerability to autoimmune disorders, such as rheumatoid arthritis, type 1 diabetes, Ankylosing Spondylitis (AS), and MS . furthermore, prior investigations for Li *et al*. (41) and Liao *et al*. (42) have examined the connection between *TIM-3* polymorphism and various chronic inflammatory and autoimmune illnesses produced contradictory findings. Also, some other researcher for instance, Wang et al. (43) and Song *et al*. (44) found that the intensity of the *TIM-3* – 574 AC genotype and – 574 C allele was substantially elevated in patients with Ankylosing spondylitis (AS), rheumatoid arthritis, and HIV+ non-lymphoma Hodgkin's, while Yaghoobi *et al*. (37) found the intensity of the -574 AA genotype and A allele reduced in rheumatoid arthritis. Also study of Wang *et al*. (45) suggested that *TIM-3* mRNA and protein amounts in CD4+ T cells, CD8+ T cells, and monocytes are dramatically reduced in MS individuals with polymorphism-574 AC genotype. Moreover, Pouladian *et al*. (39) discovered that the polymorphism in exon 3 of the *TIM-3* gene is connected to MS vulnerability, suggesting that polymorphism in *TIM-3* gene could be one of the essential genetic factors linked to MS vulnerability between Iranian communities. Additionally, Zhang *et al*., (11) discovered that the *TIM-3* A>C polymorphism had stable substantial connections with AD risk in the dominant and allelic modeling techniques, as well as with rheumatoid arthritis (RA) risk in the dominant and allelic. These data suggest that *TIM-3* + A>C may be a vulnerable predictor of ADs, particularly RA. More functional and clinical research on the relationship between these disorders and *TIM-3* polymorphisms is needed (46).

Conclusion

It can be concluded that *TIM-3* played a critical role in immune responses and subsequently the MS susceptibility, also patients with at least one copy of (C) allele whether in heterozygous (AC) and homozygous (CC) genotypes had a higher risk of MS.

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Conflicts of interest

There are no conflicts of interest declared by the authors.

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