Neurology Asia 2023; 28(2) : 349 – 358

Integrin alpha-4 gene polymorphism in relation to natalizumab response in multiple sclerosis patients

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Abstract

Objectives: The aim of this study was to assess the possible the association between +3061 (G>A, rs1143676) missense mutation in exon 24 of the integrin α-4 subunit (ITGA-4) gene and the response to natalizumab in a sample of Iraqi multiple sclerosis patients. Methods: A sample of 59 patients with multiple sclerosis (16 males and 43 females; mean age of 32 years; age range of 15 to 52 years) receiving natalizumab for at least 12 consecutive months were involved in the study between March and August/ 2022. The sample was categorized into two groups according to their response to natalizumab treatment (responders and non-responders). Polymerase chain reaction and Sanger's sequencing for the extracted deoxyribonucleic acid was performed to identify the polymorphism at ITGA-4 gene promoter region. *Results:* The 3061 AA and AG genotypes were present in both groups (responders and non-responders to natalizumab treatment) with the lack of the wild form GG genotype. The AG genotype was significantly present in the non-responders' group and appeared to have a significant impact on the responsiveness to natalizumab by increasing the propensity of being non-responder with a positive correlation (Phi-coefficient of 0.294) on the contrary of AA genotype.
Conclusion: The +3061 (G.A) missense mutation is related to the response to natalizumab in multiple sclerosis patients with the AG genotype, thereby increasing the likelihood of non-response significantly.

Keywords: Natalizumab, integrin α4 subunit gene polymorphism, multiple sclerosis.

INTRODUCTION

Multiple sclerosis (MS) is a neuroinflammatory and neurodegenerative disorder that is the major etiology of neurological impairment in young individuals.[1] Natalizumab (Tysabri®, Biogen-Idec Inc., NAT), a recombinant humanized monoclonal antibody (mAb) targeting the 4-integrin (ITGA4) component of the cell adhesion molecule, is one of the particularly efficient disease-modifying therapies (DMTs) for active relapsing remitting multiple sclerosis (RRMS).[2-4]

NAT is the first mAb approved for the treatment of MS in November 2004 based on the findings of one six-month periodic magnetic resonance imaging (MRI) research and 2 different Phase III clinical trials of two years duration, including, the monotherapy trial AFFIRM and the NAT versus Interferon Beta-1a (IFN-β-1a) SENTINEL trial.[5-7] NAT connects to integrin α4/β1 subunit of the very late antigen-4 (VLA4), present on the surface of lymphocytes and, via blocking its association with the corresponding ligand on endothelium, vascular cell adhesion molecule 1 (VCAM-1), prevents lymphocyte relocation across the blood-brain barrier (BBB).[8] Medically approved NAT dosage of 300 mg injected intravenously (IV) every 28 days improves disease activity on MRI, relapse rate, and proven disability deterioration after 2 treatment years compared to placebo.[9] It seems to be highly efficacious and quite well tolerated in young MS patients.[3] Progressive multifocal leukoencephalopathy (PML), one of the serious, rare opportunistic infections of the brain, is the primary significant safety issue related with natalizumab.[10]

However, NAT is one of the most expensive DMT and such costs impose the national health care system to significant economic pressure. The mean annual cost of treatment with Natalizumab is approximately USD 36,000 for the course of treatment per patient due to the high price of Natalizumab and the more frequent use (12 doses per year).[11]

If relapse occurs, etiological factors that

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Date of Submission: 26 December 2022; Date of Acceptance: 8 March 2023

https://doi.org/10.54029/2023afn

may result in failure that include revival of inflammatory activity in spite of treatment, as well as other factors should be evaluated. Although numerous studies have been conducted on the safety and effectiveness of natalizumab, limited data exist on the reasons for natalizumab nonresponse. The development of an emergent neurological impairment with NAT is seldom due to a recurrence of MS and should prompt comprehensive investigation of other etiologies, such as PML.[12] Anti-natalizumab antibodies constitute the second specific etiology of therapeutic failure.[13]

Recent study indicate that genetic variation can significantly impact the therapeutic response of MS patients.[14] Genetic mutations in the genes associated to the medications' mechanism of action or the pathological milieu of the condition may have a significant contribution.[15] Genome-wide association studies (GWAS) only recognizes variations related with disease susceptibility. Studies in MS genetics should also attempt to identify which genetic mutations are however linked with disease severity, progression, and therapeutic responsiveness.[16]

Genetic polymorphisms is associated with some variability in drug response as well as risk of experiencing a serious adverse drug reaction (ADR).[4] The reasonable candidates to investigate the effect of genetic polymorphism on NAT response include variants in the genes for the integrin α4 subunit (ITGA-4), the integrin β1 subunit (ITGB-1), or VCAM-1.[17] Because as VLA-4 integrin subunit facilitates the recruitment of leukocytes and other cells into the Brain, VLA-4 gene is a promising potential target for predisposition to MS or NAT response.[4] Pharmacogenetics and pharmacogenomics have been utilized in MS treatment as a result of the main goal of identifying genetic variants that can anticipate therapeutic response and toxicities. Consequently, polymorphism screening distinguishes individuals with an efficient drug response from those with an insufficient or poor response.[18]

Growing body of research published in the past years have shown contradictory results concerning the pharmacogenetics of MS[19-21] and other diseases[22-24] and polymorphisms in the ITGA-4 gene at position +3061(G>A, rs1143676). These studies examined the association of several single nucleotide polymorphisms (SNPs) and MS susceptibility. However, due to pharmacogenetics racial and ethnic variations resulting from the allele frequencies differences in different populations, the results of these studies varied from population to population.[25]

Genetic study is a population-level estimation that fluctuates between people and therefore cannot be applied to an individual patient. In addition, genetics can be affected by the impact of environment. Consequently, it is necessary to perform polymorphism testing in different populations.[26]

To date, no published study had investigated the effect of +3061 (G>A, rs1143676) polymorphism and the propensity for being a non-responder to NAT as ITGA-4 is its main target of action. We aimed to determine whether +3061 (G>A, rs1143676) SNP at position 878 in exon 24 of ITGA-4 gene possibly will affect the response to NAT in a sample of Iraqi MS patients.

METHODS

This is the report of a cross-sectional, single center study which has been carried out at the Medical City, Baghdad Teaching Hospital, Department of Neurology from March to August/ 2022. This was a component of a larger study undertaken using a convenient sample of seventy Iraqi MS patients. Since it was the exclusive site that administers NAT for MS patients, this clinic serves patients from all over the country, including city, rural, and urban populations in Iraq. The inclusion criteria stipulate that clinical proof of central nervous system (CNS) involvement, brain magnetic resonance imaging (MRI), and other diseases exclusion must be done to confirm the diagnosis of MS by specialized neurologists.[27] Also, the patients must had taken NAT by intravenous infusion, regularly every 28 days for at least 12 previous consecutive months. The exclusion criteria included patients who were using NAT for less than 12 months, using MS medications other than NAT, and patients with co-existent other neurological diseases. The research protocol has been approved by College of Pharmacy Scientific and Ethical Committee, University of Baghdad, also from the Neurology Department of the Baghdad Teaching Hospital (approval number: RECAUBCP25102021A; approval date: 25/10/2021).

In total, 87 patients with relapsing remitting MS using NAT as a monotherapy and fulfilled the inclusion criteria were investigated. Only 78 patients consented to be involved in the study, from them 70 patients met the research requirements.

Data collection

Demographic data including age, gender, duration of disease, NAT doses and relapse frequency in the last 12 months were collected from the patients by direct interview utilizing an information chart designed by the researchers. The baseline (before NAT treatment) Expanded Disability Status Scale (EDSS) were collected from the patients' files, while the current EDSS was estimated by the neurologist.

Patients' groups and clinical assessment

Fifty nine patients (43 females and 16 males, with mean age 32.02±1.076 years and age range between 15 and 52 years) were categorized into two groups; the 1st group (group A, n:29) include MS patients who clinically responded to NAT. While the 2nd group (group B, n:30) include MS patients who did not to respond to NAT as in Figure 1. The categorization into the groups depended on the clinical response as measured by EDSS and relapse rate to assess the response to NAT for at least 12 months.

The responsiveness of NAT-treated patients was determined by the presence of a steady or decreased EDSS and relapse absence. In contrast, NAT-unresponsive MS patients were diagnosed on the basis of an elevated EDSS despite treatment alone or combined with the occurrence of relapses during the course of therapy.[28,29]

The development of anti- NAT neutralizing antibodies was investigated by a specific ELISA kit to exclude antibody positive patients from the NAT unresponsive group. Four patients from group B were NAT antibody positive and they were excluded from the study. Additionally, seven patients were excluded (5 from responders and 2 from non-responders) due to the unconfirmed genetic sequencing results.

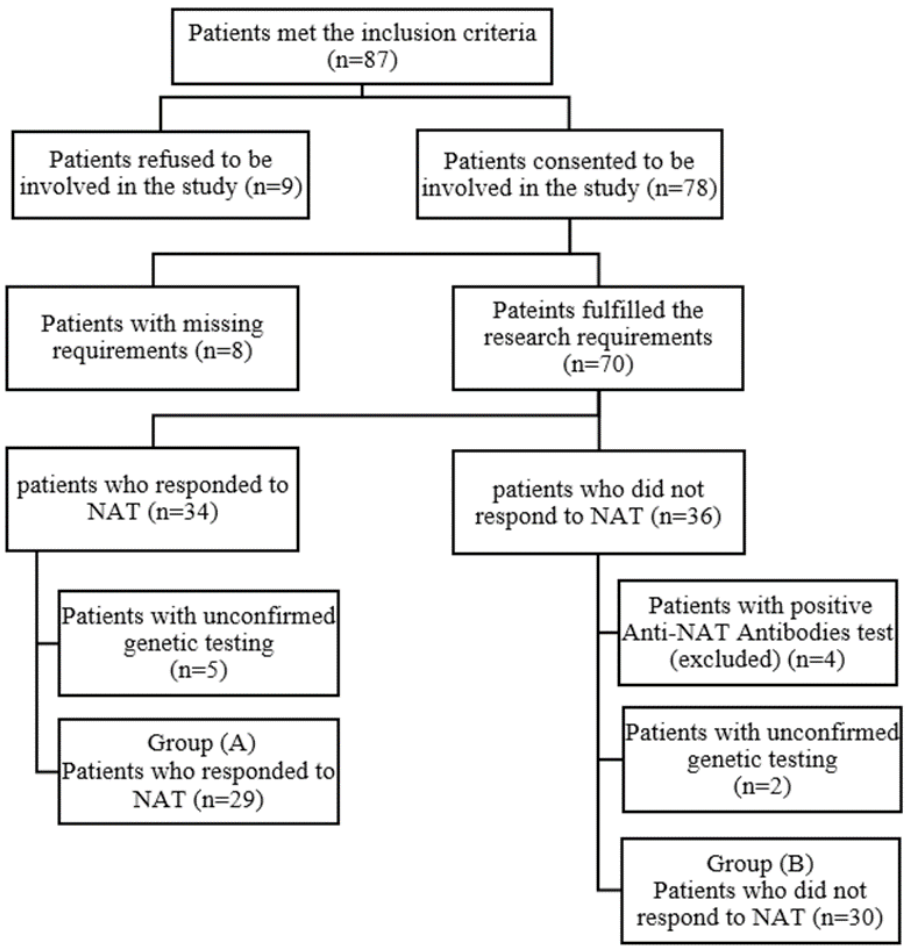

Figure 1. Study flow chart

Sample collection and deoxyribonucleic acid (DNA) extraction

Venous blood (5ml) was obtained from the forearm veins of each patient, then divided into 2 ml of blood transferred to a DNA extraction tube containing ethylenediaminetetraacetic acid (EDTA), and 3 ml of blood transferred to gel tube and centrifuged at 5000 round per minute (rpm) for 10 minutes to obtain serum.

The ReliaPrepTM Blood gDNA Miniprep System protocol (Promega, USA) was used to extract Genomic DNA from the blood sample. The Master Taq polymerase enzyme and a hybrid thermal cycler were the component of polymerase chain reaction (PCR) used to perform enzymatic amplification. The concentration of extracted DNA was measured by the Quantus Fluorometer (Promega, USA).

Primers and primers optimization

The database from the National Center for Biotechnology Information (NCBI) GenBank was used to get the ITGA-4 gene DNA sequence. The PCR primers were generated by Primer Premier 3 software (Table 1). These primers were supplied by Macrogen Company (Macrogen, South Korea).

To establish the primers' best annealing temperature, the DNA template was amplified at 55, 58, 60, 63, and 65°C utilizing the corresponding primer set (forward and reverse). As shown in Figure 2, the optimal primer annealing temperature was 65°C.

PCR amplification and sequencing of PCR products

Volumes of 20µl containing 10μl GoTaq Green Master Mix (2X) were used to perform PCR amplifications. Each primer (1μl); nuclease free water (6μl) and template DNA (2μl) were used. The PCR Express (Thermal Cycler, BioRad, USA) was used to complete PCR cycling with the specific temperature program as follows: 4 min denaturation at 94^{o}C then denaturation at 94°C for 30 seconds (30 cycles); 30 seconds of annealing at temperatures of 55, 58, 60, 63 or 65°C; and finally, extension at 72°C for 30 seconds. The final extension incubation was for 7 min at 72^{o}C, followed by incubation at 4^{o}C for 10 min in order to stop the reactions.

Macrogen Corporation's ABI3730XL, an automated DNA sequencing device, was employed to sequence PCR products by the Sanger method. The results were sent through email and evaluated using Geneious Prime software (Biomatters Ltd., New Zealand).

Anti- NAT neutralizing antibodies ELISA kit

NAT antibody screening (qualitative) enzyme-linked immunosorbent assay (ELISA) kit (ELISA Genie corporation, Ireland) was used to assess the occurrence of antibodies against NAT in serum samples of MS patients]. The test based on the sandwich principle and the results was evaluated using cut-off value.

Statistical analysis

Version 24 of Statistical Package for the Social Sciences (SPSS) (IBM Corp., USA) was utilized to perform statistical analysis. Mean ± Standard Deviation (SD)were used to represent continuous variables. Numbers and frequencies were used to express alleles and genotypes. The normality of the results was tested by Shapiro-Wilk test. Normally distributed demographic data and parameters were tested by unpaired t-test to establish the differences between responders and non-responders. Chi square test was applied to investigate the data frequency difference between the groups. The correlation between genotypes and the non-response to NAT was evaluated by using Phi correlation coefficient (phi). A p value <0.05 was considered to be a statistically significant.

RESULTS

Demographic and baseline clinical characteristics

Demographic data and disease clinical variables are shown in Table 2.

Table 1: Primers' sequence with annealing temperature and base pair size

Primer	Sequence 3`-5`	Annealing Temperature (˚C)	Size (bp)
rs1143676-F	TGTAAAACGACGGCCAGTGAACCTATATCCAACCTGCTGTAA	65	653
rs1143676-R	CAGGAAACAGCTATGACGAACTCCTGACCTTGTGATCTG		

rs1143676-F: Forward primer, rs1143676-R: Reverse primer.

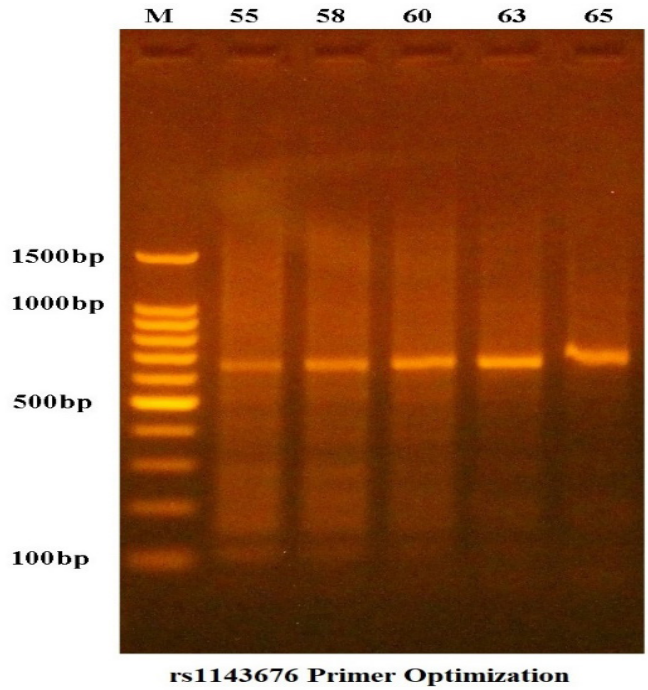

Figure 2. rs1143676 primer optimization at 55, 58, 60, 63, and 65°C annealing temperature.

Prevalence of +3061 (G>A) polymorphism genotypes and alleles

Genotypes and alleles frequencies in the two groups are presented in Table 3. Notably, the AA genotype has the higher prevalence than AG genotype. While the wild form GG genotype incidence was zero in the sample tested. The A allele represent about four times of G allele incidence.

The results also showed the genotypes allocation between the two groups (Table 4). A

Table 2: Demographic and clinical data of the groups

Category	Group A (Responders) n=29			Group B (Non-responders) n=30			p-value
	n	%	Mean ± SD	n	%	Mean ± SD	
Age (year)			30.66±7.39			33.33±8.97	0.216[a]
Gender							
Male	5	17.2		11	36.7		0.093[b]
Female	24	82.8		19	63.3		
Disease Duration (year)			7.38±4.06			8.73±4.79	0.349[c]
Number of NAT Doses			29.83±14.67			18.8±4.11	0.001*[c]
Baseline EDSS			2.57±1.37			2.82±1.88	0.866[c]
EDSS after at least 12 Months of NAT Treatment			1.67±1.41			4.87±1.67	<0.0001*[c]
EDSS change			-0.897±0.49			1.98±1.28	<0.0001*[c]
Relapse no. in the past 12 months	0	0		15	50		<0.0001*[b]

SD: Standard deviation; NAT: Natalizumab; EDSS: Expanded Disability Status Scale; a: Independent 2 sample t-test; b: Chi-square test; c: Mann Whitney U test; *: Significant difference between the groups.

Table 3: Frequency of genotypes and alleles of +3061 (G>A) polymorphism

Genetic variants	AA	AG
No. (%)	34 (57.6)	25 (42.4)
Alleles	G	A
No. (%)	25 (21.2)	93 (78.8)

Frequencies are represented by number and percentage.

significant difference in genotypes prevalence between the two groups was found, AG genotype was more frequent in the non-responders' group, while AA genotype had a higher incidence in the responders group.

Concerning the difference in the prevalence of alleles between the two groups, the results revealed a non-significant difference of A and G alleles between responders and non-responders.

Correlations between +3061 G>A SNP genotypes and the incidence of NAT non-response

Correlation between the incidence of non-response to NAT and each genotype was examined by the use of the phi coefficient analysis. The genotype seemed to increase the likelihood of NAT treatment failure was the AG genotype of +3061 G>A SNP (Table 5). On contrary, the AA genotype showed a negative significant correlation with the non-response prevalence.

Correlations between +3061 G>A SNP genotypes and EDSS change

Table 6 revealed a significant difference between the genotypes and the change in EDSS over the NAT treatment period.

Correlations between +3061 G>A SNP genotypes and relapse rate in non-responders

The results showed a non-significant association between the 3061 G>A genotypes and the occurrence of disease relapse in non-responders during the last 12 months of NAT therapy as shown in Table 7.

DISCUSSION

Pharmacogenomics employs the genomic profiles of individuals to recognize those who have a greater risk for adverse drug reactions or unresponsiveness.[30]

Variation in DMTs responsiveness among MS patients is a serious therapeutic concern. Furthermore, patients may encounter delays in having a treatment to which they can maximally respond, predisposing them to adverse effects without real benefit and imposing the healthcare systems to a significant burden.[31] Moreover, due to the risk of PML, there is a major impact of the biomarkers identifying individual at risk for having this complication.[32]

Particular response to therapy can be influenced by gene polymorphisms in genes that interact with drug effects and metabolism either directly or indirectly.[33] The migration of immune cells into the CNS is facilitated by the ITGA-4 molecule, NAT blocks ITGA-4, and prevents relocation of lymphocytes across the BBB. Hence, ITGA-4 gene may be deemed as a plausible candidate gene for MS susceptibility or NAT reactivity.[4] The current study investigated the association of the ITGA-4 gene polymorphism at position +3061 (G>A) and

Table 4: Variation in genotypes and alleles prevalence between the groups

+3061 G>A SNP	Group A (Responders) n=29	Group B (Non-responders) n=30	p-value
Genotypes	No. (%)	No. (%)	
AG	8 (27.6)	17 (56.7)	0.024*
AA	21 (72.4)	13 (43.3)	
Alleles			
G	8 (13.8)	**17 (28.3)**	0.053
A	50 (86.2)	**43 (71.7)**	

*The statistical difference between the groups was determined using a Chi-square test. *: Significant difference between the groups.

Table 5: Correlations between genotypes and NAT treatment failure likelihood

+3061 G>A SNP Genotypes	Phi-coefficient	p-value
AG	0.294	0.024*
AA	-0.294	0.024*

*The association between each genotype and the likelihood of becoming a non-responder was determined using the phi-correlation coefficient. *: Significant difference between the genotypes.

NAT ineffectiveness in a sample of MS patients.

The demographic characters of the participants were comparable to several studies conducted on Iraqi MS patients. The mean age, the preponderance of females over males and baseline EDSS were consistent with studies included MS patients for treatment satisfaction, quality of life, and diagnosis predictors.[34-37]

Almost all protein therapies could be rendered ineffective by the development of neutralizing antibodies.[4] Approximately 4.5% to 11.4% of NAT-treated MS patients can generate anti-NAT antibodies during the early phases of treatment varying between transient and persistent antibodies.[6,7,38] Persistent anti-NAT antibodies decreased serum NAT levels and compromised therapy responsiveness.[39] The postulated mechanism is the development of NAT-antibodies immunological complexes, which result in increased clearance and decreased functional serum concentrations of the drug.[40] This fact necessitated the exclusion of anti-NAT antibodies positive patients from the non-responder group according to findings from the AFFIRM study, the relapse rate among persistent antibody-positive patients was similar to that of placebo-treated patients.[6] The reason for not diagnosing antibodies positive patients earlier in the treatment course is the lack of anti-NAT antibodies routine testing in the MS healthcare center in our study.

With regard to genetic polymorphism results, the results of this study which included fifty nine MS patients treated with NAT showed the presence of +3061 (G>A, rs1143676) missense mutation in ITGA-4 gene. Numerous studies investigated this SNP and its association with increased sensitivity to MS (19–21). However, no published study evaluated the correlation between this SNP and the response to NAT in MS patients.

The prevalence of genotypes showed the predominance of AA genotype over AG genotype. Notably, none of the sample patients had the wild form GG genotype. The 3061A allele was the most prevalent than the 3061G (78.8% vs. 21.2% respectively). One of the earliest studies that investigated the prevalence of +3061 mutation genotypes revealed the abundance of 3061A allele compared to the frequency of 3061G allele which was significantly lower (0.69 and 0.31, respectively).[41] These results vary from than what is expected based on the Hardy–Weinberg equilibrium (HWE) for allele inheritance, various factors may cause these deviations including mutation, natural selection, non-random mating, genetic drift, and gene flow.[42]

Table 6: Correlations between +3061 G>A SNP Genotypes and EDSS Change

+3061 G>A SNP Genotypes	EDSS change (Mean ± SD)	p-value
AG (n=25)	**1.08±1.67**	**0.029***
AA (n=34)	**0.191±1.73**	

*SD: Standard deviation; EDSS: Expanded Disability Status Scale; Mann Whitney U test was used to determine the difference between genotypes; *: Significant difference between the groups.

Table 7: Correlations between +3061 G>A SNP Genotypes and Relapse Rate in Non-Responders

+3061 G>A SNP Genotypes In Non-responders	Relapse Rate		p-value
	n	%	
AG (n=17)	6	35.3	0.065
AA (n=13)	9	69.2	

*The difference between the genotypes was determined by Chi square test.

Genotypes distribution between the study groups showed a significant difference with AA genotype being more prevalent in the responder group. On the contrary, AG genotype was the predominant in the non-responder group. There was a positive correlation (Phi-coefficient of 0.294, P-value of 0.024) between AG genotype and the likelihood of being non-responder to NAT treatment. A point mutation that results in the transversion of arginine (CGG) to glutamine (CAG) at 3061 in exon 24, can lead to the formation of two variants for the α4 subunit, the G variant named α4mas, and the A variant named α4tex.[21] The 3061G variants in MS patients could change the conformations of α4 component resulting in an increase in the affinity of binding to VCAM-1.[19] This can attribute to NAT non-responsiveness in MS patient holding the G allele.

No previous studies looked for the +3061 G>A mutation correlation with the propensity of NAT treatment failure. Though, several studies correlated this mutation with MS and other diseases susceptibility. One study evaluated the polymorphisms of VLA-4 gene mutations at positions -269 (C>A, rs113276800), +3061 (G>A, rs1143676) and rs3135388 mutation labeling the HLA-DRB1*15:01 allele in Slovak population. The results indicated that the +3061AG genotype frequency in MS patients showed to be significantly higher in relative to the controls.[19] However, genotyping of 269 C>A and 3061 G>A showed no association of the examined VLA-4 polymorphisms to MS risk in Italian origin.[20] Another study investigated rs1143676 polymorphism, the polymorphism was found to be associated with MS risk in Iranian population.[21]

Studies evaluated +3061 (G>A) polymorphism in other diseases. A genetic association have been found between ITGA4 +3061A/G mutation and the increased risk and possible pathological involvement of Alzheimer disease that was described to be novel and independent.[22] The +3061 (G>A) missense mutation has no effect on antibody-mediated rejection in heart transplant patients.[23] Furthermore, it has no significant effect on increasing the risk of melanoma.[24]

The +3061 (G>A) mutation was found to have a significant impact on the EDSS change (p-value 0.029) with AG genotype having a significantly higher increase in EDSS compared to AA genotype (Table 6).

In the AFFIRM study, the primary outcomes for NAT treatment was annual relapse rate at the first year, and the cumulative disability progression, defined as an increase of ≥1.0 on the EDSS from (≥1.0) baseline or an increase of ≥1.5 from (0) baseline at 2 years. NAT was confirmed to reduce the relapse rate and disability progression at 1 and 2 years respectively.[6,43,44]

However, the current study could not confirm a significant association between this missense mutation and the relapse rate in non-responder group (p-value 0.065). The fact that NAT has been shown to decrease the relapse rate[6,43], these results are not consistent with the finding that AG genotype increase the propensity of NAT treatment failure. This can be attributed to the limited sample size in our study. When considering the abundance of relapses in the non-responder group only, it can be explained by the utilization of relapse occurrence as a categorization criterion in this study.

There are a number of limitations in the present study, the major one is the small sample size due to the limited number of NAT-treated MS patients who fulfilled the inclusion criteria. Also, the present study did not estimate the association between the response and number of doses of NAT. Additionally, the results of this study cannot be generalized to all Iraqi MS patients without caution since it is a single center study. Even though, this center serves MS patients from all Iraqi governorates. Moreover, this study could not be expanded to include a suitable follow up period for patients with specific genotypes because of limited facilities and lack of financial support.

In conclusion, the occurrence of +3061 (G>A) missense mutation in MS patients is associated with the NAT response. The AG genotype was shown to significantly increase the likelihood of being a non-responder to NAT and it can be used as a valuable NAT responsiveness biomarker for Iraqi MS patients to predict the tendency of ineffectiveness before administration of NAT.

DISCLOSURE

Financial support: None

Conflicts of interest: None

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