Elsevier Editorial System(tm) for Multiple Sclerosis and Related Disorders
Manuscript Draft

Manuscript Number: MSARD-D-20-00347

Title: Interleukin 10 gene polymorphism in some Iraqi patients with multiple sclerosis

Article Type: Original Article

Keywords: Multiple Sclerosis, Interleukin 10, Polymorphism, Amplified Refractory Mutation System, Relapsing-Remitting Multiple Sclerosis

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Abstract: Background: Multiple sclerosis (MS) is a type of autoimmune disease in which immune cell attacks our cells mistakenly; its severity is measured by expanded disease status scale (EDSS). This study aims to investigate the -1082 polymorphism of interleukin-10 (IL-10) as one etiology of the disease.
Methods: In this case-control study, 40 relapsing-remitting MS (RRMS) male patients, which fulfills McDonald criteria and 40 healthy controls, with matched sex and age, were compared depending on the -1082 (G / A) polymorphism in the IL10 gene by allele-specific -polymerase chain reaction (AS-PCR) method.
Results: The frequency of A allele of IL-10 was considerably higher in male MS patients than healthy control males (51.25 Vs. 42.5%). Genotype distributions of the single nucleotide polymorphism (SNP) -1082 fulfills the Hardy-Weinberg equilibrium in both cases and controls. Both homozygous (AA) and Heterozygous (GA) were non-significantly positively associated with MS male patients (OR = 1.467, 95% CI = 0.521 to 4.129, P = 0.467) and (OR = 1.692, 95% CI = 0.589 to 4.855, P = 0.326) respectively.
Conclusion: The distribution of the -1082 (G/A) polymorphism is not significantly different in our case/control study in the north of Iraq, but EDSS is significantly higher in A alleles carrier genotypes.

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Respected sir…

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2. The significant findings of this article are:
   A. The genotype frequencies of this IL-10 promoter gene -1082 G/A SNP is not a significant risk factor predisposing in developing MS disease susceptibility in our population.
   B. EDSS scores are higher in carrier A alleles (GA and AA genotypes), which means that this polymorphism may increase the severity of the disease.
3. The paper should be of interest to readers in the areas of Genetics, Immunology and Neurology.

Please address all correspondence concerning this manuscript to me at abbas.salihi@su.edu.krd. Thank you for your consideration of this manuscript.

Your sincerely

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Interleukin 10 gene polymorphism in some Iraqi patients with multiple sclerosis

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ABSTRACT

Background: Multiple sclerosis (MS) is a type of autoimmune disease in which immune cell attacks our cells mistakenly; its severity is measured by expanded disease status scale (EDSS). This study aims to investigate the -1082 polymorphism of interleukin-10 (IL-10) as one etiology of the disease.

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Conclusion: The distribution of the -1082 (G/A) polymorphism is not significantly different in our case/control study in the north of Iraq, but EDSS is significantly higher in A alleles carrier genotypes.

Keywords: Multiple Sclerosis, Interleukin 10, Polymorphism, Amplified Refractory Mutation System, Relapsing-Remitting Multiple Sclerosis

1. Introduction

The most common neurological disease also regarded as an autoimmune disease in which the insulating layer on the axon of neurons have degenerated and

the axons are damaged is MS (Huang et al., 2015; Özenci et al., 2000). MS affects more than two million people around the world with the onset age between 20-40 years. Cognitive changes, fatigue, spasticity, paresis, dizziness, and tingling are among the most common symptoms of MS (Goldenberg, 2012). The severity of the disease is measured by EDSS. The etiology of this disease still is unknown, but scientists suppose that both genetic and the environment have a role in the pathogenesis of this disease (Gourraud et al., 2012). Disruption between pro-inflammatory and anti-inflammatory cytokine balance has been thought of as a cause for increasing the probability to have this disease (Navikas and Link, 1996). The international MS Genetics Consortium (IMSGC) and genome-wide association study (GWAS) has identified 50 risk loci for MS such as tumor necrosis factor-alpha (TNF-α), IL-7, IL-2, human leukocyte antigen (HLA), TNF-RSF14, CD58, CD86, TNF-RSF14, IL-12RB1, IL-22RA2, and IL-10 (International Multiple Sclerosis Genetics Consortium, 2011).

Amongst those interleukins, IL-10 as an immune regulator cytokine has a great role in maintaining the balance and the magnitude of the immune system and it is decrease regarded as one of the causes that involved in the pathology of MS (Gourraud et al., 2012). The locus of IL-10 is at chromosome 1q32.1, in which it consists of 5 exons producing IL-10 protein consists of 178 amino acids (Moore et al., 2001). As a function, it has a role in preventing the expression of both (T-helper 1 (Th1) cytokines and class II antigens of MHC, also it has a role in lowering the generation of many immune-active agents like TNF- α, IL-12, and metabolites of reactive nitric oxides. IL-10 might also boost the proliferation, survival and antibody production of B-lymphocyte (Moore et al., 2001).

Multiple sclerosis (MS) patients showed a decrease in the amount of IL-10 mRNA expression (Van Boxel- Dezaire et al., 1999), this is linked to the

polymorphisms that may be present in the promoter region which results in cytokine production (Weiner, 2009). Therefore SNPs play a crucial function that may affect the vulnerability to MS (Smith and Humphries, 2009).

Three SNPs -592 (A / C), -819 (T / C) and -1082 (G / A) are clustered in the promoter region of IL-10 forming three haplotypes ACC, ATA, and GCC respectively, which they are linked to the level of IL-10 expression (Wergeland et al., 2005). In general, the haplotype containing –1082*G-allele, specifically GCC / GCC, is known to be correlated with high IL-10 expression; while GCC / ACC and GCC / ATA have a medium expression relationship, finally, ACC / ACC, ATA / ACC, and ATA/ATA are associated with low expression (Mihailova et al., 2005).

A study that done previously showed high IL-10 expression and cytokine production in those people that carry -1082G allele (Miteva and Stanilova, 2008). Even as, several researchers have investigated the relationship between -1082 G / A SNP and MS susceptibility (Azarpira et al., 2010; Galehdari et al., 2015). So our aim in this research is to investigate the relationship -1082 G/A SNP in IL-10 and MS susceptibility in another ethnic group which is the Kurdistan region/ Iraq.

2. Materials and Methods

A total of five ml peripheral blood samples in EDTA tube of 40 MS patients collected from the department of neurology, Rzgari hospital in Erbil city, Iraq, who diagnosed according to 2005 revised McDonald criteria (Polman et al., 2005). Forty male controls were age-matched with MS patients. The mean age of MS patients was 34.85 ± 1.442 years, and of the control group was 32.25 ± 1.375 years; yielding no statistically significant difference in their ages. The mean of EDSS in MS patients (3.250 ± 0.353).

Human blood samples were taken from peripheral veins using the five-milliliter syringe. The blood put into K2EDTA (5.4mg) tube for direct DNA extraction. The DNA was extracted using a spin column method (AccPrep Genomic DNA extraction Kit- Bioneer, South Korea), depending on the manufacturer's instructions. Then the concentration and purity of genomic DNA extracted from each human blood samples were determined using Nano-Drop ™ (Thermo Scientific, USA) spectrophotometer by recording the concentration ranged (11.05-61.60 ng/µl) and purity (1.69-2.27) for each sample. Demographic profiles of both MS patients and healthy control had shown in table 1.

Genotyping of the SNP at position -1082G>A in the promoter region of the IL-10 gene performed by a rapid and cost-effective technique called AS-PCR method. In this technique each subject underwent allele-specific amplification in two separate reactions using three primers: one reaction used for amplification of the wild type allele: the primers were 5'- CAG TGC CAA CTG AGA ATT TGG -3' (common Forward primer) and 5'- CTA CTA AGG CTT CTT TGG GAG -3'(reverse primer specific (G). The other reaction used for amplification of the mutant allele: 5'- CAG TGC CAA CTG AGA ATT TGG -3' (common Forward primer) and 5'- ACT ACT AAG GCT TCT TTG GGA A -3' (reverse primer specific (A)) (Perrey et al., 1999).

The total of 20 µl volume of PCR master mix reaction achieved containing 2µl of genomic DNA, 10µl of Taq DNA Polymerase 2x Master Mix RED (Ampliqon, Danish) and 1µl of each forward and reverse primers. Then the mixture was completed by adding 6µl of nuclease-free water. The program of PCR amplification consisted of an initial denaturation at 94°C for 5 minutes followed by the next 35 cycles 94°C for 30 seconds (denaturation), 62°C for 30 seconds (annealing),72°C for 30 seconds (elongation) and the final step at 72°C for 5

minutes to extend all PCR fragments. After PCR amplification, the PCR DNA amplicons separated on 2.0% agarose gel then the separated bands were stained with ethidium bromide to visualize under UV light (Bio-Rad UV-trans-illuminator) (Brown, 2016). The 258 bp mean the appearance of IL-10 gene polymorphism in both G and A alleles.

3. **Statistical Analysis**

Graph Pad Prism 6 statistical software used for doing statistical analysis. The independent t-test used to compare the demographic characteristics parameters between MS patients and healthy controls. Genotype and allele frequencies of IL-10 MS patients and healthy controls were analyzed using the Chi-square (χ^2) test. Both genotype and allelic odds ratio (OR) and 95% confidence interval (CI) calculated to determine the association of the SNP -1082 (G>A) in the promoter region of the IL-10 gene polymorphisms with MS. Simple logistic regression and one way ANOVA were used to know the association and difference between Mn-SOD gene polymorphisms and EDSS score. A p-value of less than 5% ($p < 0.05$) set to be statistically significant.

4. Results

All subjects in both groups 40 RRMS patients and 40 healthy volunteers were genotyped for –1082 G/A SNP in the IL10 gene successfully and approximately 10% of randomly selected DNA samples were re-analyzed without finding any discrepancies. The observed distribution of genotypes in controls and patients did not show significant difference with Hardy–Weinberg equilibrium (P = 0.428, P = 0.114 respectively, χ^2 test). The genotype distribution in the study population of RRMS cases and controls were different (AG Vs. GG, P = 0.467; AA Vs. GG, P = 0.326), without reaching the statistical significance. Also, a tendency for the

elevation of A-allele was observed among cases compared to controls (A Vs. G; P = 0.267). Consequently, the AA genotype is overrepresented in RRMS patients than controls (32.50% vs. 15%). The carrying of GA-genotype and AA-genotype was associated with higher risk of susceptibility to diseases (OR = 1.467, 95% CI = 0.521 to 4.129, P = 0.467) and (OR = 1.692, 95% CI = 0.589 to 4.855, P = 0.326) respectively, without reaching the statistical significance. The G-allele could be accepted as protective factors for RRMS (OR = 0.703, 95% CI = 0.376 to 1.312, P = 0.267).

Expanded disability status scale (EDSS) the number assigned to MS patients depending on the severity of the disease ranged from 0 to 10. Comparison by one-way ANOVA revealed that there are significant differences between genotypes (GG, GA, and AA) (p-value < 0.0001). In Dunnett's multiple comparisons tests showed that the mean of EDSS score by GA genotypes was significantly lower if compared to GG genotypes (GA VS GG: 3.433 VS 0.666), besides, the mean of EDSS score by AA genotypes was also significantly lower if compared to GG genotypes (GA VS GG: 4.154 VS 0.666) (figure 1a). Regarding the difference of EDSS in G and A alleles, the mean of EDSS in patients with A alleles recorded more (3.685 ± 0.377) if compared to G alleles (2.204 ± 0.3513), their differences are significant (0.006) (figure 1b).

5. Discussion

Multiple sclerosis is a neurodegenerative disease (Biernacka-Lukanty et al., 2015), that is characterized by an imbalance between inflammatory and anti-inflammatory cytokines (Özenci et al., 2000; Slavin et al., 2010). Among cytokine, the Interleukin-10 is the best-studied and most prominent in human anti-inflammatory cytokine, and it is the crucial target candidate-gene for genetic

association studies of developing MS susceptibility implicates (Tizaoui, 2018; Wong et al., 2011).

The proximal promoter region of the IL-10 gene is highly polymorphic with multiple SNP sites, including (−1082A/G, −819T/C and −592A/C) are related to the level of IL-10 expression, and the risk of MS susceptibility is related to SNPs of IL-10 gene (Jiang et al., 2015; Wergeland et al., 2005).

In the present study (genetic case-control studies), the association of –1082 G/A SNPs in IL-10 genes with MS in Erbil province Kurdistan Region-Iraq investigated by comparing the frequency of alleles or genotypes between the cases and controls. The Genotypes expressed as AA (A) and GG (G) in the homozygote alleles while AG in the heterozygous allele. In the IL-10 −1082 G/A SNPs position, these genetic models compared A/A genotypes to G/A + G/G genotypes in the dominant model and A/A + G/A to G/G genotypes in the recessive model.

According to the result of the present study, –1082 G/A polymorphism represents a statistically no significant association observed between the IL-10 polymorphism and MS susceptibility. But the polymorphic AG genotype and AA-genotype frequencies of IL-10 SNP was elucidated to be non-significantly increased in MS patients. Meanwhile, the frequencies of A and G alleles were also not found to be related to MS.

Our results agree with previous reports which recommended that RRMS with GA and AA genotypes are at high risk to develop MS and the G allele accepted as protective factors for MS in the Kurdish population. The conflicting results from different communities reported. Most studies on IL-10 gene polymorphisms represented no association with MS. Our observation is in concordances with the studies by Izad et al. in an Iranian population and by Mirowska-Guzel et al. in a Polish community, which did not reveal any differences in IL-10 the promoter gene −1082 G/A SNPs haplotypes and genotypes frequency

between patients and controls (Izad et al., 2010; Mirowska-Guzel et al., 2011). Additionally, Luomala et al. exhibited that −1082 SNP was not significantly associated with the manifestation of MS (Luomala et al., 2003). Similar to us, both Pickard et al. and Myhr et al. studies declared that no associations were ascertained for any IL10 gene promoter polymorphisms when the MS cases compared with controls with regards to disability (Myhr et al., 2002; Pickard et al., 1999). On the other hand, the genetic meta-analysis also showed and realized none significant association of –1082 G/A SNPs in IL-10 genes with MS (Nikolopoulos et al., 2011; Ramakrishnan et al., 2017; Tizaoui, 2018).

Whereas the conflicting results with association were obtained such as the studies in Europeans were done by Galehdari et al. which explained that the result showed a significant relationship between this IL-10 the promoter gene −1082 G/A polymorphism and MS risk (Galehdari et al., 2015; Nikolopoulos et al., 2011). Besides, Shahbazi et al. revealed that IL-10 polymorphisms seen to be associated with susceptibility to MS (Shahbazi et al., 2017).

However, theoretically, IL-10 must be decreased in RRMS patients; it means that AA genotypes should be predominant in RRMS patients since this genotype is responsible for lower production of IL-10. The AA genotypes in this study were higher in patients without reaching a significant level. The Rieckmann et al. (1995) showed that IL-10 is up-regulated in stable than active RRMS but Van Boxel- Dezaire et al. (1999) found that it down-regulated in patients during attacks, one of the causes of decreasing IL-10 in MS is due to polymorphism of -1082 G/A SNPs and changing G allele in the promoter region of IL-10 to A allele (Yılmaz et al., 2005). If IL-10 decreases due to -1082 G/A SNPs, the Th will shift to Th1, cell-mediated immunity will predominate, inflammation will rise, the MHC-Ⅱ will be up-regulated, the myelin basic protein (MBP) antigen will be presented by macrophage to Th lymphocyte and consequently, the auto-immune

disease is flared up (Abbas et al., 2019). When the IL-10 decreases, the epitope spreading, the appearance of cryptic antigen and bystander activation of the immune cell will happen which cause further aggravates of the disease (Abbas et al., 2019)

6. Conclusions

To be concluded, the genotype frequencies of this IL-10 promoter gene -1082 G/A SNP is not a significant risk factor predisposing in developing MS disease susceptibility in our population. However, EDSS scores are higher in carrier A alleles (GA and AA genotypes), which means that this polymorphism may increase the severity of the disease. Due to the small size of the sample, polymorphism of IL-10 without serum IL-10 determination, and homogenous ethicality of the population (Since all MS patients are Kurds), this study cannot explain the critical role of IL-10 promoter gene -1082 G/A SNP in the pathogenesis of MS.

Funding/support

This research received no specific grant from any funding agency in public, commercial, or non-profit sectors.

Declaration of Competing Interest

None of the authors has any financial conflict of interest relating to this manuscript.

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Figure caption

Figure 1: EDSS in different genotypes (a) and alleles (b) of –1082 G/A SNPs in IL-10 genes in MS patients.

Funding/support

This research received no specific grant from any funding agency in public, commercial, or non-profit sectors.

Declaration of Competing Interest

None of the authors has any financial conflict of interest relating to this manuscript.

Figure
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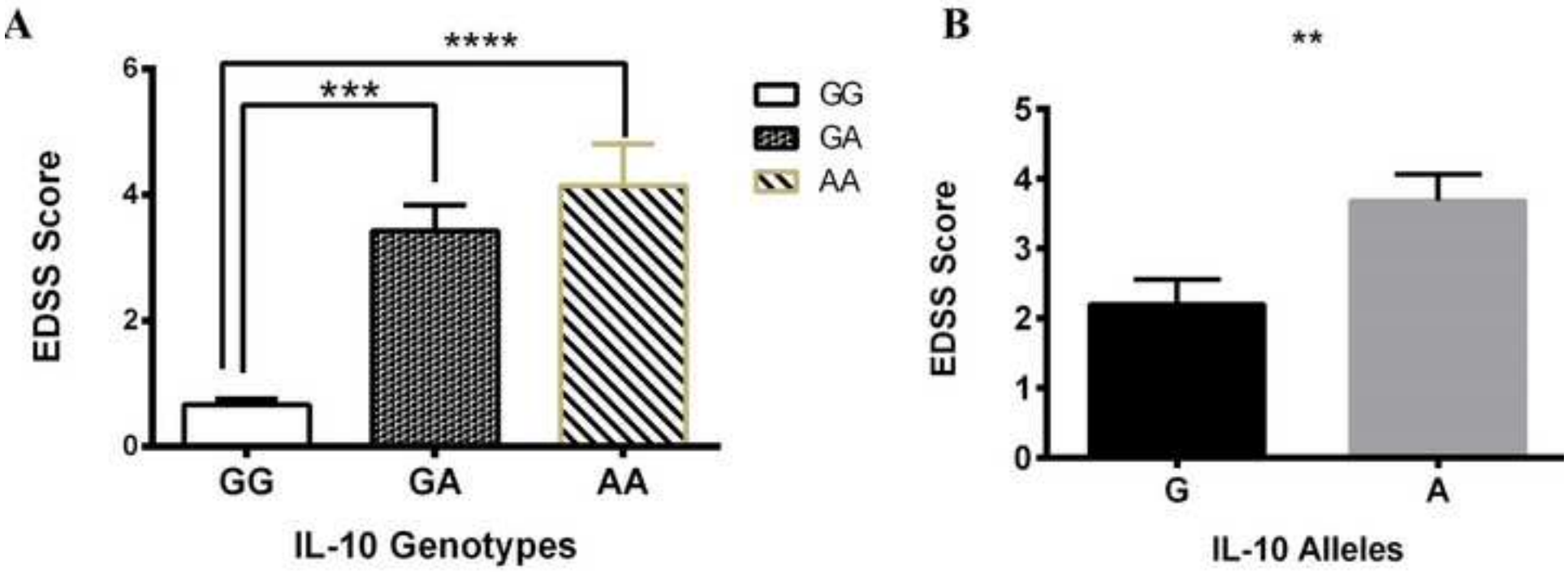

Table 1: Demographic parameters of both MS patients and healthy control participants

Variable	MS Patients Mean ± SE	Controls Mean ± SE	p-Value
MS Patients	34.85 ± 1.442	32.25 ± 1.375	0.196
EDSS	3.250 ± 0.354		
Duration of disease	4.227 ± 0.503		
Number of attacks	3.400 ± 0.395		

Table 2: Association of MS with carriage of alleles/genotypes of IL-10

Polymorphism	ASDs (N=40)		Control (N=40)		OR	95% CI	P-value
	No	%	No	%			
GG	12	30	12	30			-
AG	15	37.50	22	55	1.467	0.521 to 4.129	0.467
AA	13	32.50	6	15	1.692	0.589 to 4.855	0.326
AG+AA	28	70	28	70	0.785	0.296 to 2.085	0.627
GG+AG	27	67.50	34	85	1.344	0.573 to 3.150	0.495
G	39	48.75	46	57.50	0.703	0.376 to 1.312	0.267
A	41	51.25	34	42.50			
HWE P-Value	0.428		0.114				