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# Association study of *Interleukin 10* gene polymorphisms in Iraqi patients with multiple sclerosis

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ABSTRACT

Multiple sclerosis (MS) is a type of autoimmune disease where immune cell attacks our cells mistakenly; its severity is measured by expanded disease status scale (EDSS). The study aims the investigation of −1082 polymorphism in interleukin 10 (IL-10) as one of the etiologies that develops the disease. This is a case-control study that allele-specific polymerase chain reaction (AS-PCR) were provided to compare 100 relapsing-remitting MS (RRMS) patients, which fulfills McDonald criteria with 100 healthy controls depending on the −1082 (G/A) polymorphism of the gene encoding IL-10. The A allele frequency of IL-10 gene has been considerably less in MS patients compare to healthy control (60.50 Vs. 81%). Genotype distributions of the single nucleotide polymorphism (SNP) -1082 fulfills Hardy-Weinberg equilibrium in cases (P = 0.155) but it doesn't in controls (P < 0.0001). In MS patients, Heterozygous (GA) genotypes were non-significantly associated with MS (OR = 0.834,95% CI = 0.6890 to 1.29, P 0.706) but homozygous (AA) were significantly associated with this condition (OR = 3.420, 95% CI = 1.450 to 8.065, P = 0.0037). To conclude, the genotype distribution of −1082 (G/A) polymorphism has been showed a significant difference in the case/control study recruited in Erbil province-Iraq, and EDSS is significantly higher in A allele's carrier genotypes. There was non-significance association AA genotypes and duration of the disease.

## 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 multiple sclerosis (MS) (Özenci et al., 2000; Huang et al., 2015). Above two million people were diagnosed with Multiple sclerosis MS worldwide, between the ages of 20–40. MS includes the most prevalent symptoms which are Cognitive changes, fatigue, spasticity, paresis, dizziness, and tingling (Goldenberg, 2012). The disease severity of MS is measured by EDSS. The etiology of this disease still is unknown, but scientists suppose both genetic and the environmental factors play a part in the disease pathogenesis (Gourraud et al., 2012). Disruption between pro-inflammatory and anti-inflammatory cytokine balance has been thought of as a cause for

*Abbreviation:* IL-10, interleukin 10; MS, multiple sclerosis; EDSS, expanded disease status scale; GWAS, genome-wide association study; AS-PCR, allele-specific polymerase chain reaction.

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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 hundreds of risk loci for MS such as tumor necrosis factor-alpha (TNF-α), human leukocyte antigen, *IL-7*, *IL-2* (Arabi et al., 2018), *TNF-RSF14*, *CD58*, *CD86*, *TNF-RSF14*, *IL-12RB1*, *IL-22RA2*, and *IL-10* (International Multiple Sclerosis Genetics Consortium, 2011).

Among 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 MS and its pathology (Gourraud et al., 2012). The Locus of *IL-10* is at chromosome 1q32.1, in which it consists of 5 exons producing 178 amino acids made IL-10 (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 the production of cytokine (Weiner, 2009). Therefore SNPs have a crucial function which may affect the vulnerability to MS (Smith and Humphries, 2009).

In the *IL-10* promoter, three SNPs −592 (A/C), −819 (T/C) and −1082 (G/A) are clustered that forms three haplotypes ACC, ATA, and GCC respectively, which they are linked to the expression rate of *IL-10* gene (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 moderate expression relationship, finally, ACC/ACC, ATA/ACC, and ATA/ATA are associated with low expression (Mihailova et al., 2005). A single study that had been 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 of −1082 G/A SNP with vulnerability to MS (Azarpira et al., 2010; Galehdari et al., 2015). Thus, our aim in this research is the study of the relationship of −1082 G/A SNP in *IL-10* and MS susceptibility within another ethnic group in Kurdish population in Iraq. This study aimed to investigate the relationship between *IL-10* gene polymorphism at −1082 (G/A) and MS susceptibility within another ethnic group in Kurdish population in Iraq.

## 2. Materials and methods

### 2.1. Sample collection and DNA analysis

An overall five ml of peripheral blood samples in EDTA tube of 100 MS patients (50 males and 50 females) collected from the department of neurology, Rzgari hospital in Erbil city, Iraq, who diagnosed according to the 2005 revision of McDonald criteria (Polman et al., 2005). The blood was also collected form 100 healthy controls (50 males and 50 females). The MS patients who were recruited with mean age of 34.85 ± 1.442 years and of the control group was 32.25 ± 1.375 years. Statistically, the ages showed non-significant difference and also the EDSS was measured for the patients with the mean of (3.250 ± 0.353).

Human blood samples were taken from peripheral veins using the five-milliliter syringe. The blood put into K2EDTA (5.4 mg) tube for direct DNA extraction. The DNA was extracted using a spin column method (AccPrep Genomic 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. MS patients and healthy control group with their Demographic profiles are shown in Table 1.

**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 | | |

Genotyping of the SNP in promoter region at position −1082G > A of IL-10 gene performed by a rapid and cost-effective technique called AS-PCR method. In this technique we did allele-specific amplification for all subjects by two reactions with three DNA primers. One of the reactions is used to amplify 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) (https://www.ncbi.nlm.nih.gov/tools/primer-blast/)). 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 2 μl of genomic DNA, 10 μl Taq DNA Polymerase 2× Master Mix RED (Ampliqon, Danish) and 1 μl for each forward and reverse primers. Then 6 μl of nuclease-free water is added to complete the mixture. The PCR program started with initial denaturation step which was set at 94 °C for 5 min and after that the next 35 cycles 94 °C for 30 s (denaturation), 62 °C for 30 s (annealing), 72 °C for 30 s (elongation). The final step (extension step) was set at 72 °C for 5 min 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. If 258 bp was found in separated reactions of a sample, it was heterozygote (AG). If 258 bp was only seen in A allele reactions of a sample, it was mutant homozygote (AA), If 258 bp was only seen in G allele reactions of a sample, it was wild homozygote (GG).

### 2.2. Statistical analysis

Graph Pad Prism 6 and SPSS statistical softwares used for doing statistical analysis. All data fulfilled the criteria of performing tests of normality (Kolmogorov-Smirnov, Shapiro-Wilk and D'Agostino), so parametric tests were applied. To compare MS patients with healthy control group in the aspect of demographic characteristic parameters, the Independent *t*-test was used. The Chi-square ($\chi^2$) test was used to analyze and compare the two variables which are IL-10 genotype and allele frequency in both MS patients and healthy controls. We calculate both odds ratio (OR) and 95% confidence interval (CI) for both genotype and allele to estimate the correlation of the polymorphism in IL-10 promoter region specifically the SNP -1082 (G > A) with MS. Binary logistic regression used to define the correlation of genotype with both EDSS and duration of the disease.

The *t*-test and one-way ANOVA were used to know the difference between IL-10 gene polymorphisms and EDSS score in alleles and genotypes. A statistically significant *p*-value of less than 5% ($p < 0.05$) was determined.

## 3. Results

All subjects were genotyped in both groups 100 RRMS patients and 100 healthy volunteers for SNP –1082 G/A in the IL-10 gene successfully and approximately 10% of randomly selected DNA samples were re-analyzed without finding any discrepancies. In control group, the noticed genotypes distribution was not harmony but in patients are

harmony with Hardy–Weinberg equilibrium ($P < 0.0001$, $p = 0.155$ respectively, $\chi^2$ test). The genotype distribution in the study population of RRMS cases and controls were different (GA Vs. GG, $P = 0.707$; AA Vs. GG, $P = 0.004$), reaching the statistical significance in AA-genotype but they don't in GA-genotype. Also, a tendency for the difference of A-allele was observed among cases compared to controls (A Vs. G; $P < 0.0001$). Consequently, the AA genotypes increase susceptibility to the disease by 3.420 folds. The carrying of GA-genotype was not but AA-genotype was associated significantly with higher risk of susceptibility to diseases (OR = 0.834, 95% CI = 0.6890 to 1.29, P 0.706) and (OR = 3.420, 95% CI = 1.450 to 8.065, $P = 0.004$) respectively. The A-alleles could be accepted as risk factors for RRMS (OR = 2.783, 95% CI = 1.769 to 4.379, $P < 0.0001$). Regarding haplotypes, we found significant association in dominant model comparison (GA/AA Vs. GG: OR = 3.857, 95% CI: 2.133 to 6.974; $P \leq 0.0001$), but a non-significant association was found in the recessive model (GG/GA Vs. GG: OR = 2.111, 95% CI: 0.927 to 4.806; $P = 0.071$) (Tables 2 & 3).

### 3.1. EDSS comparisons and associations in different genotypes and alleles

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 higher if compared to GG genotypes (GA VS GG: 3.194 + 0.250 VS 1.286 + 0.368), similarly, the mean of EDSS score by AA genotypes was also significantly higher if compared to GG genotypes (AA VS GG: 5.833 + 0.188 VS 1.286 + 0.368) (Fig. 1b). Regarding the difference of EDSS in G and A alleles, the mean of EDSS in patients with A alleles recorded more (3.685 ± 0.264) if compared to G alleles (2.204 ± 0.246), their differences are significant ($p$-value <0.0001) (Fig. 1a).

Binary logistic regression revealed that the AA genotypes associated with the severity of disease score (EDSS) significantly (OR = 1.087, 95% CI = 0.771 to 1.532, $P = 0.001$), but there was non-significant association with the duration of the disease (OR = 2.671, 95% CI = 1.461 to 4.885, $P = 0.634$) (Table 4).

## 4. Discussion

Multiple sclerosis is defined as a neurodegenerative disease (Biernacka-Lukanty et al., 2015), that is characterized by balance interruption between pro-inflammatory (*TNF alpha* and *IL-1*) and anti-inflammatory cytokines (*IL-4*, *IL6*, and *IL-10*) (Özenci et al., 2000; Slavin et al., 2010; Smail et al., 2020). Among cytokines, 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 (Wong et al., 2011; Tizaoui, 2018).

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

In the present study (genetic case-control studies), association of −1082 G/A SNPs of *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 dominant model and also in the recessive model compared A/A + G/A to G/G genotypes.

According to the result of the present study, −1082 G/A polymorphism represents a statistically significant association observed between the polymorphism of *IL-10* gene and susceptibility to MS. Furthermore, the polymorphic AG genotypes of *IL-10* SNP were elucidated to be non-significantly but AA-genotype frequencies increased in MS patients. Meanwhile, the frequencies of A were also found to be a risk factor 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 A allele accepted as risk factors for MS in the Kurdish population. The conflicting results from different communities reported. Nearly all studies on SNPs of *IL-10* gene represented non-significant association with MS. Our observation contradicted the studies done by Izad et al. in Iranian community and Mirowska-Guzel et al. in a Polish community, which had not shown differences of *IL-10* promoter region −1082 G/A SNPs haplotypes and genotypes frequency between MS patients and control group (Mirowska-Guzel et al., 2011; Izad et al., 2010). Additionally, Luomala et al. exhibited that −1082 SNP represented non-significant association with MS pathogenesis (Luomala et al., 2003). In contrast to us, both Pickard et al. and Myhr et al. studies declared that there were no confirmed correlation between polymorphism in *IL-10* gene promoter and disability in MS cases (Pickard et al., 1999; Myhr et al., 2002). On the other hand, the genetic meta-analysis also showed and realized statistically none-significant association between −1082 G/A SNPs of *IL-10* genes and MS (Nikolopoulos et al., 2011; Ramakrishnan et al., 2017; Tizaoui, 2018).

Whereas the similar 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 of *IL-10* promoter gene

**Table 3**
EDSS comparisons in different genotypes and alleles.

| Polymorphism | MS | P=Value |
|---|---|---|
| | EDSS Mean | |
| GG | 1.286 + 0.368 | <0.0001 |
| GA | 3.194 + 0.250 | |
| AA | 5.833 + 0.188 | |
| G | 2.204 ± 0.246 | <0.0001 |
| A | 3.685 ± 0.264 | |

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

| Polymorphism | MS ($N = 100$) | | Control (N = 100) | | OR | 95% CI | $P$ value |
|---|---|---|---|---|---|---|---|
| | No | % | No | % | | | |
| GG | 19 | 19 | 10 | 10 | – | – | – |
| GA | 41 | 41 | 18 | 18 | 0.8341 | 0.689 to 1.290 | 0.707 |
| AA | 40 | 40 | 72 | 72 | 3.420 | 1.450 to 8.065 | 0.004 |
| GA + AA | 81 | 81 | 90 | 90 | 2.111 | 0.927 to 4.806 | 0.071 |
| GG + GA | 60 | 60 | 28 | 28 | 3.857 | 2.133 to 6.974 | <0.0001 |
| G | 79 | 39.50 | 38 | 19.00 | 2.783 | 1.769 to 4.379 | <0.0001 |
| A | 121 | 60.50 | 162 | 81.00 | | | |
| HWE P-Value | 0.155 | | <0.0001 | | | | |

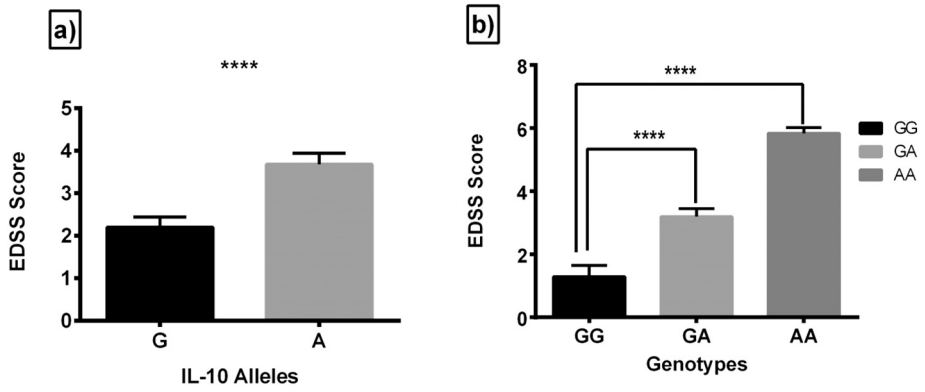


**Fig. 1.** EDSS in different genotypes (b) and alleles (a) of −1082 G/A SNPs in IL-10 genes in MS patients.

**Table 4**
Logistic regression analysis to associate of EDSS and duration of disease with genotypes.

| Variable | OR | CI | p-Value |
|---|---|---|---|
| EDSS | 1.087 | 0.771–1.532 | 0.001 |
| Duration of disease | 2.671 | 1.461–4.885 | 0.634 |

−1082 G/A polymorphism and the risk of MS (Nikolopoulos et al., 2011; Galehdari et al., 2015). Besides, Shahbazi et al. revealed the role of *IL-10* polymorphisms in the development of MS (Shahbazi et al., 2017). The AA genotypes are predominant in RRMS patients since this genotype take the responsibility of lowering the *IL-10* synthesis.

The AA genotypes in this study were higher in patients and reaching a significant level may cause low level of *IL-10* in the serum of the patients with RRMS. 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 G allele changed in *IL-10* promoter region 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).

The EDSS scores the severity of the disease; logistic regression uncovered signification association with AA genotypes; similarly, ANOVA tests confirmed that there was signification difference between A allele carrying genotypes and non A allele carrying genotypes. Furthermore, logistic regression showed non signification association between AA genotypes and other genotypes (GA and GG).

To be concluded, both the genotype and haplotypes, besides the allele frequency of *IL-10* promoter gene −1082 G/A SNP is a significant risk factor predisposing in developing MS disease susceptibility in our population. Additionally, EDSS scores are higher in carrier A alleles (GA and AA genotypes), which means that this polymorphism may increase the severity and susceptibility 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 and association of *IL-10* promoter gene −1082 G/A SNP in the pathogenesis of the MS.

## CRediT authorship contribution statement

All the authors have equally contributed.

## Ethical approval

We have followed all ethical approvals for this study.

## Informed consent

All authors have read and approved the contents and manuscript.

## Declaration of competing interest

The authors have declared that no competing interests exist.

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*Ethical approval and consent to participate*

The present study was authorized and approved by the Human Ethics Committee of Salahaddin University-Erbil. Patients provided informed consent.

*Patient consent for publication*

All patients provided written informed consent for the publication of data in this study.

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