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**INTERNATIONAL JOURNAL OF  
 ADVANCED RESEARCH (IJAR)**

Article DOI: 10.21474/IJAR01/1718  
 DOI URL: <http://dx.doi.org/10.21474/IJAR01/1718>



### RESEARCH ARTICLE

#### WHAT IS THE IMPACT OF *IL12B*<sub>+1188</sub> SINGLE NUCLEOTIDE POLYMORPHISM ON IL-12 SERUM LEVEL OF MULTIPLE SCLEROSIS IRAQI PATIENTS?

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#### Manuscript Info

##### Manuscript History

Received: 12 July 2016  
 Final Accepted: 19 August 2016  
 Published: September 2016

##### Key words:-

Multiple sclerosis, IL-12 serum level,  
 Single nucleotide polymorphism.

#### Abstract

Multiple sclerosis (MS) is a chronic neurodegenerative disease that results from interaction between genetic, epigenetic, and environmental factors, and affects especially young adults. Serum level and single nucleotide polymorphism (SNP) of Interleukin-12 (IL-12) were determined in 68 relapsing-remitting MS Iraqi patients and 20 healthy individuals, matched patients for ethnicity, age and gender. The patients were distributed into IFN $\beta$  pre- and post-medicated patients, in addition, extended disability status scale (EDSS) was also applied as a parameter for distribution. The results indicated that there were significant differences in serum levels of IL-12 among the three investigated groups; pre- and post-medicated patients and controls ( $35.9 \pm 1.6$  vs.  $29.5 \pm 2.4$  vs.  $22.7 \pm 2.9$  pg/ml, respectively). However, there was no significant effect of EDSS on the level of IL-12 in pre- and post-medicated patients. *IL12B* gene SNP at position +1188 was presented with three genotypes (AA, AC and CC), which showed a significant difference between the observed and expected genotype frequencies ( $p \leq 0.01$ ) in patients, while a good agreement with Hardy-Weinberg equilibrium was observed in controls. However, there was no significant variation between patients and controls in the distribution of *IL12B*<sub>+1188</sub> allele and genotype frequencies; although CC genotype was observed with a frequency of 17.9% in MS patients, while none of the controls possessed this genotype, and the odds ratio of such difference was 9.23. A significant impact of *IL12B*<sub>+1188</sub> SNP on IL-12 serum level was recorded in MS patients carrying CC and AC genotypes ( $27.1 \pm 3.1$  vs.  $35.9 \pm 2.4$  pg/ml, respectively), while no such impact was observed in controls.

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#### Introduction:-

Multiple sclerosis (MS) is a neurodegenerative autoimmune disease that causes axon demyelination leading to plaque formation in white matter of central nervous system (Shirani and Tremlett, 2010). Genetic, epigenetic and environmental factors were suggested to trigger MS episodes, in which immunological factors are also involved; especially cytokines (Fragoso *et al.*, 2014; Fukaura, 2014; van den Elsen *et al.*, 2014).

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IL-12 is one of these cytokines, composes of two subunits; p35 and p40. It regulates both innate and adaptive immune responses, and produced mainly by phagocytes and dendritic cells upon infection with various pathogens. Such production results in stimulating natural killer (NK) cells and cytotoxic T-lymphocytes (CTLs) to secrete IFN- $\gamma$  (Xu *et al.*, 2010). Additionally, IL-12 activates T-cell differentiation and antibody production by B-cell, increases the cytotoxicity and differentiation of naive CD4<sup>+</sup> T-cells to Th1 cells, and enhances the expression of MHC II and adhesion molecules on Th1 cells (van Wanrooij *et al.*, 2012).

*IL12B* gene is located on chromosome 5q31-33, and encodes the p40 subunit that is responsible for proliferation and differentiation of Th1 cells through enhancing IFN- $\gamma$  production. The gene has been suggested to have relevance to many autoimmune diseases including MS (Zhang *et al.*, 2015). *IL12B*<sub>+1188</sub> SNP has been suggested to have a significant role in MS predisposition (Shokrgozar *et al.*, 2009), but a recent meta-analysis by Huang *et al.* (2016) failed to confirm such finding in Caucasian and some Asian patients. Accordingly, the present investigation aimed to determine the role of IL-12 in pathogenesis of MS in Iraqi patients in terms of serum level and genetic polymorphism of *IL12B* at locus +1188.

## Materials and methods:-

### Patients:-

Sixty eight Iraqi Arab Muslim patients were diagnosed as relapsing-remitting multiple sclerosis (RRMS) patients by physicians according to McDonald criteria 2010 revision (Milo and Miller, 2014) at Multiple Sclerosis Clinic (Baghdad Teaching Hospital). The patients were distributed as: 30 pre-medicated patients (did not receive the immunomodulatory drug IFN $\beta$  or corticosteroids) and 38 patients received IFN $\beta$  (post-medicated). The post-medicated patients were treated with 8MI4 dosage of IFN $\beta$  every other day administered subcutaneously (SC). The patients were also distributed according to the extended disability status scale (EDSS); < 3 and  $\geq$  3. Scores from 0 to less than 3 (< 3) means that the patient is fully ambulant, while score equals or higher than 3 ( $\geq$  3) is given to patients with moderate to high disability. Some detailed informations about patients are given in table 1.

### Controls:-

The controls (20 subjects) were obtained from Teaching Laboratories of Medical City personnels who were not receiving any non-steroidal anti-inflammatory drugs (NSAIDs) for at least 48 hours, non-smokers, and had no history of any autoimmune disease, and were apparently healthy. They were Iraqis and distributed as 5 males (age mean: 37.0  $\pm$  4.9 years) and 15 females (age mean: 34.3  $\pm$  2.2 years).

**Table 1:-** Details of multiple sclerosis patients and their distributions.

Characteristics		Multiple Sclerosis Patients (No. = 68)			
		Pre-medicated (No. = 30)		Post-medicated (No. = 38)	
		Males (No. = 10)	Females (No. = 20)*	Males (No. = 13)	Females (No. = 25)**
Age Mean $\pm$ SE (Years)		34.9 $\pm$ 3.1	33.3 $\pm$ 2.3	34.5 $\pm$ 2.2	36.1 $\pm$ 2.1
Extended Disability Status Score	< 3	7	7	9	17
	$\geq$ 3	3	10	4	7

\*EDSS was missing from three cases; \*\*EDSS was missing from one case

### Sample collection:-

The blood (5 ml) of 68 MS patients and 20 controls was withdrawn into two kinds of tubes: plain and K<sub>2</sub>-EDTA tubes. The plain tube blood was left to clot, and then centrifuged at 3000 rpm and the serum was collected and frozen at -20°C until assessment of IL-12 serum level. The K<sub>2</sub>-EDTA blood was frozen at -20°C until isolation of DNA for *IL12B*<sub>+1188</sub> SNP determination.

### Measurement of IL-12 Serum Level:-

Serum level of IL-12 was assessed in sera of MS patients and controls by means of ELISA (enzyme linked immunosorbent assay) principles. The assessment was carried out by using mini-ELISA kit that was produced by PeproTech Company (U.K.), and the manufacturer instructions were followed. The sample results were calculated by interpolation from a standard curve that was performed in the same assay as that for the samples by using standard curve fitting equation for IL-12. The equation and drawing of the standard curve were carried out using Microsoft Excel 2010.

**DNA isolation:-**

Genomic DNA was isolated from frozen EDTA blood by using ReliaPrep™ gDNA MiniPrep System Kit, U.S.A. The concentration and purity of the DNA samples were estimated by using NanoDrop technology at optical densities of 260/280 nm wavelength.

***IL12B*<sub>+1188</sub> Genotyping:-**

Single nucleotide polymorphism for *IL12B*<sub>+1188</sub> gene was analysed by using Cytokine CTS-SSP-PCR Tray kit provided by Collaborative Transplant Study (CTS) for genotyping of cytokine polymorphisms, Heidelberg, Germany. The genotyping was performed according to the instruction supplied with the kit, and then agarose gel electrophoresis was performed by using 2% agarose at 170 V for 25 minutes. The amplification signal was identified positive or negative through UV transilluminator (312nm) and the results were interpreted according to the manual supplied with kit.

**Statistical analysis:-**

Serum level of IL-12 was statistically analyzed using SPSS (Statistical Package for Social Sciences) version 13. The data were given as mean  $\pm$  standard error (S.E.), and differences between means were assessed by ANOVA (Analysis of Variance), followed by LSD (Least Significant Difference) or Duncan test. A Pearson Chi-square was also used to test the significant differences between EDSS within patients' group.

Allele frequencies of *IL12B*<sub>+1188</sub> gene was calculated by direct gene counting method, while significant departure from Hardy-Weinberg equilibrium (HWE) was estimated using online H-W calculator for two alleles (<http://www.had2know.com/academics/hardy-weinberg-equilibrium-calculator-2-alleles>). Allele and genotype of *IL12B*<sub>+1188</sub> were presented as percentage frequencies, and significant differences between their distributions in MS patients and controls were assessed by two-tailed Fisher's exact probability (*p*). In addition, odds ratio (OR), etiological fraction (EF) and preventive fraction (PF) were also estimated to define the association between alleles and genotypes with the disease (Ad'hiah, 1990; Abramson, 2011).

**Results and Discussion:-****IL-12 serum level:-**

Pre-medicated MS patients showed the highest level of IL-12 ( $35.9 \pm 1.6$  pg/ml), followed by post-medicated patients ( $29.5 \pm 2.4$  pg/ml), and finally controls ( $22.7 \pm 2.9$  pg/ml), and these differences were significant. However, there was no significant effect of EDSS on the level of IL-12 between pre- and post-medicated patients (Table 2).

**Table 2:-** Serum level mean of IL-12 in pre-medicated and post-medicated multiple sclerosis patients and controls.

Groups		IL-12 Serum Level Mean $\pm$ S.E. (pg/ml)		
		Patients (No. = 68)		Controls (No. = 20)
		Pre-medicated (No. = 30)	Post-medicated (No. = 38)	
Total		$35.9 \pm 1.6^A$	$29.5 \pm 2.4^B$	$22.7 \pm 2.9^C$
EDSS	< 3	$35.7 \pm 2.1^A$	$28.8 \pm 2.7^A$	$22.7 \pm 2.9^B$
	$\geq 3$	$37.3 \pm 2.6^A$	$32.6 \pm 5.1^A$	$22.7 \pm 2.9^B$
<i>p</i> $\leq$		Not significant	Not significant	-

Different superscript letters: Significant difference between means of rows.

*p*: Probability of difference between means of extended disability status scale groups.

Dimisianos *et al.* (2014) showed a significant increased level of IFN- $\gamma$ , TNF- $\alpha$ , and IL-2 in sera of IFN- $\beta$  responder MS patients compared to untreated patients. A similar observation was also made for IL-12 (Musabak *et al.*, 2011); therefore, IL-12 might be involved in MS immunopathology, especially in pre-medicated patients of present study. It has been suggested that IL-12 is the main regulator of Th1 cell response through IFN- $\gamma$  production as it is one of the CD8+ cells signals and also contributes to CD4+ cells survival (Del Vecchio *et al.*, 2007). Elevated level of this cytokine was interpreted as a dysfunction of NK cells activity and imbalance of Th1/Th2 ratio. Accordingly, this cytokine might be considered as one of the markers for MS pathogenesis (Wong *et al.*, 2015). The investigated IL-12 was also evaluated in terms of EDSS, and the results suggested that serum level of this cytokine might not be influenced by EDSS.

**IL12B Gene SNP at +1188 Position (rs3212227):-**

The *IL12B* gene SNP at position +1188 was presented with two alleles (A and C) and three genotypes (AA, AC and CC). These genotypes showed deviation from HWE in MS patients, because there was a significant difference between the observed and expected genotype frequencies ( $p \leq 0.01$ ), while a good agreement with HWE was observed in controls (Table 3). The results revealed that genotype frequencies of AA (52.2% vs. 60.0%) and AC (29.9% vs. 40.0%) showed variations between MS patients and controls, but the differences were not significant. The third genotype (CC) was observed with a frequency of 17.9%, while none of the control subjects possessed this genotype. Such difference scored OR value of 9.23, and the associated etiological fraction (EF) was 0.16. In addition, the mutant allele (C) was also observed to have an increased frequency in patients compared to controls (32.8% vs. 20.0%; OR = 1.96; EF = 0.16; 95% C.I. = 0.84 - 4.55). However, none of these differences in genotype and allele frequencies attended any significant level (Table 3).

Reviewing the literature, it was revealed that *IL12B*<sub>+1188</sub> SNP in MS was the subject of five studies; four in Caucasians (Hall *et al.*, 2000; van Veen *et al.*, 2001; Forte *et al.*, 2006; Shokrgozar *et al.*, 2009), and one in Asians (Liu *et al.*, 2014). Several significant and non-significant genetic associations with this SNP were reported in these studies; but the general theme was in favor of *IL12B*<sub>+1188</sub> genotypes and alleles have no association with MS risk.

To clarify the role of *IL12B*<sub>+1188</sub> in MS predisposition, Huang *et al.* (2016) performed a meta-analysis of data reported in the seven case-control studies. Although the pooled analysis showed an association between *IL12B*<sub>+1188</sub> and MS in all study subjects under dominant model and allelic comparison model, in subgroup analysis based on ethnicity, they did not find an association between *IL12B*<sub>+1188</sub> and MS risk in Caucasians under dominant, recessive, homozygote, and allelic comparison models, and the same finding was also recorded for the single Asian study. Accordingly, the present study shares the conclusion of these studies, and *IL12B*<sub>+1188</sub> might have no influence on MS risk.

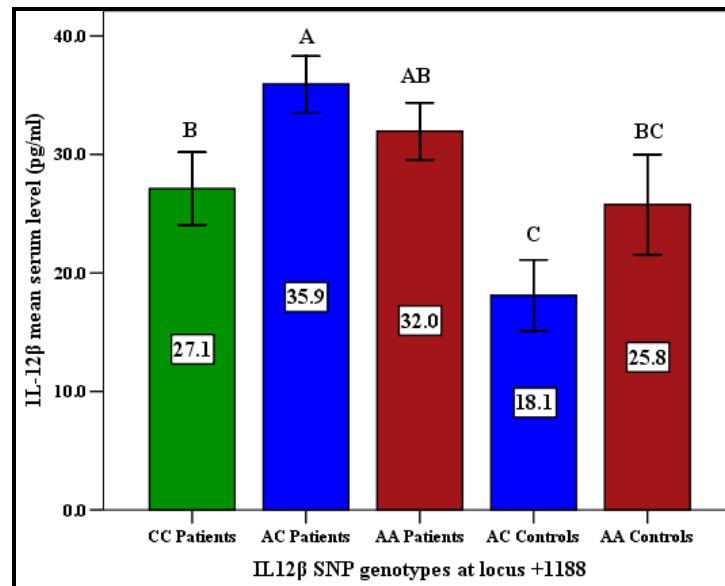
**Table 3:-** Observed and expected genotype and allele frequencies of *IL12B*<sub>+1188</sub> in multiple sclerosis patients and controls.

Groups			<i>IL12B</i> gene at position +1188 (dbSNP-ID: rs3212227)					
			Genotypes			HWE	Alleles	
							A	C
			AA	AC	CC	$p \leq$		
MS patients (No. = 67) (ND=1)	Observed	No.	35	20	12	<b>0.01</b>	90	44
		%	52.2	29.9	17.9		67.2	32.8
	Expected	No.	30.2	29.6	7.2		Not estimated	
		%	45.1	44.1	10.8			
Controls (No. = 20)	Observed	No.	12	8	0	N.S.	32	8
		%	60.0	40.0	0.0		80.0	20.0
	Expected	No.	12.8	6.4	0.8		Not estimated	
		%	64	32	4			
Odds Ratio (OR)			0.73	0.64	9.23		0.51	1.96
Etiological Fraction (EF)			-	-	0.16		-	0.16
Preventive Fraction (PF)			0.16	0.15	-		0.39	-
Fisher's Exact Probability			N.S.	N.S.	N.S.		N.S.	N.S.
95% Confidence interval (C.I.)			0.27-1.97	0.23-1.76	0.56-152.57		0.22-1.19	0.84-4.55

HWE: Hardy-Weinberg Equilibrium; N.S.: Not significant; ND: Not determined

**Impact of *IL12B*<sub>+1188</sub> SNP genotypes on IL-12 Serum Level:-**

The *IL12B*<sub>+1188</sub> CC genotype recorded the lowest serum level of IL-12 among MS patients ( $27.1 \pm 3.1$  pg/ml), but a significant difference was reached when the comparison was made with the mean of AC genotype in patients ( $35.9 \pm 2.4$  pg/ml). In controls, there was no significant impact of *IL12B*<sub>+1188</sub> genotypes on IL-12 level (Figure 1). It was indicated that IL-12 levels are affected by some functional polymorphisms like that in the 3' UTR region of *IL12B* gene (Peng *et al.*, 2006; Gerenova *et al.*, 2016). Nevertheless, some studies referred to the association of *IL12B* gene functional polymorphisms to some autoimmune diseases (Gerenova *et al.*, 2016). Correspondingly, it was stated that polymorphism within *IL12B* gene may influence the responses of Th-1 cells which can be attributed to increase pathogenesis or become beneficial for some autoimmune diseases (Morahan *et al.*, 2001).



**Figure 1:-** Impact of *IL12B*<sub>+1188</sub> genotypes on serum level of IL-12 in multiple sclerosis patients and controls (Different letters: significant difference between means).

### Conclusion:-

The current study indicated to that AC genotype of *IL12B*<sub>+1188</sub> SNP influenced the serum level of IL-12 in a sample of MS Iraqi patients, which may contribute to the pathogenesis of MS. In addition, despite the non-significant variation in allele and genotype frequencies between MS patients and controls, CC genotype of *IL12B*<sub>+1188</sub> SNP can be of risk nine-fold in carriers as compared to none carriers, and makes it as a risk genotype for MS.

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