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Evaluation of Interleukin-6 Gene Expression and Serum Level Related with Medications and Course of Disease in Sample of Multiple Sclerosis Iraqi Patients

[1]Omar A. Ali, [2]Bushra J. Mohammed

[1]Department of Microbiology, College of Medicine, University of Anbar
[2]Institute of Genetic Engineering and Biotechnology for Postgraduate Studies, University of Baghdad

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Abstract: Multiple sclerosis (MS) is a common autoimmune neurological illness. Many investigations have elucidated the aberrant production of particular cytokines over the course of disease may effected via the type of medications. This study aimed to evaluate gene expression and serum level of interleukin-6 (IL-6) related with medications and courses of disease that probably contributing in susceptibility of MS. Blood samples were obtained from 75 MS Iraqi patients, whom received various medications as betaferon, gilenya, rituximab and tysabri. Also the study included control group consist of 75 healthy individuals, the estimation of IL-6 expression was achieved via quantitative real time polymerase chains reaction (qRT-PCR), while evaluation of IL-6 serum level was done by using the Enzyme-linked Immunosorbent Assay (ELISA) The statistical analysis was done by Statistical Analysis System (SAS) to identify the impact of different factors on research parameters. The results revealed that the most common MS course was Relapsing Remitting MS (RRMS) 53(70.7%), as well as, the mainly medication used in treatment of MS Iraqi patients was Betaferon 36(48%), also the results showed that IL-6 gene expression was increased in MS patients (1.87±0.12 folds) as compared to the control group (0.51±0.04 folds), furthermore IL-6 serum level was noteworthy increased in patients group (15.28+0.40 pg/ml) compared to the control group (4.40+0.22 pg/ml), the findings of impact of duration of disease on IL-6 levels revealed that highest IL-6 level was in PPMS followed by SPMS and RRMS, while the result of medications impact on IL-6 levels in MS patients indicated greatest IL-6 averages in patients whom received interferon-ß followed by fingolimod, retuxan, and natalizumab, in general there were diminish in IL-6 serum level in all patients whom used any type of mentioned medications.

Keywords: Multiple Sclerosis; IL-6 serum level; MS Course; MS Medications; IL-6 gene expression.

Corresponding author: (E-mail: dr.bushrajassim@ige.uobaghdad.edu.iq).

Introduction

Multiple sclerosis (MS) is an autoimmune, central nervous system (CNS) disease, where inflammatory cells infiltrate to the CNS and subsequently cause neurodegeneration due to demyelination and loss of oligodendrocytes (1). T- cells are a major player in MS, although other types of immune cell, such as B- cells, macrophages, microglia, as well as, non-immune cells like endothelial cells and neurons, Interferes to disease pathogenesis and many degradative processes such as pro-inflammatory cytokine production, including Interleukin-6 (IL-6), which is an influential inducer of acute immune response, IL-6 receptor, on microglia and some leukocytes, while the soluble

IL-6 receptor (sIL-6R) which consist a complex with IL-6 that inducing downstream signaling by reacting with β-receptor glycoprotein (gp) 130, which is ubiquitously expressed in many cell types, such as astrocytes and neurons, this trans signaling is crucial for pro-inflammatory features of IL-6 (1, 2). The augmented levels of IL-6 in MS patients clarify its pathogenic role in the onset of neuronal destruction and advancement of demyelination. IL-6 released peripherally by inflammatory cells and directly sensitizes nociceptors and enhances T- cell functions via facilitating its proliferation and infiltration to CNS by augmentation of cell adhesion molecule 1 on the vascular endothelial cells, with the presence of transforming growth factor- β, IL-6 also promotes the differentiation of CD4+ T cells to inflammatory T-helper (Th) 17 cells, that produce IL-17 and subsequently stimulate astrocytes to provide IL-6, as well as, reactive oxygen species, and nitric oxide presence in astrocytes enhances IL-6 expression and cause further harms to oligodendrocytes and myelin sheath. In this way leading to worse of MS symptoms, ultimately in the presence of IL-23 resulting in full development of MS (3, 4), hence and because of the implications of MS health problem, the present research was carried out to evaluate IL-6 gene expression and serum level related with medications and courses of disease in some MS Iraqi patients.

Materials and methods

This study was conducted in the laboratories of Institute of Genetic Engineering and Biotechnology for Postgraduate Studies/ University of Baghdad through the period from May 2022 to the end November 2023. Blood samples were collected from 75 MS Iraqi patents who came to MS clinic in Saad ALWatri hospital for neurological sciences in Baghdad and Al-Yarmouk teaching hospital, who taken various treatments as betaferon, gilenya, rituximab and tysabri, Also, the study included 75 gender-age matched healthy volunteers as a control group. All subjects with age ranged between (22-58) years.

IL-6 gene Expression

Two hundred and fifty microliter of blood was obtained from each subject and it was put into 750 µl of trizol preservation for RNA extraction using RNA purification kit (Promega/USA). Estimation of RNA concentration and purity was done according to Mohammed (5) using nanodrop (Bioneer/Korea). Expression of IL-6 gene was estimated by using a two-step RT-qPCR method. In the first step, RNA was converted to cDNA using the AddScript reverse transcriptase kit (addbio, Korea) according to the program shown in (Table 1). Subsequently, in the second step, the RT-qPCR was carried out following the method outlined by Mohammed (5), the specific primers provided by Macrogen (Korea) (Table 2) were used. The PCR amplification was done by using RT-qPCR (Molecular System / Australia) according to the program which clarified in (Table 3). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as a reference gene.

IL-6 serum level

Evaluation of IL-6 serum level was done via taking 2 ml of blood from every participant in gel tube then it was left for 10- 15 min to allow clotting, then it was centrifuged for 10 min at 3000 rpm for serum separation,

which was kept in -20° C. Estimation of IL-6 serum level was conducted via enzyme linked immunosorbent assay (ELISA) and human IL-6 ELISA Kit (Invitrogen/ USA), results were achieved by taking the average of the absorbance values of each set of duplicate standards, while the standard curve was created via plotting the mean absorbance of each standard tube against its human IL-6 concentration on the abscissa. Microsoft Excel was used to create a table of two columns, the first column for the absorbance (OD) and the second for the concentration (pg/ml), after that a curve was drawn where the absorbance column was chosen to be the Y axis while concentration column was X axis, the option of display equation on Chart was activated in the format trendline to add the curve line equation to the chart. The X value (Concentration) for the samples was calculated from the curve line equation.

Statistical analysis

The results were presented as mean ± S.E., while the statistical analyses of data was conducted using IBM SPSS– version 28. The T-test was used for significant compare between two samples, while the significance of different percentages was tested using pearson Chi-square test.

Table (1): PCR program for cDNA conversion

Step	Temp°C
Priming	25 for 10 min
Reverse transcription	50 for 60 min
RT inactivation	80 for 5 min
hold	12 ∞

Table (2): Primers of IL-6 and GAPDH reference gene

Primers	Sequences 5-3`	References
IL-6 F	CCAATCTGGATTCAATGAGGAG	6
IL-6 R	GGTCAGGGGTGGTTATTGCATC	
GAPDH F	CCATGAGAAGTATGACAAC	
GAPDH R	GAGTCCTTCCACGATACC	

Table (3): Thermocycler protocol for IL-6 gene expression.

qPCR step	Temperature	Time	Repeat cycle
Initial Denaturation	95 °C	5min	1
Denaturation	95 °C	20sec	45
Annealing	**60 °C**	**30sec**	
Melting curve temperature	**60-95°C**		

Results and discussion

The results of current study indicated that the most prevalent MS course was relapsing remitting MS (RRMS) which represented as 53(70.7%), followed by secondary progressive MS (SPMS) as 16 (21.3%) then primary progressive MS (PPMS) as 6(8 %) with high significant differences (P= 0.001) (Table 4). These results were closely aligned with results of local study of Al-Araji and Mohammed, (7) whom reported that RRMS was the common course in Iraqi MS patients, as well as, Egyptian study of Hamdy *et al*. (8) identified RRMS as the most frequent phenotype of MS. A meta-analysis in the Middle East and North Africa indicated RRMS as 71%, SPMS as 12%, and PPMS as 8% (9). The successful management strategies of MS may have led to a reduction in

the severity of MS and degrease of the development towards SPMS and PPMS phenotypes. However, recent investigations have demonstrated that several factors (including clinical features and MRI) are predictive of long term progression, such as higher age, sex and symptoms at disease onset, early brain atrophy, shorter time to second relapse, as well as, number of relapses in RRMS phase (10), which may leads to improve the disease condition and prevent its exacerbation.

Table (4): Distribution of MS patients according to courses of the disease.

Courses of MS	No.	Percentage (%)
RRMS	53	70.67 % a
SPMS	16	21.33 % b
PPMS	6	8.00 % c
Total	75	100%
Chi-square	15.44	
P-value	0.001	
**** (P ≤0.01), different letters in same column mean differed significantly**		

The MS patients whom participants in present study, received a variety of medications under the umbrella of the Ministry of Health, some since they were initially diagnosed, some have used one medicine and switched to another over time according to the severity of the condition and the medicine´s benefits, current study demonstrated that majority of patients were received interferon-ß medicine with brand name beta feron 36 (48%), followed by fingolimod with brand name gilenya 18 (24%), retuxan with brand name rituximab 16 (21.33%), and natalizumab with brand name tysabri drug as 5 (6.67%) with high significant (P=0.0001) (Table 5). The results confirmed that interferon-ß (Beta-Feron) exhibited a greater response compared to fingolimod (gilenya) therapy, with the extent of response being dependent on the length of treatment and the expanded disability status scale (EDSS) score, in this line the current results were aligned with local study of Hammood and Mohammed (11). The IFN-β (Betaferon) mediated the shift from Th1/Th17 toward Th2 an anti-inflammatory profile that reduces neuronal demyelination, and benefit the blood-brain briar (BBB) via downregulation of pro-inflammatory cytokines, as well as, upregulation of anti-inflammatory cytokines (12). In patients with relapsing MS, gilenya reduced the risk of disability progression compared to interferon-β (13), whilst retuxan activate apoptosis in pro-inflammatory CD3+ T cells expressing CD20, Leads to depletion of peripheral B-cell that associated with reductions in the relapses number, lesion and disability accumulation (14). Tysabri drug is a valuable tool in the managing of the relapsing forms of MS, it is a monoclonal antibody that impedes lymphocyte infiltration to the CNS (15).

Table (5): Distribution of MS patients according to courses of medications

Treatments	No.	Percentage (%)
interferon-ß (beta feron)	36	48.00 %a
fingolimod (gilenya)	18	24.00 %b
retuxan (rituximab)	16	21.33%b
natalizumab (tysabri)	5	6.67 %c
Total	75	100%
Chi-square	26.38	
P-value	0.0001	
**** (P ≤0.01), different letters in same column mean differ significantly**		

The fundamental result of this study was an elevated IL-6 gene expression in MS patients (1.87±0.12 folds) as compared to the control group (0.51±0.04 folds) with highly significant differences (P = 0.006) (Table 6) and also elevated IL-6 serum level in MS patients (15.28±0.40 pg/ml) when compared to the control group (4.40±0.22 pg/ml) with highly significant differences (P = 0.0001) (Table 7). Several previous reports have identified notable differences in IL-6 levels between MS patients and control groups (16, 17). Increased levels of IL-6 were observed also in MS active plaques (18). IL-6 was additionally emphasized after the discoverer of IL-6 deficient animals were resistant to experimental autoimmune encephalomyelitis (EAE) (19), and undergo defective TH17 differentiation (20). Moreover, treatment with an IL-6R blocking antibody or inhibition of trans signaling in the periphery lead to reduction of symptoms in EAE (1). Current findings indicated that IL-6 has a role in demyelination and pathogenesis progression in MS, as evidenced by its upregulation and elevated serum levels in all MS patients. The activation of NF-κB indicates the expression of pro-inflammatory cytokines including IL-6, that produced by different immune cells including T, B lymphocytes, macrophages, microglia, as well as, non immune cells like endothelial cells and neurons (1, 21), the upregulation in IL-6 levels aligns with its neuroinflammatory role in MS, the heightened levels of IL-6 impedes the maturation of regulatory T cells (Tregs), and increasing the Th17/Treg balance which correlated to the breakdown of immune tolerance and creates the circumstances for the development of autoimmune disorder and inflammatory responses.

Table (6): Comparison between IL-6 gene expression in MS patients and control.

Groups	Mean±SE				
	CT(U6)	**CT(IL-6)**	**ΔCT**	**ΔΔCT**	**IL-6 Fold**
Patient	14.11	18.79	2.74	0.052	1.87±0.12
Control	14.08	25.02	5.59	3.41	0.51±0.04
T-test					2.89
P-value					0.006**
**** (P≤0.01)**					

Table (7): Comparison between serum level of IL-6 in MS patients and control groups.

Group	Mean ± SE of IL-6 (pg/ml)
Patients	15.28±0.40
Control	4.40±0.22
T- test	23.53
P- value	0.0001**
**** (P≤0.01)**	

The Current study demonstrated that patients with the PPMS phenotype had significantly higher gene expression and serum levels of IL-6 followed by SPMS and then RRMS with high significant differences as clarified in (Table 8), these results were consistent with previous studies (22-24). The increasing levels of IL-6 was in line with the clinical status from RRMS toward PPMS phenotypes, which may provide substantial support for its impact on neuronal destruction development and demyelination advancement.

Table (8): Relationship of IL-6 gene expression and serum level with MS courses.

Courses of MS	RRMS	SPMS	PPMS	Chi-square	P-value
IL-6 gene expression (Folds)	1.37±0.52	1.65±0.91	1.92±0.73	4.02	0.026*
IL-6 serum level (pg/ml)	9.41±0.82	12.76±0.68	16.43±1.03	9.50	0.003**
* (P≤0.05), ** (P≤0.01)					

The findings of medications effect on gene expression and serum level of IL-6 in MS patients revealed that the highest levels of IL-6 was in patients whom used interferon-ß followed by fingolimod, retuxan, and natalizumab (Table 9). In general there was diminish in IL-6 serum level in all patients whom used any type of mentioned medications, these result were anticipated due to earlier research demonstrated that mentioned medications commonly suppress levels of inflammatory cytokines, particularly IL-6 in variety of cells (25- 27). The diminish of IL-6 secretion offer a novel possibilities for the management of autoimmune diseases, the mentioned medications are known to suppress inflammation rapidly and have been widely used in the management of MS, thus, the observed decrease in the levels of IL-6 could be the direct consequence of anti-inflammatory and anti-demyelination properties of these medications, including T-cell deactivation, prevents adhesion and penetration into the CNS through the BBB, as well as, mediated the shift from Th1/Th17 toward Th2 profile (an anti-inflammatory and anti-demyelination profile) (10, 28).

Table (9): Relationship of IL-6 gene expression and serum levels with types of MS medications.

Medications	Interferon-ß	Fingolimod	Retuxan	Natalizumab	Chi-square	P-value
IL-6 gene expression (Fold)	1.40± 0.44	1.52± 0.41	1.74± 0.53	2.03± 0.31	4.72	0.022*
IL-6 serum level (pg/ml)	6.35± 0.83	7.09± 0.10	10.63±0.60	13.18± 0.49	5.94	*0.017
* (P≤0.05)						

Conclusion

The present findings disclosed that RRMS was the predominant MS course and the largest percentage of MS patients used Betaferon. The fundamental result of this study was an elevated gene expression and serum level of IL-6 in all MS patients when compared with control, which indicate the involvement of IL-6 in the MS pathogenesis, the production of IL-6 was increased in line with the clinical status from RRMS toward PPMS phenotypes, and the received medications generally diminished IL-6 serum levels, therefore, IL-6 may be

present as an interesting prognostic biomarker for the diagnosis of MS and follow up of the disease condition.

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