The correlation between IFNγ serum level and \textit{IFNG}_{+874} SNP in a multiple sclerotic Iraqi patients

Meelad A. Al-Nasiri *, Ehab D. Salman, Ali H. Ad’hiah

\textsuperscript{1}Sera and Vaccine Institute, Ministry of Health, Baghdad, Iraq.
\textsuperscript{2}Department of Biotechnology, College of Science, University of Baghdad, Baghdad, Iraq.
\textsuperscript{3}Tropical-Biological Research Unit, College of Science, University of Baghdad, Baghdad, Iraq.

Abstract

Multiple sclerosis (MS) is a neurodegenerative autoimmune disease causes a demyelination in neuronal axon and multifocal lesions in especially white matter. Genetic, epigenetic and environmental factors play a pivotal role in the pathogenesis of MS. IFNγ is a proinflammatory cytokine that enhances Th1-cell immune response upon infection and is considered as exacerbation factor in relapsing-remitting MS (RRMS) patients. The current study aimed to find the association between serum level of IFNγ and single nucleotide polymorphism (SNP) genotypes/alleles at locus +874 of \textit{IFNG} gene. Sixty eight Iraqi Arab Muslim patients infected with RRMS were enrolled in this study and they were categorized into two group: IFNβ pre-medicated and IFNB post-medicated patients. Twenty Iraqi healthy individuals were chosen as control group. ELISA assay was used to measure IFNγ serum level and sequence specific primers –polymerase chain reaction (SSP-PCR) technique was used for genotyping \textit{IFNG}_{+874} SNP. The results showed insignificant difference between IFNβ pre-medicated and IFNβ post-medicated patients, while there was a significant decreased (\( P=0.015 \)) mean serum levels in RRMS patients pre-medication and post-medication RRMS patients as compared to control group (68.8 ± 3.3 and 68.5 ± 4.46 vs. 91.8 ± 10.5pg/ml) respectively. The results demonstrated that there was nonsignificant difference between observed and expected genotype frequencies of \textit{IFNG} gene SNPs at position +874. In addition, there was insignificant variation between patients and controls in the distribution of these genotypes and alleles. The results also revealed that there was no impact of \textit{IFNG} gene SNP at locus +874 on the serum level of this cytokine in RRMS patients. Meanwhile, a significant increase of control group serum level corresponding TT genotype compared to that corresponding AA genotype (114.8±24.03 vs. 66.67±10.87) (\( P = 0.004 \) pg/ml) respectively. Furthermore, it was observed that a significant decrease (\( p=0.042, p=0.08 \)) of patients’ IFNγ serum level corresponding both AT and TT genotypes as compared to those in control group (64.9±4.33 vs. 90.42 ± 12.58, 77.72± 7.54 vs. 114.8±24.03 , pg/ml respectively. Briefly, the results indicated that \textit{IFNG}_{+874} SNP has no effect on serum level of RRMS Iraqi patients and also has no effect on MS risk. The low levels of IFNγ in RRMS patients may be attributed to the remitting state of the patients.

Keywords: Multiple sclerosis, cytokine , single nucleotide polymorphism

*Email: melodyalaziz@gmail.com
العلاقة بين المستوى المصمي ل $\text{IFN}_\gamma$ والتغافر الوراثي احادي النكميوتيدة ل $\text{IFNG}^{+874}$ عند مرضى عراقيين بالتصمب العصبي اللويحي المتعدد

علي عبد السلام الناصري* 1، ايهاب داود سممان 2، عمي حسين أدحية 3

1 معهد المصول والمقاح ، وزارة الصحة ، بغداد ، العراق
2 قسم التقنيات الإحصائية لكلية العلوم ، جامعة بغداد ، بغداد ، العراق
3 وحدة البحوث البيولوجية للمناطق الحارة ، جامعة بغداد ، بغداد ، العراق

الخلاصة

يعتبر التصمب العصبي المويحي المتعدد مرض تحمل عصبي من امراض المناعة الذاتية الذي يصيب 8% من المحور العصبي متسببا ببقع متعددة في المادة البيضاء في الدماغ. تمعب الوراثة الجينية والوراثة اللاجينية والعوامل البيئية دورا حيويا في امراضية التصلب العصبي المتعدد. IFN-$\gamma$ هو حركي خموي بدائي للالتهاب والذي يساهم بتحفيز الاستجابة المناعية الخلايا المناعية المساعدة من النوع الأول والذي يعد عامل زيادة لشدة المرض في مرضى التصلب العصبي. IFN-$\gamma$ ايجاد العلاقة بين مستوى IFNG في موقع $+874$ عند الموقع $\text{IFN}_\gamma$ والتغافر الوراثي احادي النكميوتيدة لجين $\text{IFNG}$ عند الموقع $+874$. شملت الدراسة ثمان وستون مريض عراقيا مسمما مصابا بالتصمب العصبي المتعدد من نوع $\text{MS}$، تم تقسيم المرضى إلى مجموعتين: مجموعتي المرضى كما بعد العلاج و مجموعة قبل العلاج، وقد تم اختيار عشرون شخصا من الاصحاء واعتبرت كمجموعة سيطرة. لقد اظهرت النتائج بعدم وجود فروق معنوية بين مجموعتي المرضى ما قبل العلاج وما بعد العلاج في حين لوحظ بان هناك انخفاضا معنيا (p=0.015) في المستوى المصمي ل $\text{IFN}_\gamma$ عند مجموعتي المرضى مقارنة مع مجموعة السيطرة ($68.8 \pm 3.3$ مقابل $68.5 \pm 4.46$ مقابل $91.8 \pm 10.5$, بيكوغرام / مل عمى التوالي). كما لوحظ وجود نقصا معنيا (p=0.008; p=0.04) في المستوى المصمي بين المرضى ومجموعة التوبولوجيا $\text{IFNG}^{TT}$ ($77.72 \pm 12.58$ مقابل $90.42 \pm 4.33$, بيكوغرام / مل عمى التوالي). بالإضافة إلى عدم وجود خطر للإصابة بمرض التصلب العصبي المتعدد ، قد يعزى انخفاض المستوى المصمي الى فترة التحسن عند المرضى.

Introduction

Multiple sclerosis (MS) is a neurodegenerative autoimmune disease that results in multifocal lesions in white matter and less dominant in grey matter. MS is a multifactorial disorder that caused by interaction of environmental factors with genes in a controversial mechanism [1], incomplete penetrance of disease heritability may explain the correlation of environmental factors to MS susceptibility [2]. IFN-γ is a cytokine produced mainly by Th1 and NK cells, and considered as an antiviral agent and enhancer for expression of MHC-class II molecules on APCs promoting T-cell differentiation and expression of adhesion molecules [3]. It has been reported that the expression is determined by transcription factors and epigenetic parameters including methylation of CpG islands and histone methylation of IFNG promoter within CD8 + T-cells [4].
With respect to MS, it has been demonstrated that IFN-γ is an exacerbation factor during RRMS, and is concentrated around CNS lesions [5]. In addition, the level of IFN-γ has been reported to be increased during active phase in blood and CNS lesions, while it is decreased during remission in PBMCs [6]. However, low levels of IFN-γ production after IFN-β administration has been recorded, and it was suggested to be a positive sign for drug responsiveness. On the other hand it was noticed that IFN-γ level is raised two weeks before MS relapse [7].

IFNG gene is located on chromosome 12q24, and a SNP at +874 locus of intron 1 and other promoter SNPs were found to be associated with the risk of some autoimmune diseases, and they also influenced the serum level of IFN-γ production [8]. Regarding MS, [9] demonstrated a significant difference in genotype frequencies of IFNG_+874 SNP between MS patients and controls and the heterozygous genotype AT showed a significant increase among MS patients; an observation that may suggest its MS predisposing effect. In contrast, other studies revealed no significant correlation between +874 A>T SNP and MS risk [6, 10, 11]. The goal of current research is to detect the impact of IFNG_+874 SNP on IFNγ serum level in a sample of relapsing-remitting Iraqi patients.

**Materials and methods**

**Subjects**

Sixty eight MS patients were enrolled in the study during the period of December 2013 - March 2014. They were referred the Multiple Sclerosis Clinic at Baghdad Teaching Hospital for diagnosis and treatment. The diagnosis was made by the consultant medical staff at the clinic according to the revised McDonald criteria of 2010 [12]. The diagnosis was based on clinical examination, magnetic resonance imaging (MRI) findings, evoked potential (EVP) test and blood testing for complete blood picture (CBP), erythrocyte sedimentation rate (ESR), anti-phospholipid antibody, anti-double strand DNA antibody, brucellosis and HLA typing for B27 antigen. In some advanced cases, the cerebral spinal fluid (CSF) was also examined. Patients that had stroke, neuromyelitis optica, Lyme disease or brucellosis were excluded. For each patient, an information sheet was filled under the supervision of the consultant.

The controls (20 subjects) were obtained from Teaching Laboratories of Medical City personnel 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 Iraqi Arab Muslims and were distributed as 5 males (age mean: 37.0 ± 4.9 years) and 15 females (age mean: 34.3 ± 2.2 years).

**Sample Collection**

From each participating subject, 5 ml of venous blood was collected using 5ml disposable syringe. The blood was distributed into two aliquots. For the first, 3 ml of blood was dispensed in plain tube, and after clotting, the tube was centrifuged (3000 rpm for 15 minutes) to collect serum. The serum was distributed into 8 aliquots in 0.5ml Eppendorf tubes, and by then, the tubes were frozen at -20 °C until assessment of cytokine serum level. The remaining blood (2 ml) was dispensed in EDTA tube, and frozen at -20 °C until extraction of DNA for single nucleotide polymorphism (SNP) genotyping of cytokines.

**Measurement of Serum Cytokine Levels**

The level of IFN-γ 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 for each cytokine 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 equations for IFN-γ. The equation and drawing of the standard curve were carried out using Microsoft Excel 2010.

**DNA Isolation**

The DNA was isolated from whole EDTA blood that was frozen at -20 °C by using a ready kit (ReliaPrep™ Blood gDNA Miniprep System; Promega Corporation, USA). The Nanodrop UV spectrophotometer was employed to assess the DNA purity and concentration. The optical density of DNA sample (2 μl) was measured at two wavelengths (260 and 280 nm). In most samples, DNA purity gave A260/A280 ratio between 1.7 and 2.0, which was considered to be suitable for a further analysis in determining cytokine gene polymorphisms.
Cytokine gene polymorphism

*IFNG* SNP at locus +874 genotypes were assigned by using CTS-PCR-SSP kit from University of Heidelberg, Germany. Thermocycling process was optimized according to company instructions and the bands were visualized at transilluminator (312nm) after a 2% agarose gel electrophoresis and 170 volt for 25 minutes showing PCR-SSP genotyping for *IFNG*.

**Statistical analysis**

Serum level of IFNγ was statistically analysed using the computer programme SPSS (Statistical Package for Social Sciences) version 13[13]. Their data were given as mean ± standard error (S.E.), and differences between means were assessed by ANOVA (Analysis of Variance), followed by LSD (Least Significant Difference) to determine the significant differences among groups. Allele frequencies of cytokine genes and significant departure from Hardy-Weinberg (H-W) equilibrium were calculated using H-W calculator for two alleles, which is available free online at [14]. Alleles and genotypes of cytokines 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, relative risk (RR), etiological fraction (EF) and preventive fraction (PF) were also estimated to define the association between cytokine alleles and genotypes with the disease [14]. These estimations were calculated by using the WINPEPI computer programs for epidemiologists [15].

**Results**

**IFN-γ serum level**

IFN-γ serum level mean was significantly decreased in pre- and post-medicated MS patients (68.8 ± 3.3 and 68.5 ± 4.46) pg/ml respectively compared to controls (91.8 ± 10.5 pg /ml).

**IFNG SNP at +874 locus**

The *IFNG* gene SNP at position +874 was presented with three genotypes (TT, AT, and AA) that were corresponding to two alleles (A and T). These genotypes and corresponding alleles showed no significant difference between observed and expected genotype frequencies (Table-1). In addition, there was no significant variation between patients and controls in the distribution of these genotypes and alleles (Table-2).

**Impact of IFNG +874 SNP genotypes on IFNγ serum levels**

It was demonstrated that there were insignificant differences among patients IFNγ serum level of related SNP genotype with the highest serum level recorded for carriers of TT genotype in both patients and control group (77.72 ± 7.54, 114.8±24.03) pg/ml respectively. Accordingly, a significant increase of control group serum level corresponding TT genotype compared to that corresponding AA genotype (114.8±24.03 vs. 66.67±10.87, P = 0.004) pg/ml respectively. Furthermore, it was observed that a significant decrease of patients’ IFNγ serum level corresponding both AT and TT genotypes as compared to those in control group (64.9±4.33 vs. 90.42 ± 12.58 , P=0.042; 77.72± 7.54 vs. 114.8±24.03 , P=0.008) pg/ml respectively (Figure-1).

**Table 1**-Observed and expected genotype and allele frequencies of *IFNG*+874 in multiple sclerosis patients and controls

<table>
<thead>
<tr>
<th>Groups</th>
<th>IFNG gene at position +874 (dbSNP-ID: rs2430561)</th>
<th>Genotypes</th>
<th>HWE P ≤ 0.05</th>
<th>Alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TT</td>
<td>AT</td>
<td>AA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Observed</td>
<td>No. 14</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>% 21.21</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Expected</td>
<td>No. 13.19</td>
<td>32.63</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>% 19.98</td>
<td>49.44</td>
</tr>
<tr>
<td>Controls (No. = 20)</td>
<td></td>
<td>Observed</td>
<td>No. 7</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>% 35</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Expected</td>
<td>No. 5.51</td>
<td>9.98</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>% 27.56</td>
<td>49.88</td>
</tr>
</tbody>
</table>

HWE: Hardy-Weinberg Equilibrium; N.S.: Not significant; ND: not determined
Table 2 - Epidemiological parameters of IFNG+874 genotypes and alleles association to multiple sclerosis

<table>
<thead>
<tr>
<th>IFNG genotypes/alleles</th>
<th>Epidemiological parameters</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>95% Confidence interval (C.I.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Odds ratio (OR)</td>
<td>EF</td>
<td>PF</td>
<td>Fisher's Exact Probability</td>
<td>N.S.</td>
<td>0.17-1.46</td>
</tr>
<tr>
<td>TT</td>
<td>0.5</td>
<td>-</td>
<td>0.18</td>
<td>N.S.</td>
<td>0.17-1.46</td>
<td></td>
</tr>
<tr>
<td>AT</td>
<td>1.64</td>
<td>0.18</td>
<td>-</td>
<td>N.S.</td>
<td>0.6-4.54</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>1.09</td>
<td>0.03</td>
<td>-</td>
<td>N.S.</td>
<td>0.38-3.16</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>1.37</td>
<td>0.15</td>
<td>-</td>
<td>N.S.</td>
<td>0.68-2.76</td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>0.73</td>
<td>-</td>
<td>0.14</td>
<td>N.S.</td>
<td>0.36-1.47</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1- Impact of IFNG+874 genotypes on serum level of IFN-γ in multiple sclerosis patients and controls (Different letters: significant difference between means).

Discussion

A reduced level of IFN-γ was associated with the disease, and therapy had no effect. However, conflicting results concerning IFN-γ serum level in that some studies showed that the serum level increases during relapse in RRMS patients, whereas other studies showed a decreased level of this cytokine in patients compared to control group [6, 15, 16]. The low levels of IFNγ might refer to the remission state in MS patients.

It was reported in some studies that IFNβ-MS treated patients showed lower levels of IFNγ than in healthy subjects [6] which was consistent with our results. Anyway, IFN-γ is considered as a functional cytokine of NK cells and when increased in expression, may reveal an increase in the cytotoxic activity of NK cells and may accompany relapse phase and disease severity [17]. However, IFN-γ is also a signature cytokine of Th1 cells, the absolute dominance of Th1 response in MS patients has been suspected, especially when there was no observation concerning Th1/Th2 shifting in the demyelination sites[19].

The lowest genotype frequency was observed for TT genotype in patients (21.21%) whereas the lowest genotype frequency was observed for AA genotype in healthy individuals (30%). Moreover, the results indicated that there was insignificant difference in allele / genotype distribution among this...
Iraqi sample, plus AT, AA genotypes and A allele comprised attributed factors for MS predisposition (OR=1.64,1.09,1.37; EF=0.18,0.03,0.15) respectively.

Comparing to current results [6] observed that there was no significant difference in the frequencies of IFNG+874 SNP genotypes among Iranian MS patients and that AA genotype frequency was higher in patients than control group that might explains the decreased production level of this cytokine, whereas TT genotype showed decreased level in the patients, therefore, these results were compatible to our findings. Some studies focused on the impact of this SNP on MS susceptibility [6, 9]. Nevertheless, it was suggested that there was a correlation between 12-CA repeats allele within +874 SNP locus of IFNG and T allele in T1D this meets the role of NF-κB binding site which is important in constitutive expression of IFNG [20].

IFNγ gene expression depends on T-cell receptor (TCR) stimulation and other cytokines (IL-2, IL-12, IL-18); the mapping of the gene core promoter revealed that many regulatory elements and factors determine the expression levels of this cytokine including: cis-acting regulatory sites like TATA box, proximal and distal regions that regulate the transcription of this cytokine, and the other non-core promoter regions were found to have sites to bind transcription factors like STATs, NFκB, nuclear factor activated T-cell (NFAT), besides to T-bet –the fundamental transcription factor for Th1 differentiation and IFNγ production [21]. Several epigenetic mechanisms participate in IFNγ expressing CD8+ T-cells; of these mechanisms: CpG island methylation of IFNγ promoter and post-transcriptional histone modification, hence, IFNγ promoter function was assayed by using luciferase reporter gene that exhibits the methylation of the whole IFNγ promoter vector which results in suppression of the transcriptional activity [4].

Signal transduction of IFNγ starts when this cytokine binds to IFNγR causing conformational changes of the receptor, JAK pathways will be then autophosphorylated, followed by the phosphorylation of tyrosine residues of IFNγR1 chain. The STAT pathway will be activated and STAT1 homodimer binds to the receptor, the complex will in turn phosphorylated and STAT1 homodimer dissociates, transfers to the nucleus, and finally binds to the promoter of IFNγ activation site to regulate the expression of IFNγ [22].

Conclusion

IFNG SNP at +874 has no effect on the progression of MS and it is favoured to enlarge sample size and further genetic studies on IFNG expression and sequencing candidate gene region that might have role in MS pathogenesis.

Reference


