Tumor Necrosis Factor Alpha Gene −376 Polymorphism in Egyptian Patients with Multiple Sclerosis

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ABSTRACT

Background: Tumor necrosis factor α, a proinflammatory cytokine, was found to play an important role with the clinical activity of relapsing–remitting multiple sclerosis and the development of progression. Dysregulation in the expression of tumor necrosis factor gene α had been suggested in the pathogenesis of multiple sclerosis. Objective: Our aim was to investigate the relation between tumor necrosis factor α −376 polymorphism with disease susceptibility and clinical course of multiple sclerosis in Egyptian patients. Methods: Polymerase chain reaction and restriction fragment length polymorphism were carried out on 36 primary progressive multiple sclerosis patients, 36 age and sex-matched remitting relapsing multiple sclerosis patients (Diagnosed according to McDonald Diagnostic criteria) and 30 age and sex-matched healthy controls. Results: The GG genotype and the guanine allele (G) were detected significantly more often in the primary progressive multiple sclerosis group as compared with the healthy control group (p = 0.002; p = 0.004). Conclusion: The “G” allele in the examined position in tumor necrosis factor alpha might have a role as regards susceptibility in MS. The “G” allele may be one of the factors responsible for progression in primary progressive multiple sclerosis. (Egypt J Neurol Psychiat Neurosurg. 2010; 47(2): 311-316)

Key Words: Multiple sclerosis (MS); Primary progressive multiple sclerosis (PPMS); Tumor necrosis factor alpha (TNF-α) gene polymorphism.

INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the CNS. A number of genetic variations have been shown to increase the risk of developing MS. The genetic region most clearly associated with MS is the major histocompatibility complex (MHC)². Multiple sclerosis is a heterogeneous disease, which results in different clinical manifestations. In a minor subset of patients, the disease progresses from the beginning where the relapsing phase is not observed (primary progressive MS)².

Tumour necrosis factor (TNF), a proinflammatory cytokine, is involved in the regulation of a broad spectrum of biological processes and TNF signaling has several important functions within the CNS including injury-mediated microglial and astrocyte activation, and regulation of BBB permeability, glutamatergic transmission, and synaptic plasticity and scaling⁶.⁷. TNF is involved in the pathogenesis of infectious and autoimmune disorders, including MS. Evidence that implicates TNF in the underlying pathology of MS includes observation at autopsy that MS patients have elevated TNF levels at the site of active MS lesions⁸; CSF and serum TNF levels are elevated in patients with MS compared to unaffected individuals and TNF levels correlate to the severity of the lesions⁹,¹⁰; and evidence that mononuclear cells in peripheral blood of MS patients just prior to symptom exacerbation have elevated TNF secretion after stimulation compared to cells from the same patients during remission⁹,¹⁰,¹¹.

The human TNF gene maps to chromosome 6p21.3 in the MCH region. A number of polymorphisms had been described for the TNF-α locus. TNF-α is associated with clinical activity in RRMS and the development of the progressive form of the disease⁶,¹⁰. The most widely investigated single nucleotide polymorphisms (SNPs) of the TNF-α gene are at positions −238, −308, and −376, all of which are guanine (G) to adenine (A) substitutions and are of different ethnic bases compared to Egyptians¹²-¹⁷. The aim of this study was to investigate the possible influence of the TNF-α (−376) polymorphism on the susceptibility to MS in Egyptians and to compare the PPMS genotype with those of RRMS patients and healthy controls (HC) to detect its relation with disease course.

PATIENTS AND METHODS

Study Design and Patients’ Selection

This study is a case-control study conducted between February 2009 and October 2009. The study included 72 non-relative Egyptian MS patients (46 females and 26 males) with clinically definite MS according to McDonald criteria¹⁸ and thirty age and sex-matched healthy control subjects (HC). All participants were recruited from outpatient neurology clinic at Cairo University hospitals and from inpatient department of neurology at Cairo University hospitals, Egypt. All patients signed a written informed consent.

Patients were further subdivided into two groups:
A- Thirty six patients with RRMS.
B- Thirty six patients with PPMS: PPMS patients had at least one year of disease progression and with a positive brain or spinal cord MRI. Healthy control subjects were included from a general population base where they were age and sex matched to the patients group and no family history of MS or any other genetic or inflammatory disease was a criterion for inclusion in this study.

Methods:

All the 72 MS cases showed clinical, radiological and electrophysiological [visual evoked potential (VEPs) and brainstem auditory evoked potentials (BAEPs)] findings that confirmed diagnosis of MS according to criteria of McDonald.

I- Clinical evaluation:

A- Clinical data included sex, the date of birth, year of diagnosis, age of onset of the disease, disease duration.
B- Expanded Disability status scale (EDSS).
The EDSS scores the disability by determining the degree of neurological impairment; it has scores from zero (normal) to 10 (death due to MS).
C- The progression index (PI): The PI corresponds to the ratio between the EDSS and the disease duration in years.

II- Neurophysiological:

A- Visual evoked potential (VEPs).
B- Brainstem auditory evoked potentials (BAEPs).

III- Neuroradiological:

Magnetic resonance imaging (MRI) of the brain was surveyed, and it was performed using General Electric Medical System Signal 1.5 Tesla, and the results of T1-, T2-weighted spin echo images, PD and FLAIR pulse sequences were obtained, and all included patients had radiological criteria for definite multiple sclerosis according to Barkhof criteria.

IV- Cerebrospinal fluid examination:

Eight patients out of included patients performed CSF analysis to confirm the diagnosis and all had positive oligoclonal bands.

V- Genetic studies:

**Detection of TNF α - gene polymorphism at position -376:**

Three ml venous blood were withdrawn from all patients and controls and kept on EDTA treated vacutainers at -70 °C deep Freezer. DNA isolation: Genomic DNA was isolated from 3 ml EDTA-treated blood. The extraction kit used was Axyprep TM DNA extraction kit (AXYGEN BIOSCIENCES; catalogue number: AP-MN-BL-GD-MA-50).

*Polymerase chain primers and conditions:*

The relevant fragments of 224 base pairs were amplified by using the forward primer 5'-TTTCTGAAGCCCTCCCCAGTC-3' and the reverse primer 5'-TACCCCTACAATCCCCCATCC-3'. For each sample, PCR was performed in 20 ul reaction in PCR –Gold Master –Mix beads kit (Cat-No: 10020-96) containing 96 tubes. PCR Gold is a new, powerful, and ready to use PCR reagent optimized for more accurate PCR amplification.

*Experimental protocol:*

Add 3u of the template DNA to 1u of the forward primer and 1 u of reverse primer in the PCR tube. Concentration of DNA and primer (20 µl reaction): DNA5-50 ng and Primer 5-10 pmol. Add 15 u Distilled water to PCR tube to a total volume of 20 µl. Dissolve the lyophilized pellet by vortexing and briefly spin down. The PCR cycles: Cycling conditions were; 95 °C for 5 min. followed by 30 cycles of 95°C for 1 min., 63 °C for 1 min, 72°C for 1 min, with a final polymerization at 72°C for 5 minutes. All PCR cycles were performed in Thermal cycler (Perkin-Elmer-9600). PCR products, 10 ul were loaded on agarose gel (2%) and electrophoresis was carried out, the gel is visualized under ultraviolet light after staining with ethidium bromide. DNA was run together with 50 base pairs ladder.

*Allelic-Discrimination Restriction Fragment Length Polymorphism (RFLP):*

The primers created a restriction site for the Restriction enzyme (Fast Digest Tsp509 I (TasI) #FD 1354 Lot: 00041454) (5'-^AATT-3'). Eight u of the amplified PCR products were digested by 1u TasI enzyme at 65c together with 1.5 u buffer. The digestion products were resolved on a polyacrylamide gel (3%) & detected under ultraviolet light after staining with ethidium bromide. The relevant fragments of 224 base pairs were loaded on agarose gel (2%) and electrophoresis was carried out, the gel is visualized under ultraviolet light after staining with ethidium bromide. DNA was run together with 50 base pairs ladder.

Statistical Methods:

Data were statistically described in terms of range, mean±standard deviation (±SD), median frequencies (number of cases) and percentages when appropriate. Comparison of quantitative variables between study groups was done using Student t test for independent samples in comparing two groups when normally distributed and Mann Whitney U test for independent samples when not normally distributed. Comparison of age was done using one-way analysis of variance (ANOVA) test with posthoc multiple 2-group comparisons. For comparing categorical data, Chi-square (x^2) test was performed. Exact test was used instead when the expected frequency is less than five. A probability value (p value) less than 0.05 was considered statistically.
significant. All statistical calculations were done using computer programs Microsoft Excel 2003 (Microsoft Corporation, NY, USA) and SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 15 for Microsoft Windows.

RESULTS

The characteristic clinical data of the three examined groups are presented in Table (1). As MS comprises different disease subtypes, it is important to evaluate whether MS patients with certain genotypes have an altered MS disease course. The distributions of the genotype and allele frequencies of the TNF-α −376 polymorphism in the MS patients (RR and PPMS) and the HC are displayed in Table (2).

Genotypes: -AA genotype: We could not detect the AA homozygote genotype in either the MS patients or the HC, so it was not included in the statistics. -GG genotype: As regards the GG genotype, a statistically significant higher level was found in the PPMS group as compared with the HC group (P=0.002; OR =0.088; CI = 0.018–0.438). In addition, a statistically significant higher level was found in the RRMS group as regard GG genotype when compared with the HC group (P =0.015; OR = 0.188; CI = 0.053-0.668). -GA genotype: As regards the GA genotype, it was underrepresented in both PPMS and RRMS groups of patients relative to the HC group (P = 0.002; OR = 0.088; CI = 0.018–0.438) (P=0.015; OR = 0.188; CI = 0.668–0.053) respectively. No significant differences in genotypes (GG and GA) were found between the RRMS and PPMS groups (p = 0.670).

Alleles: As for the G allele, a significant difference was observed between the PPMS and HC groups (p = 0.004; OR = 0.114; CI = 0.024–0.534). In addition, a statistically significant higher level was found in the RRMS group when compared with the HC group (p = 0.024; OR = 0.088; CI = 0.025–0.306). As regards the A allele, it was underrepresented in both PPMS and RRMS groups of patients relative to the HC group (p = 0.013; OR = 0.114; CI = 0.024–0.534) (OR = 0.088; CI = 0.025–0.306) respectively. The distributions of the alleles (G and A) in the two groups of patients were similar (p = 0.677).

The genotype did not influence the clinical characteristics. No association was found between the genotype status of the TNF-α−376 polymorphism and the age at onset, the disease duration, EDSS, PI (Table 3).

Table 1. Socio-demographic data of the three examined groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Male/ Female Ratio</th>
<th>Age (years)</th>
<th>Age at onset (years)</th>
<th>Disease Duration (years)</th>
<th>EDSS</th>
<th>PI</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPMS(n=36)</td>
<td>16/20</td>
<td>32.56, (5.348)</td>
<td>27.83, (3.598)</td>
<td>4.72, (2.160)</td>
<td>4.28, (1.74)</td>
<td>1.037</td>
</tr>
<tr>
<td>RRMS(n=36)</td>
<td>10/26</td>
<td>31.72, (5.804)</td>
<td>27.67, (4.323)</td>
<td>4.06, (2.506)</td>
<td>4.33, (1.177)</td>
<td>1.446</td>
</tr>
<tr>
<td>HC(n=30)</td>
<td>13/17</td>
<td>32.70, (5.364)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P-value</td>
<td>0.294</td>
<td>0.731</td>
<td>0.859</td>
<td>0.231</td>
<td>0.842</td>
<td>0.011</td>
</tr>
</tbody>
</table>

EDSS expanded disability status score, HC healthy control, PPMS primary progressive multiple sclerosis, PI progression index, RRMS relapsing-remitting multiple sclerosis Values are given as mean (SD)

Table 2. Genotype and allele frequencies of TNF-α−376 gene Polymorphism in Egyptian MS patients.

<table>
<thead>
<tr>
<th>TNF-α gene</th>
<th>PPMS(n=36)</th>
<th>RRMS(n=36)</th>
<th>HC(n=30)</th>
<th>P-value-1</th>
<th>P-value-2</th>
<th>P-value-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>34 (94.4 %)</td>
<td>32 (88.9 %)</td>
<td>18 (60 %)</td>
<td>0.670</td>
<td>0.002*</td>
<td>0.015*</td>
</tr>
<tr>
<td>GA</td>
<td>2 (5.6 %)</td>
<td>4 (11.1 %)</td>
<td>12 (40 %)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AA</td>
<td>0 %</td>
<td>0 %</td>
<td>0 %</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Allele</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>70 (97.2 %)</td>
<td>68 (94.4 %)</td>
<td>48 (80 %)</td>
<td>0.677</td>
<td>0.004*</td>
<td>0.024*</td>
</tr>
<tr>
<td>A</td>
<td>2 (2.8 %)</td>
<td>4 (5.6 %)</td>
<td>12 (20 %)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

HC healthy control, PPMS primary progressive multiple sclerosis, RRMS relapsing-remitting multiple sclerosis * p<0.05=
Significant, p<0.01 =highly significant P-value-1, between the two diseased groups P-value-2, between PPMS and control, P-value-3, between RRMS and control.
Table 3. Relationship between genotype status and clinical characteristics in Egyptian MS patients.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Age (years)</th>
<th>Age at onset (years)</th>
<th>Disease duration (years)</th>
<th>EDSS</th>
<th>PI</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG (n=66)</td>
<td>32.24</td>
<td>27.83</td>
<td>4.41</td>
<td>4.31</td>
<td>1.2492</td>
</tr>
<tr>
<td></td>
<td>(5.703)</td>
<td>(4.029)</td>
<td>(2.379)</td>
<td>(1.163)</td>
<td>(0.709)</td>
</tr>
<tr>
<td>GA (n=6)</td>
<td>31.00</td>
<td>26.83</td>
<td>4.17</td>
<td>4.25</td>
<td>1.1650</td>
</tr>
<tr>
<td></td>
<td>(3.742)</td>
<td>(3.061)</td>
<td>(2.137)</td>
<td>(1.332)</td>
<td>(0.454)</td>
</tr>
<tr>
<td>AA (n=0)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**P value**

| Value | 0.683 | 0.560 | 0.885 | 0.901 | 0.639 |

DISCUSSION

The possibility that genetic susceptibility to MS might be influenced by TNF gene polymorphism has been repeatedly suggested. TNF α levels are under genetic control, and are determined in part by the alleles of TNF. Previous studies, focusing on -308 and -238 TNF α promoter polymorphisms, yielded negative results. There are only few papers concerning the −376 TNF α SNP. Our findings demonstrated that the GG genotype and the G allele at position −376 of TNF-α gene were more frequent in PPMS group and RRMS than the control suggesting a possible genetic predisposition to MS in Egyptian Patients. This is in agreement with the results found in the study done by Losonczi et al. on the Hungarians which is considered the first published study on an association between PPMS and TNF-α gene −376 SNP currently available. They found that the GG genotype and the guanine allele (G) were detected significantly more often in the PPMS as compared with the HC group. TNF α -376 polymorphism was also analyzed by Huizinga et al. but its frequency in the Dutch population was found to be very low both in MS patients and controls. A SNP at position -376 of the TNF α gene has been also associated with susceptibility to MS in Spain and this was confirmed twice, first in 2001 and in a replication study in 2004 confirming the association. Earlier, Fernandez et al. (1999) reported that there was a statistically significant increase of A at position -376 in the TNF α gene in MS patients. However, no association was found between TNF -376 in USA and the Netherlands and recently Argentinian patients. In another sample composed primarily of people of German, Scandinavian, and British descent, there is no apparent excess of TNFA-376 α allele polymorphism in patients with MS compared with controls rigorously matched for ethnicity. De Jong et al. and Wirz et al. also did not detect any association between TNF promoter gene polymorphisms at different positions including -376.

In the present study, as regards A allele, it was underrepresented in both PPMS and RRMS groups of patients relative to the HC group. On the contrary, Losonczi et al. reported that the PPMS group displayed a lower “A” allele frequency than in the HC group, indicating that carriage of this less common allele may decrease the risk of the development of this progressive subtype. Previous investigations of the TNF-α gene in MS from around the world failed to afford definitive evidence that disease association may be a consequence of the differences in the HLA region in different ethnic groups. No association was found in present work between the genotype status of the TNF-α −376 polymorphism and the clinical characteristics including age at onset, the disease duration, EDSS, PI. This is consistent with the results of certain studies relating to EDSS. In our study, the frequency of the rare “A” allele was 2.8%, which is similar to data on the Hungarian population with PPMS (2% to 8%) This differs from the data on the Sardinian population, where the A allele frequency is markedly high. The AA genotype was not observed in our subjects, similarly as in other studies. This is the second investigation of the SNP of TNF-α at position −376 in PPMS following the study done on the Hungarian patients by Losonczi et al. in 2009.

In conclusion, our results suggest that the G allele in the examined position might have a role as regards susceptibility in MS. Because the TNF α -376 polymorphism is located within a regulatory region of the TNF α gene, a functional role is possible, and this polymorphism may therefore play a direct role as a susceptibility factor in MS. Alternatively, the -376 polymorphism may be a useful genetic marker for another functional polymorphism nearby.

2. Lassmann H, Brück W, Lucchinetti CF. The immunopathology of multiple sclerosis: an
overview, Brain Pathol. 2007; 17: 210–8.
31. Weinsonker BG, Hebrink DD, Atkinson E, Kantarci OH. Association of a tumor necrosis factor

الملخص العربي
تأثير التحور الجيني لعامل التكزر الورمي ألفا 376 – في مرضى التصلب الورمي المتتاثر المصريين

مقدمة: يعد مرض التصلب الورمي المتتاثر من الأمراض العصبية المزمنة غير معروفة الأسباب حيث تظهر بقع التصلب في الدم والدماغ الشوكي نتيجة تلف وفقدان غشاء الميلين. عامل نخر الأورام α قد وجد أن يلعب دورا هاما في تطور مرض التصلب الورمي المتتاثر والتدور الجسدي فيه تعبير الجين عن عامل نخر الورم قد اقترح في النتائج أن تصلب الورم المتتاثر هذه الدراسة إلى دراسة العلاقة بين جين عامل نخر الورم 376α تعدد الأشكال مع احتمال الاصابة بالأمراض والمطع السريري للمرضى التصلب المتتاثر في مصروف أجريت هذه الدراسة على 72 مريض مرض التصلب الورمي المتتاثر المزمن 30 من العمر والجنس مطابقة صحيحة للمجموعة الضابطة

النتائج: وراثي العوامل (G) تم الكشف عن أكثر من ملحوظ في كثير من الأحيان في مجموعة التصنيفي الابدائي مماثلة للتصنيف مقارنة مع المجموعة الضابطة.

الختام: إن أي لين في الوقوف المفسر في عامل نخر الورم ألفا قد يكون له دور فيما يتعلق بحدود مرض التصلب العصبي المتتاثر ووراثي G قد يكون واحدا من العوامل المسؤولة عن التقدم في الابدائي التصنيفي في مرض التصلب الورمي المتتاثر.