Genetic Association of Promoter FOXP3 Gene Polymorphism with Behcet's Disease in Egyptians Patients

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Abstract

T regulatory (T reg) cell is one of the immune system cells involved in the pathogenesis of BD, defined by the expression of CD4, CD25 and the transcription factor forkhead box (FOXP3) which is master regulator for the development and function of Treg cells.

Forkhead box P3 (FoxP3) gene encodes a transcription factor with crucial roles in the development and function of Treg cells. This pilot study was designed to investigate the association between FOXP3 gene polymorphism (-3499A/G and -3279C/A) with Behcet's disease. Genotyping polymorphism is performed using restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR) in 69 Egyptian patients with BD and 84 normal controls. No association between any genotype of Foxp3 (-3499 A/G) and (-3279 C/A) polymorphism and the susceptibility to BD.

It was found that male patients with BD had significantly lower frequency of -3279 C allele (P <0.05) than healthy control. Also BD patient totally have lower frequency of total C allele. C allele is negatively correlated with BD patient (r= 0.495; P<0.05), it might be protective allele.

Also we found Significant association between Foxp3 (-3279 C/A) A allele and neural involvement in BD was found that patients with neural involvement had significantly higher frequency of -3279 A allele (P= 0.048) than patients without and neural involvement is positively correlated with -3279 A allele (r=2.31).

Taking into consideration the fact that genetic polymorphisms are population specific, our data stressed the importance of FOXP3 gene polymorphism in developing Behcet’s disease. To the best of our knowledge, this study is the first one that examines FOXP3 polymorphism in Behcet’s disease. Additional prospective studies on larger population are needed to confirm our findings.

Keywords: Behcet’s disease; Polymorphism; FOXP3; Treg
Introduction

Behcet’s disease (BD) is a chronic relapsing multisystemic inflammatory disorder characterized by four major symptoms (oral aphthous ulcers, genital ulcers, skin lesions, and ocular lesions) and occasionally by five minor symptoms (arthritis, gastrointestinal ulcers, epididymitis, vascular lesions, and central nervous system). Patients with BD may manifest all or only some of these clinical features.

Regulatory T (Treg) cells are known to play an important role in immune regulation. Treg cells whose main functions are attenuation of inflammation and suppression of effector T cells aid in keeping the balance between immunity and autotolerance. They mainly characterized by CD4+/CD25+/FOXP3+ expression [1]. Treg is believed to play a critical role in tumor immune evasion [2]. Its increase has been reported in a wide array of human malignancies, including HCC [3-8]. HCC patients had a high frequency of Tregs, and high numbers of Tregs correlated with a poor prognosis [4].

Forkhead box P3 (FOXP3) is a major regulatory factor for the development and functioning of Treg cells [7]. It acts as a main regulator in the development and function of Treg cells. In fact, the absence of a functional FOXP3 gene product has been revealed to cause an abnormal production of Treg cells [9]. The FOXP3 polymorphism is associated with autoimmune disease such as allergic rhinitis Graves’ disease, and psoriasis, as well as breast cancer and HCC [2,10-19].

The FOXP3 gene is positioned at the Xp11.23 locus on the X chromosome. There are five SNPs in the promoter region of FOXP3. These are-924A/G (rs2232365), -1383C/T (rs2232364), -2383C/T (rs3761549), -3279C/A (rs3761548) and -3499A/G (rs3761547) polymorphisms, and they may affect the expression and activity of FOXP3 decreasing or eliminating functional Treg. Studies on the association between FOXP3 polymorphism and HCC are very rare [1,11,12,19-30]. Thus, this study was conducted to investigate the genotype distribution of FOXP3 SNPs (-3499A/G and -3279C/A) in Egyptian patients in order to clarify their implication on the development of BD.

Materials and Methods

Study population

Sixty nine patients with BD were recruited from Department of Rheumatology at El-Kasr El-Aini hospital (52 men and 17 women). They were diagnosed according to (ISG for Behcet’s Disease, 1990). Eighty four age and gender matched healthy control subjects were included in the study. Patients who had other autoimmune disease, infection or malignancy were excluded from the study. Informed consent was obtained from all subjects. The onset of the syndrome was defined as the time when the patient fulfilled the diagnostic criteria.

All investigations were done in accordance with the Ain-Shams University, Health and Human Ethical Clearance Committee guidelines for Clinical Researches. Local ethics committee approved the study protocol. All BD patients were subjected to proper full history recording, thorough clinical examination, routine laboratory investigations.

DNA Extraction

Five ml sample of venous blood was collected from each subject into a sterilized vacutainer tubes containing EDTA as anticoagulant. DNA was extracted from whole blood-EDTA samples by Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, USA) according to manufacturer’s protocol. The purified genomic DNA has a single band more than 30 kb is seen after 1%agarose gel electrophoresis and ethidium bromide staining. The extracted DNA was stored at -20°C until further use (Figure 1).

Genotyping

Genotyping of -3499 A/G, -3279 C/A and -2383 C/T were performed by PCR-based restriction fragment length (RFLP) method [30]. Primer sequences were summarized in Table (1).

Genotyping of FOXP3 (-3499 A/G) polymorphism: The reaction was done in one tube with 15 μl final reaction volume. PCR mixtures consisted of Dream Taq Green PCR Master Mix (2x) (Fermentas Life Science, Thermo Fisher Scientific Inc,. MA, USA), 10 pmoles of each primer, and 160ng of DNA. All reactions were carried out in a Biometra thermal cycler (Biometra GmbH, Germany). Amplification conditions were as follows: 94°C for 5 min and 30 cycles of denaturing at 94°C for 30s, annealing at 55°C for 30s, and extension at 72°C for 30s and a final single extension at 72°C for 10 min. The PCR product (158bp) was digested by Pvull (Fermentas). The presence of the G allele resulted in the digestion of the applicant to136 and 22-bp products (Figure 2). The digested PCR products were visualized by 4% agarose gel in 0.5X Tris-acetate-EDTA (TAE) buffer with ethidium bromide staining (10 mg/ml) under an ultraviolet
Genotypic of FOXP3 (-3279C/A) polymorphism: The reaction was done in one tube with 25 μL final volume for PCR reaction. PCR mixtures consisted of Dream Taq Green PCR Master Mix (2×) (Fermentas), 10 pmols of each primer, and 200 ng of DNA. Amplification conditions were as follows: 94°C for 5 min and 30 cycles of denaturing at 94°C for 30 s, annealing at 68°C for 30 s, and extension at 72°C for 30 s and a single final extension at 72°C for 10 min. The PCR product (300 bp) was digested by addition of PstI (Fermentas). The presence of the A allele resulted in the digestion of the applicant to 159 and 141-bp products (Figure 3). The digested PCR products were visualized by 3% agarose gel and were determined relative to the migration of a 100bp step ladder (Fermentas).

All statistical analyses were performed using the Statistical Package for Social Science (SPSS) version 19 (LEAD Technology Inc.). For analyzing of the genotype and allele frequency, the HCC patients and healthy controls were divided into female and male groups because allele frequencies of the FOXP3 gene, located on X chromosome, were different in the two sexes [31]. The comparison of genotype and allele frequency between HCC patients and controls were analyzed by Chi-square test.

Results

Characteristics of the Study Population

Patient characteristics and clinical features are summarized in Table (1). Of the 69 BD patients, 52 were men and 17 were women, 84 healthy controls, with mean age 32.6±13.3.

<table>
<thead>
<tr>
<th>Fo xp3 gene</th>
<th>Control group</th>
<th>BD group</th>
<th>p</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/A</td>
<td>36 (90%)</td>
<td>16 (94.1%)</td>
<td>Ns</td>
<td>1.77 (0.184-17.19)</td>
</tr>
<tr>
<td>A/G</td>
<td>4 (10 %)</td>
<td>1 (5.9%)</td>
<td>Ns</td>
<td>0.563(0.058-5.44)</td>
</tr>
<tr>
<td>G/G</td>
<td>0 (0 %)</td>
<td>0 (0 %)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AG or GG</td>
<td>4(10 %)</td>
<td>1 (5.9 %)</td>
<td>Ns</td>
<td>0.563(0.058-5.44)</td>
</tr>
</tbody>
</table>

Allele Frequency for female

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>G</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>76 (95%)</td>
<td>4 (5%)</td>
</tr>
<tr>
<td>97.1%</td>
<td>0%</td>
<td>1.737 (0.187-16.13)</td>
</tr>
</tbody>
</table>

Table 1: Association between FOXP3 polymorphisms and BD.

F o x p3 (-3499 A/G) (rs3761547) Genotyping

F o x p3 (-3499 A/G) was genotyped using RFLP. The PCR product was 158 bp. The size of PCR products was determined relatively to the migration of a 25 bp step ladder F o x p3 (-3499 A/G) genotypes and allele frequencies in patients with BD and healthy controls are shown in Table (2). Three genotypes were seen in F o x p3 (-3499 A/G). Analysis of F o x p3 (-3499 A/G) SNPs in the promoter region revealed that AA genotype was found more frequently in BD patients and healthy controls and increase in the frequency of A allele over G allele. Analysis of F o x p3 (-3499 A/G) SNP genotypes revealed insignificant change in the distribution of female genotypes between BD patients and healthy controls (AA 94.1% , 5.9% AG and 0% GG versus 90% AA , 10% AG and 0% GG respectively).

Table 2: Genotype distribution and allelic frequency of the Foxp3 (-3499 A/G) (rs3761547) in patients with BD and normal controls.

<table>
<thead>
<tr>
<th>Allele Frequency for male</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>42(95.4%)</td>
<td>52(100%)</td>
</tr>
<tr>
<td>G</td>
<td>2(4.6%)</td>
<td>0(0%)</td>
</tr>
</tbody>
</table>

**Total allele frequency**

<table>
<thead>
<tr>
<th>Allele Frequency</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>118 (70.2%)</td>
<td>85 (61.6%)</td>
</tr>
<tr>
<td>G</td>
<td>6 (3.6%)</td>
<td>1 (0.7%)</td>
</tr>
</tbody>
</table>

- P (p value)
- OR (odd ratio)

**Foxp3 (-3279 C/A) (rs3761548) Genotyping**

Foxp3 (-3279) was genotyped using RFLP. The size of PCR products was 300 bp was determined relatively to the migration of a 100 bp step ladder.

Foxp3 (-3279 C/A) genotypes and allele frequencies in patients with BD and healthy controls are shown in Table (11). Analysis of Foxp3 (-3279 C/A) SNPs in the promoter region revealed that increase in the frequency of A allele over C allele in both BD and control group. There was insignificant change in the distribution of -3279 C/A female genotypes between healthy controls and BD patients (20% CC, 17.5% CA and 62.5% AA versus 23.5% CC, 5.9% CA, 70.6% AA).

It was found that male patients with BD had significantly lower frequency of -3279 C allele (P <0.05) than healthy control. Also BD patient totally have lower frequency of total C allele . C allele is negatively correlated with BD patient (r= 0.495; P<0.05), it might be protective allele.

<table>
<thead>
<tr>
<th>Foxp3 gene</th>
<th>Control group</th>
<th>BD group</th>
<th>P</th>
<th>OR (95% CI)</th>
</tr>
</thead>
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<tr>
<td></td>
<td>(N= 84)</td>
<td>(N= 69)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Foxp3 (-3279 C/A) Female Genotype (N, %)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C/C</td>
<td>8 (20%)</td>
<td>4 (23.5%)</td>
<td>Ns</td>
<td>1.23 (0.315-4.807)</td>
</tr>
<tr>
<td>C/A</td>
<td>7 (17.5%)</td>
<td>1 (5.9%)</td>
<td>Ns</td>
<td>0.295 (0.033-2.603)</td>
</tr>
<tr>
<td>A/A</td>
<td>25 (62.5%)</td>
<td>12 (70.6%)</td>
<td>Ns</td>
<td>1.44 (0.423-4.89)</td>
</tr>
<tr>
<td>CA or AA</td>
<td>32 (80%)</td>
<td>13 (76%)</td>
<td>Ns</td>
<td>0.813(0.208-3.17)</td>
</tr>
<tr>
<td><strong>Allele Frequency for female</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>23 (28.7%)</td>
<td>9 (26.5%)</td>
<td>Ns</td>
<td>0.892(0.362-2.2)</td>
</tr>
<tr>
<td>A</td>
<td>57 (71.3%)</td>
<td>25 (73.5%)</td>
<td>Ns</td>
<td>1.12(0.454-2.764)</td>
</tr>
<tr>
<td><strong>Allele frequency for male</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>18(41%)</td>
<td>10 (20.2%)</td>
<td>0.04</td>
<td>0.414(0.18-0.951)</td>
</tr>
<tr>
<td>A</td>
<td>26(59%)</td>
<td>42 (79.8%)</td>
<td>Ns</td>
<td>1.615(0.884-2.95)</td>
</tr>
<tr>
<td><strong>Total allele frequency</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>41 (24.4%)</td>
<td>19 (13.8%)</td>
<td>0.02</td>
<td>0.495(0.272-0.9)</td>
</tr>
<tr>
<td>A</td>
<td>83(49.4%)</td>
<td>67(48.6%)</td>
<td>Ns</td>
<td>0.996(0.616-1.516)</td>
</tr>
</tbody>
</table>

Table 3: Genotype distribution and allele frequency of Foxp3 -3279 C/A in patients with BD and normal controls.

**Association between Clinical Findings and Foxp3 Gene Polymorphisms**

In order to investigate the association between clinical findings and foXP3 gene polymorphisms in BD, the patients were classified according to the clinical features of the disease. As shown in Table (4).

It was found that BD patients with neural involvement had significantly heigher frequency of -3279 A allele (P 0.048) than patients without neural involvement. and is positively correlated with -3279 A allele (r= 2.31, P<0.05).
Discussion

Treg cells are defined as T cells in charge of suppressing potentially deleterious activities of Th cells and are mainly characterized by the expression of FOXP3+ transcriptional factor. FOXP3+ acts as a main regulator in the development and function of Tregs [1,2,9,11-30,32,33]. The absence of a functional FOXP3 gene product has been revealed to cause an abnormal production of regulatory T cells [9].

To the best of our knowledge this study is first to be done on Behçet’s disease. According to this study, we found no difference in genotype and allele frequencies of -3499 A/G polymorphism among BD patient and healthy control. Female genotypes in BD patients and healthy control were found (AA 94.1%, 5.9% AG and 0% GG versus 90% AA, 10% AG and 0% GG) respectively, total frequency of A allele (118 (70.2%) in healthy control versus 90% AA, 10% AG and 0% GG) respectively, total frequency of G allele 6 (3.6%) in healthy control versus 85 (61.1%) in BD patient and total frequency of G allele 6 (3.6%) in healthy control versus 1 (0.7%) in BD patient.

Our results were in agreement with who studied Japanese patients with autoimmune thyroid disease (Graves and Hashimotos thyroiditis) and found no difference in genotype and allele frequencies of -3499 A/G among any groups [30]. Also our results in agreement with who found no difference in genotype and allele frequencies of -3499 A/G among patient with systemic lupus erythromatosus and healthy control. Also found that the A allele is a risk of psoriasis vulgaris in indian patient with psoriasis vulgaris.

Analysis of Foxp3 (-3279 C/A) SNPs in the promoter region revealed there was insignificant change in the distribution of -3279 C/A female genotypes between healthy controls and BD patients (20% CC, 17.5% CA and 62.5% AA versus 23.5% CC, 5.9% CA, 70.6% AA respectively) .But we found that the male patients with BD (18 (41%) have significantly lower frequency of -3279 C allele (P<0.05) than healthy control 10(20.2%). Also the total frequency of C allele in BD patient 41 (24.4%) is lower in the healthy control 19 (13.8%). The C allele is significantly negatively correlated with BD patient (r= 0.495; P<0.05), it might be protective allele.

Our results were in agreement with Inoue et al., (2010) who studied Japanese patients with Hashimoto’s disease and found no difference in genotype and allele

<table>
<thead>
<tr>
<th>Oral Ulcer</th>
<th>Genital Ulcer</th>
<th>Other Skin</th>
<th>Ocular Involvement</th>
<th>Vascular Involvement</th>
<th>Neural Involvement</th>
<th>Arthritis Involvement</th>
<th>Chest Involvement</th>
<th>IT Involvement</th>
<th>Activity</th>
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<td>no</td>
<td>yes</td>
<td>No</td>
<td>yes</td>
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<td>yes</td>
<td>no</td>
<td>yes</td>
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</tr>
<tr>
<td>Number</td>
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<td>64</td>
<td>5</td>
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Foxp3 -3499 A/G

<table>
<thead>
<tr>
<th>A allele</th>
<th>85</th>
<th>0</th>
<th>79</th>
<th>49</th>
<th>40</th>
<th>30</th>
<th>58</th>
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<th>15</th>
<th>24</th>
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<th>4</th>
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<th>3</th>
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<th>21</th>
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<tbody>
<tr>
<td></td>
<td>99.30%</td>
<td>0%</td>
<td>1.70%</td>
<td>38.30%</td>
<td>37.10%</td>
<td>30%</td>
<td>63%</td>
<td>37%</td>
<td>55.90%</td>
<td>44.10%</td>
<td>70.60%</td>
<td>29.40%</td>
<td>65%</td>
<td>35%</td>
<td>50%</td>
<td>50%</td>
<td>62.50%</td>
<td>37.50%</td>
<td>62.50%</td>
<td>37.50%</td>
</tr>
</tbody>
</table>

| G allele | 1 | 0 | 1 | 127 | 70 | 1 | 91 | 0 | 34 | 0 | 34 | 0 | 40 | 0 | 8 | 1 | 7 | 0 | 56 |
|----------|----|---|---|-------|----|---|----|---|----|---|----|---|----|---|---|---|---|---|---|---|
|          | 0.70% | 0% | 0.80% | 99.20% | 0% | 70% | 1.10% | 98.90% | 0% | 100% | 0% | 100% | 0% | 100% | 0% | 100% | 0% | 100% |

Foxp3 -3279 C/A

| C allele | 19 | 0 | 19 | 109 | 8 | 62 | 14 | 78 | 4 | 30 | 2 | 32 | 9 | 31 | 1 | 7 | 2 | 6 | 7 | 49 |
|----------|----|---|----|------|---|----|---|----|---|----|---|----|---|----|---|---|---|---|---|---|---|
|          | 12.10% | 0% | 4.40% | 91.60% | 11.40% | 88.60% | 55.20% | 48.80% | 11.80% | 88.20% | 5.90% | 94.10% | 22.50% | 77.50% | 12.50% | 87.50% | 25% | 75% | 12.50% | 87.50% |

<table>
<thead>
<tr>
<th>A allele</th>
<th>67</th>
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<th>61</th>
<th>67</th>
<th>32</th>
<th>38</th>
<th>45</th>
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<tbody>
<tr>
<td></td>
<td>77.90%</td>
<td>0%</td>
<td>7.70%</td>
<td>52.30%</td>
<td>5.70%</td>
<td>54.30%</td>
<td>88.90%</td>
<td>51.10%</td>
<td>44.10%</td>
<td>55.90%</td>
<td>64.70%</td>
<td>35.30%</td>
<td>42.50%</td>
<td>37.50%</td>
<td>62.50%</td>
<td>50%</td>
<td>50%</td>
<td>50%</td>
<td>50%</td>
<td></td>
</tr>
</tbody>
</table>

Table 4: Association of the most common clinical findings of the disease and foxp3 gene polymorphisms in the patients with BD. P<0.05 is considered significant (in bold) *(P<0.05).
Our results also are contradictory with who studied Chinese Population and found the genotype AA was significantly increase in the unexplained spontaneous recurrent abortion (URSA) group than the control group which suggest that the AA genotype may contribute to occurrence of URS. Our result also are contradictory with who found that the A allele was a risk factor for Systemic lupus erythematosus and for allergic rhinitis respectively. Our results also is disagree with who found that the females homozygous allele (A/A) are protected against allergic rhinitis; otherwise, females who are either wild types (C/C) or heterozygote carriers (C/A) of the rare allele are more susceptible to allergic rhinitis in the Hungarian female population [34-36].

Conclusions

In conclusion, although the sample size of the present association study is likely insufficient for detecting all the associations between these SNPs and BD, our study points to t1- There is no association between any genotype of Foxp3 (-3499 A/G) and (-3279 C/A) polymorphism and the susceptibility to BD, The C allele (-3279 C/A) is negatively correlated with susceptibility to Behcet’s Disease, The neural involvement is positively correlated with A allele (-3279 C/A). A precise genetic association study involving multiple centers and regions may provide a sound foundation for further research into the involvement of the FOXP3 gene with liver cancer. Further research is also required to elucidate the molecular mechanism by which FOXP3 affects the development of HCC.

References


(rs3761548) in breast cancer progression: a study from India. Tumour Biol 35(4): 3785-3791.


