Association of Higher Defensin β-4 Genomic Copy Numbers with Behçet’s Disease in Iraqi Patients

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ABSTRACT: Objectives: Behçet’s disease (BD) is an immune-mediated small vessel systemic vasculitis. Human β-defensins are antimicrobial peptides associated with many inflammatory diseases and are encoded by the β-defensin family of multiple-copy genes. However, their role in BD necessitates further investigation. The aim of the present study was to investigate the possible association of BD in its various clinical forms with defensin β-4 (DEFB4) genomic copy numbers. Methods: This case-control study was conducted from January to September 2011 and included 50 control subjects and 27 unrelated Iraqi BD patients registered at Baghdad Teaching Hospital, Baghdad, Iraq. Copy numbers of the DEFB4 gene were determined using the comparative cycle threshold method by duplex real-time polymerase chain reaction technology at the Department of Dermatology of Jena University Hospital, Jena, Germany. Results: DEFB4 genomic copy numbers were significantly higher in the BD group compared to the control group (P = 0.010). However, no statistically significant association was found between copy numbers and clinical variables within the BD group. Conclusion: The DEFB4 copy number polymorphism may be associated with BD, however, it is not associated with different clinical manifestations of the disease.

Keywords: Behçet Disease; beta-Defensins; Genetic Polymorphisms; Gene Copy Numbers; Iraq.

DEFB4 is a member of the β-defensin family, a group of antimicrobial peptides that are encoded by multiple-copy genes. They are associated with many inflammatory diseases. However, their role in BD needs further investigation. The present study aimed to investigate the possible association of BD with the DEFB4 gene copy numbers by conducting a case-control study from January to September 2011, involving 50 control subjects and 27 unrelated Iraqi BD patients registered at Baghdad Teaching Hospital. The copy numbers of the DEFB4 gene were determined using the comparative cycle threshold method by duplex real-time polymerase chain reaction technology at the Department of Dermatology, Jena University Hospital, Jena, Germany. The results showed that DEFB4 genomic copy numbers were significantly higher in the BD group compared to the control group (P = 0.010). However, no statistically significant association was found between copy numbers and clinical variables within the BD group. Therefore, the DEFB4 copy number polymorphism may be associated with BD, but it is not associated with different clinical manifestations of the disease.
of BD remains unknown, genetic, immunological and environmental factors have been suggested to contribute to its development. A genetic basis for BD is supported by its high prevalence in certain geographic areas, the familial aggregation of cases and a strong association with human leukocyte antigen-B51. Moreover, single nucleotide polymorphism studies have revealed associations with several genes, including interferon regulatory factor 1 and tumour necrosis factor-a (TNF-α). Thus, it is possible that variations in the DEFB4 gene might influence an individual’s susceptibility to immune-mediated diseases. The antimicrobial and proinflammatory nature of these β-defensins has led many researchers to hypothesise that variations in gene dosage may affect the pathogenesis of many immunological diseases. Hence, the present study aimed to investigate genomic copy numbers of DEFB4 in Iraqi BD patients in comparison with normal healthy controls. Various clinical manifestations of BD were also studied in association with DEFB4 genomic copy numbers.

Methods

This case-control study was conducted between January and September 2011 and included all unrelated Iraqi patients registered at the BD Clinic in Baghdad Teaching Hospital, Baghdad, Iraq (n = 27), and randomly selected healthy control subjects (n = 50). All of the BD patients fulfilled the International Study Group criteria for a diagnosis of BD, which requires confirmation that they were free from any symptoms or signs suggestive of BD.

Blood was collected from all of the subjects and genomic DNA was extracted from peripheral blood mononuclear cells by a standard phenol-chloroform method at the Department of Pathology in Baghdad Medical College, Baghdad. Samples were then stored at -20°C and delivered to the Department of Dermatology at Jena University Hospital, Jena, Germany. DNA concentrations and quality were then determined with a NanoDrop 1000 spectrophotometer (Nanodrop Technologies Inc., Thermo Fisher Scientific Inc., Wilmington, Delaware, USA) using the following settings: optical density (OD) at 260/280 = 1.8 and OD at 260/230 = 2.0–2.2. Samples were anonymised and the genomic copy number analysis was performed by individuals who were blinded to the relevant clinical information.

Diploid DEFB4 genomic copy numbers (GenBank: AF040153.1) were determined by a duplex real-time polymerase chain reaction (PCR) technique as described by Jaradat et al. Briefly, PCR was performed in 96-Well Microplates (Applied Biosystems, Thermo Fisher Scientific Inc.) in a 7500 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific Inc.). All assays were performed in quadruplicate. Each plate included templates for genomic DNA, negative controls and NA07048HM calibrator DNA (Coriell Institute for Medical Research, Camden, New Jersey, USA) at four copies per genome. Each well contained a 20 µL reaction mixture including 4 µL of genomic DNA (5 ng/µL), 10 µL of TaqMan® Genotyping Master Mix (Thermo Fisher Scientific Inc.) 1 µL of TaqMan® Copy Number Assay Mix (Applied Biosystems, Thermo Fisher Scientific Inc.), 1 µL of TaqMan® Copy Number RNase P Reference Assay mix (Applied Biosystems, Thermo Fisher Scientific Inc.) and 4 µL of nuclease-free water (Ambion® GmbH, Thermo Fisher Scientific Inc.).

The reaction conditions were as follows: 95°C for 10 minutes for initial denaturation and enzyme activation followed by 40 cycles of 95°C for 15 seconds and 60°C for one minute. Cycle threshold values were calculated using the ABI PRISM® 7700 Sequence Detection System, Version 1.3 (Applied Biosystems, Thermo Fisher Scientific Inc.). Relative quantitation was performed using Copy CallerTM Software (Applied Biosystems) to estimate the genomic copy number in each sample according to the comparative cycle threshold method (ΔΔCt).

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS), Version 16.0, (IBM Corp., Chicago, Illinois, USA).
Data were presented as the mean estimated copy numbers of DEF4. Each single genomic copy number estimate was rounded to the nearest integer number. Comparisons between the study groups were performed using an unpaired Student's t-test to assess the differences in the means ± standard deviation of DEF4 genomic copy number values. Pearson's Chi-squared test was used to analyse categorical data. All P values were two-sided and statistical significance was set at P ≤ 0.05.

This study was approved by the Ethical Committee of the Iraqi Board for Medical Specializations (approval #3) according to the principles of the Declaration of Helsinki. All subjects gave informed consent for inclusion in this study.

Results

A total of 27 BD patients were recruited along with 50 healthy controls. No significant difference in age (P = 0.33) or gender (P = 0.57) was found between the groups [Table 1]. The DEF4 integer genomic copy number range was 3–7 copies per diploid genome in BD cases compared with 2–6 copies in the control group. The mean was 4.44 ± 1.15 copies versus 3.80 ± 0.92 copies in the BD and control groups, respectively. The DEF4 genomic copy number was significantly higher in BD cases than in controls (P = 0.01) [Figure 1].

No association was found between the clinical presentations of BD and DEF4 genomic copy numbers [Table 2].

Discussion

High DEF4 genomic copy number values have been reported in patients with psoriasis and chronic obstructive pulmonary disease.17,18 Previous studies have also demonstrated increased DEF4 messenger ribonucleic acid expression of these copy number variations on the cellular level and in the mucosal tissues of the colon, upper airways and psoriatic skin.9,18–20 In a large Chinese sample, significantly higher DEF4 gene numbers were observed among patients with systemic lupus erythematosus and anti-neutrophil cytoplasmic antibody-associated small vasculitis with a mean genomic copy number of 3.98 and 4.05, respectively.21 Jansen et al. presented evidence that both genomic copy number and pro-inflammatory cytokines affect the biological levels of hBD-2.20

BD is a good example of a disease rooted in immunological disturbance mediated by cytokines derived from T-helper lymphocytes, such as TNF-α, which acts as a mediator in the initiation and propagation of BD.22 In this context, TNF-α is a common inducer of DEF4 expression.18 Possible mechanisms underlying the uncontrolled inflammatory response seen in BD to infection or other environmental triggers are either a high basal level of hBD-2 occurring in

Table 1: Demographic variables of patients with Behçet’s disease in comparison to healthy controls in Baghdad, Iraq (N = 77)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control group (n = 50)</th>
<th>BD group (n = 27)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age in years ± SD</td>
<td>34.20 ± 8.88</td>
<td>36.39 ± 10.00</td>
<td>0.33*</td>
</tr>
<tr>
<td>Gender, n</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>20</td>
<td>9</td>
<td>0.57†</td>
</tr>
<tr>
<td>Male</td>
<td>30</td>
<td>18</td>
<td></td>
</tr>
</tbody>
</table>

*Calculated using the unpaired Student’s t-test. †Calculated using the Pearson’s Chi-squared test.

Figure 1: Frequency of defensin β-4 (DEFB4) genomic copy numbers among Iraqi patients with Behçet’s disease (n = 27) in comparison to healthy controls (n = 50). The mean DEF4 genomic copy number was significantly higher in the BD group (P = 0.01).

Table 2: Defensin β-4 genomic copy numbers among patients with Behçet’s disease in Baghdad, Iraq (N = 27)

<table>
<thead>
<tr>
<th>Clinical variable</th>
<th>Present</th>
<th>Absent</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>Mean GCN ± SD</td>
<td>n</td>
<td>Mean GCN ± SD</td>
</tr>
<tr>
<td>Genital ulcer</td>
<td>17</td>
<td>4.39 ± 9.94</td>
<td>10</td>
</tr>
<tr>
<td>Ocular lesion</td>
<td>7</td>
<td>4.15 ± 0.20</td>
<td>20</td>
</tr>
<tr>
<td>Skin lesion</td>
<td>18</td>
<td>4.21 ± 1.03</td>
<td>9</td>
</tr>
<tr>
<td>Arthritis</td>
<td>23</td>
<td>4.53 ± 1.09</td>
<td>4</td>
</tr>
<tr>
<td>Positive skin pathergy test</td>
<td>20</td>
<td>4.47 ± 1.11</td>
<td>7</td>
</tr>
</tbody>
</table>

GCN = gene copy number; SD = standard deviation.
*Determined using the unpaired Student’s t-test.
genetically susceptible individuals with high DEF B4 genomic copy numbers or high levels of hBD-2 induced through the stimulation of TNF-α. It is notable that the oral epithelium, the main site of involvement in BD, has enriched hBD-2 expression. The BD patients in the present study showed significantly higher DEF B4 copy numbers than the healthy controls. Such an association between DEF B4 copy numbers and BD has not been previously reported. However, no significant relationship was observed between genomic copy number variations of DEF B4 and the various clinical manifestations of BD.

The results obtained from the current study were not consistent with previous research from South Korea, which found no significant correlation between DEF B4 copy numbers and BD. These contradictory results could be due to several reasons. It is well known that the effect of variation in β-defensin genomic copy numbers is regarded as complex, rather than simple, copy number variation; this is difficult to evaluate in small- or moderate-sized case-control studies. As a result, very few good candidate genes have been accurately studied in genomic association trials. The results of Aldhous et al. support this observation; in spite of a large sample size, they failed to replicate both the previously reported high and low genomic copy numbers of DEF B4 among patients with Crohn’s disease. In addition, discrepancies in assay reliability could play a role in explaining differences in the obtained results as well as the difference between the paralog ratio test used in the Korean study and the quantitative PCR assay used in the present study. However, Fernandez-Jimenez et al. demonstrated that both techniques can produce comparable DEF B4 results with the use of optimum DNA normalisation and high-quality genomic DNA. Nevertheless, it is known that DEF B4 copy numbers vary between ethnicities; this might account for the differences observed between the studied Iraqi and Korean populations. Genetic heterogeneity was observed in the β-defensin gene cluster among 67 populations; this may have been due to selection. Such genetic heterogeneity may differ along geographical or racial lines in different populations.

One of the limitations of this study was the small sample size. It is important to note that the current study simply presents the significantly higher counts of mean DEF B4 genomic copies in BD, drawing attention to a possible pathological association. Further research should focus on the influence of these results on hBD-2 levels both in the peripheral tissues (especially the orogenital mucosa) and serum of BD individuals. Nevertheless, the present study is the first to analyse DEF B4 in a cohort of healthy Iraqi men and women. The median genomic copy number found among this group was similar to the modal diplotype genomic copy numbers observed in seven different populations. A large-scale multicentre study is recommended to validate the results of the present study and to investigate the functional consequences of these copy number variations in terms of hBD-2 protein expression.

Conclusion

There was an association between BD and DEF B4 genomic copy numbers among the studied Iraqi subjects, with BD patients having significantly higher DEF B4 genomic copy numbers than the control group. This suggests that high DEF B4 genomic copy numbers may be associated with susceptibility to BD. However, no association was found between DEF B4 genomic copy numbers and the different clinical manifestations of BD. Among the healthy Iraqi control group, the median DEF B4 genomic copy number was comparable to numbers observed in other populations.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

References


