The Pattern of Methylation and Polymorphism in Interleukin-6 Promoter Gene Are Related to the Development of Rheumatoid Arthritis?

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Received: 08 Apr. 2017; Accepted: 12 Dec. 2017

Abstract: Rheumatoid Arthritis (RA) is a complex disease with higher level of IL-6. In order to elucidate the alterations related to IL-6 gene in RA, we evaluated the -174 G/C IL-6 gene polymorphism and methylation pattern of its promoter. A total of 120 RA patients and 120 healthy controls were recruited for polymorphism analysis, and 30 individuals of both groups were randomly selected for methylation analysis. The IL-6 gene polymorphism was analyzed by PCR-RFLP and methylation pattern was analyzed by MSP-PCR. Regarding IL-6 gene polymorphism, no significant difference was found between RA patients and controls ($P>0.05$). For DNA, two CpG regions of IL-6 promoter gene was analyzed. The first region (−1069, −1061, −1057 and −1001 CpG) did not show significant differences, whereas the second region (−628, −610, −574 and −491 CpG) showed a significant hypermethylated status ($P=0.0004$) in RA patients as compared with controls. These results, suggest a possible relationship between methylation pattern and the susceptibility to RA in our studied population.

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Keywords: Cytokine; Inflammation; Epigenetics; MSP-PCR; Single nucleotide polymorphism

Introduction

Rheumatoid Arthritis (RA) is an autoimmune disease of unknown etiology. Genetics immunological and epigenetic factors may contribute to RA susceptibility (1,2). Many cytokines are expressed in the process of this pathology (3), among them, the interleukin-6 (IL-6) that is a pleiotropic cytokine that can promote inflammation through to differentiation of T helper cells and proliferation of osteoclasts and synoviocytes (4,5). Besides this, has been documented higher levels of IL-6 in serum and synovial fluid of RA patients (6). Studies have shown that high concentrations of cytokines may be related to genetic and epigenetic variations in RA subjects and consequently affected on susceptibility and severity of the disease (1,2). Besides that, studies involving polymorphism in cytokines genes have been associated with RA susceptibility (7,8). The -174 G/C IL-6 (rs1800795) gene polymorphism, is responsible for altering levels of cytokine in vivo (9) and was associated with inflammatory diseases, as like RA (7), acute rheumatic fever (10) and osteoarthritis (11). DNA methylation is an epigenetic modification that affects gene silencing and occurs mainly in CpG-rich islands (1). Recent studies have reported that methylation of CpG motifs is a potential mechanism of regulation of IL-6 production that may be relevant to the pathogenesis of inflammatory diseases (12,13). Based on this information we hypothesized that the -174 G/C (rs1800795) IL-6 gene polymorphism and IL-6 gene promoter methylation pattern might constitute to both epigenetic and genetic susceptibility factors for RA in patients from Pernambuco, Brazil.

Materials and Methods

Study population

In our present study, 120 RA patients (cases) and
120 healthy individuals (controls) were selected from
the investigation of Division of Clinical Rheumatology
of the Hospital Oswaldo Cruz-Recife, Pernambuco,
Brazil. The patients were diagnosed according to
American College of Rheumatology/European League
Against Rheumatism classification criteria (14). Blood
samples were collected for DNA analysis. Control
subjects consisted of healthy volunteers between 20 and
80-year-old with the same study to match RA in terms
of age, gender, smoking status and proportion that
patients group. The individuals were included in control
group if their medical history did not reveal RA, other
autoimmune or infectious disease. If some of these
conditions were showed in any individual, we excluded
from the control group. The methylation analysis were
performed using 30 RA patients and 30 healthy control
subjects selected randomly. All subjects inhabited the
same geographic area and were recruited between July
2015 and May 2016. The study was approved by the
Ethics Committee of the Health Sciences Center of
Federal University of Pernambuco (protocol
1.097.918/2015). Written informed consent was
obtained from all participants.

DNA isolation
Genomic DNA was isolated from peripheral blood
leukocytes using Wizard Genomic DNA Purification Kit
(PROMEGA, Madison, USA) according to the
manufacturer’s descriptions. The isolated DNA was
stored at 20°C.

Bisulfite conversion and methylation-specific
polymerase chain reaction (MSP)
Five hundred nanograms of genomic DNA were
treated with bisulfite follows the Pappas et al., (15)
instructions, to modify unmethylated cytokines to
uracil, whereas methylated cytokines must remain
unaltered. Bisulfite-treated DNA was purified and
resuspended in 50 µl of TE (10 mM Tris -1 mM EDTA)
buffer pH 8.0. Amplification of the bisulfite-treated
DNA was performed based on Stefani et al., (13), to
assess two regions of the IL-6 promoter gene containing
eight CpG motifs (MSP1: −1069, −1061, −1057 and
−1001 and MSP2: −628, −610, −574 and −491)
(Genbank Accession No.: AY170325-1). All
amplification products were separated by electrophoresis
on 2% agarose gel. It was performed two independent
experiments to ensure the reproducibility of the

Genotyping
The -174 G/C IL-6 gene polymorphism, was detected by PCR followed by restriction enzyme fragment
analysis (PCR-RFLP). The primers used were as follows: forward 5’-
TTGTCAGACATGCAAAGTG-3’ and reverse 5’-
CTGATTGGAACCTTATTAGG-3’. The products
were digested with Hsp92II restriction enzyme, as
previously described (16). The PCRs consisted of a pre-
denaturation step of 4 min at 94°C and 40 cycles each
of 30 s denaturation at 94°C, 30 s annealing at 55°C
and 30 s elongation at 72°C. This was followed by a
post-elongation step of 7 min at 72°C. Restriction
endonuclease was obtained from Fermentas (st. Leon-
rot, Germany) and was used as described by the
manufacturer. Restriction fragments were visualized by
electrophoresis on 3% agarose.

Statistical analysis
A descriptive statistical approach was used initially
with a data set created from information such as
genotyping and methylation patterns. Univariate
analysis was performed to evaluating the association
between RA development and genotyping. In addition,
we performed a logistic regression with adjustment for
age and gender to assess the association between
development of RA and methylation patterns.

Results
Demographics and risk factors of all patients were
described (Table 1). Mean age of patients with RA was
52.86±10.33 years is 97.5% women. For the control
group, the mean age was 48.79±12.47 years being 95%
women. The RA patients selected for methylation
analysis had mean age 54.46±9.85 years, of these,
96.67% women, whereas controls group had mean age
55.44±7.95 years as 93.3% women.
Table 1. Clinical features of the patients with rheumatoid arthritis (RA)

<table>
<thead>
<tr>
<th>Variable</th>
<th>RA patients (N=120)</th>
<th>RA patients methylation analysis (N=30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender Female/ Male; n (%)</td>
<td>117(97.5)/3(2.5)</td>
<td>29(96.67)/1 (3.33)</td>
</tr>
<tr>
<td>Age; mean (range) years</td>
<td>52.86 ± 10.33</td>
<td>54.46 ± 9.85</td>
</tr>
<tr>
<td>Age at RA onset; mean (range) years</td>
<td>41.86 ± 10.18</td>
<td>43.67 ± 9.94</td>
</tr>
<tr>
<td>Disease duration, mean (range) years</td>
<td>11.00 ± 7.88</td>
<td>10.8 ± 8.14</td>
</tr>
<tr>
<td>CRP, means (range) mg/L</td>
<td>5.08 ± 10.43</td>
<td>7.98 ± 12.98</td>
</tr>
<tr>
<td>ESR; mean (range) mm/h</td>
<td>22.49 ± 12.8</td>
<td>21.9 ± 12.27</td>
</tr>
<tr>
<td>Rheumatoid factor positive, n (%)</td>
<td>117(97.5) / 3 (2.5)</td>
<td>29 (96.67) / 1 (3.33)</td>
</tr>
<tr>
<td>ACPA present, n (%)</td>
<td>15 (75)</td>
<td>--</td>
</tr>
<tr>
<td>DAS28 Low (2.6 &lt; DAS28 ≤ 3.2)</td>
<td>13 (10.83)</td>
<td>1 (3.33)</td>
</tr>
<tr>
<td>Moderate (3.2 &lt; DAS28 ≤ 5.1)</td>
<td>97 (72.5)</td>
<td>22 (73.33)</td>
</tr>
<tr>
<td>Severe (&gt; 5.1)</td>
<td>6 (5)</td>
<td>3 (10)</td>
</tr>
<tr>
<td>HAQ; mean (range)</td>
<td>1.43 ± 0.66</td>
<td>1.33 ± 0.73</td>
</tr>
<tr>
<td>CDAI; mean (range)</td>
<td>20.33 ± 10.02</td>
<td>20.5 ± 11.62</td>
</tr>
<tr>
<td>Rheumatoid factor positive, n (%)</td>
<td>53 (44.16)</td>
<td>11 (36.67)</td>
</tr>
<tr>
<td>ACPA present</td>
<td>15 (75)</td>
<td>--</td>
</tr>
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<td>1 (3.33)</td>
</tr>
<tr>
<td>Moderate (3.2 &lt; DAS28 ≤ 5.1)</td>
<td>87 (72.5)</td>
<td>22 (73.33)</td>
</tr>
<tr>
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<td>22 (73.33)</td>
</tr>
<tr>
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<td>6 (5)</td>
<td>3 (10)</td>
</tr>
</tbody>
</table>

Table 2. Genotype distribution and allele frequency of -174 G/C IL-6 (rs1800795) gene polymorphism and methylation pattern of the IL-6 promoter gene in Rheumatoid Arthritis patients and healthy controls

<table>
<thead>
<tr>
<th>-174 G/C IL-6 polymorphism</th>
<th>RA patients N=120</th>
<th>Controls N=120</th>
<th>P*</th>
<th>OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>68 (56.66)</td>
<td>72 (60.0)</td>
<td></td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>GC</td>
<td>50 (41.66)</td>
<td>43 (35.83)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>2 (1.66)</td>
<td>5 (4.17)</td>
<td></td>
<td>0.381</td>
<td>0.87</td>
</tr>
<tr>
<td>Allele a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.694</td>
</tr>
<tr>
<td>G</td>
<td>186 (77.5)</td>
<td>187 (77.92)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>54 (22.5)</td>
<td>53 (22.08)</td>
<td></td>
<td>0.97 (0.63-1.50)</td>
<td>1.000</td>
</tr>
<tr>
<td>Methylation Pattern</td>
<td>RA patients N=30</td>
<td>Controls N=30</td>
<td>P*</td>
<td>OR (95% CI)</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSP1 b</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MM</td>
<td>2 (6.67)</td>
<td>3 (10.0)</td>
<td></td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>UM</td>
<td>15 (50.0)</td>
<td>17 (56.67)</td>
<td></td>
<td>0.06 (0.29-27.3)</td>
<td>0.381</td>
</tr>
<tr>
<td>UU</td>
<td>13 (43.33)</td>
<td>10 (33.33)</td>
<td></td>
<td>1.34 (0.38-44.5)</td>
<td>0.248</td>
</tr>
<tr>
<td>MSP2 b</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UM</td>
<td>24 (80.0)</td>
<td>10 (33.33)</td>
<td></td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>UU</td>
<td>4 (13.33)</td>
<td>19 (63.34)</td>
<td></td>
<td>0.37 (0.15-0.25)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Bold means significance; P* - P value of χ²; OR – odds ratio; CI – confidence interval; P - P value of OR; MM - total methylation; UM - partial methylation; UU - unmethylation; * Univariate Analyses; b Logistic regression analyses, adjusted for age and gender.
Discussion

In relation to -174 G/C IL-6 gene polymorphism, no significant difference between RA patients and healthy controls was observed. Even though, the power of our study is not high due to the sample size (Power (1−β err prob)=0.50), similar outcomes were found in Mexican (12), Spanish (19) and Turkish (20) populations. However, literature data show contradictory results since another study in Chinese (7) and Egyptian (21) populations indicates association of the polymorphism with the risk for RA development. Indeed, a meta-analysis suggests that this polymorphism has been associated with RA risk in Asian, but not in Caucasian populations (22). Although this polymorphism has been related to altering levels of cytokines in vivo assays (6,9), there is no consensus whether RA is associated or not with this polymorphism, since lack association found in many studies can be explained due to the low frequency of C variant in different ethnics groups.

We analyze the methylation pattern in two different regions of the IL-6 gene (MSP1 and MSP2). Logistic regression analyses did not reveal difference in first region MSP1 (−1069, −1061, −1057 and −1001 CpG motifs). Similar results were found by Nile et al., (12) and Ishida et al., (23) in RA and Stefani et al., (13) studying periodontitis also showed similar methylation pattern between patients and controls. Nevertheless, for the second region MSP2 (−628, −610, −574 and −491 CpG motifs) RA patients showed higher level of methylation as compared with healthy controls. Contradictory results were found by Nile et al., (12) and Ishida et al., (23), which did not verify hypermethylation in promoter IL-6 gene. Besides this, other study with RA patients and TNF-α gene indicated hypermethylation of CpG motifs in patients group as compared with controls (24). In other diseases as obesity which involve inflammatory process, it was found a hypermethylation pattern of IL-6 gene most frequent in patients than controls group (25). These findings can be explained by different hypothesis: the persistent inflammation could cause global DNA hypermethylation of pro-inflammatory cytokines or the specific characteristics of our study population as ethnic, environmental exposures, disease duration, disease activity or laboratory features of the patients may influence the methylation pattern. Moreover, Ishida et al., (23) suggested that hypermethylation may be a result of increased DNA methyltransferases activity as a way to decrease the inflammatory process.

In conclusion, the -174 G/C IL-6 gene polymorphism did not play an important role in RA development in our population even though the hypermethylated status of Cpg motifs in the IL-6 promoter region may implicating a role in RA in the population of Pernambuco, Brazil.

Acknowledgements

We thank the patients who generously agreed to participate in this study. This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

References


