EVIDENCE OF ANGIOTENSIN CONVERTING ENZYME (ACE) INSERTION POLYMORPHISM IN RHEUMATOID ARTHRITIS FROM PAKISTANI PATIENTS

MOEEZ S.1*, IQBAL T.2, JOHN P.1 AND BHATTI A.1

1Department of Health Care Biotechnology, Atta-ur-Rahman School of Applied Biosciences (ASAB), National University of Sciences and Technology (NUST), Islamabad-44000, Pakistan.
2Department of Medicine, Shifa College of Medicine-Shifa Tameer e Millat University Pitrash Bhoukari Road, Islamabad-44000, Pakistan.
*Corresponding Author: Email- pjohn@asad.nust.edu.pk

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Abstract-

Background: The study was aimed to explore the association of I/D polymorphism genotypes in Angiotensin converting enzyme (ACE) gene in Rheumatoid Arthritis (RA) patients from the Pakistani population.

Methodology: The intron 16 of ACE gene was amplified in 200 RA patients and 200 normal healthy individuals by using a forward (ACE-F: 5’CTGGAGACCCTCCACATCTTTCT3’) and a reverse primer (ACE-R: 5’GATGTGCCCACCATTCGTCAGAT3’). PCR products were directly sequenced in an ABI prism 310 Automated Sequencer (Applied Biosystem). The data was statistically analyzed through SPSS software and association was tested through Chi-square and Z-test.

Results: We noticed that genotype II was most frequently occur (p<0.001) in cases as compare to our healthy controls. No significant association was analyzed between the homozygote (DD) and heterozygote (ID) genotypes and pathogenesis of RA. ACE I allele carriers were at higher risk to develop RA than those who were D carriers. An association of ACE I/D gene polymorphism was established with migratory arthritis (p>0.001).

Conclusion: Our present study identified an association between ACE insertion (II) polymorphism and Rheumatoid arthritis in the Pakistani population.

Keywords- Angiotensin-converting enzyme (ACE), Rheumatoid Arthritis, I/D Polymorphism, Primers, SNP, DNA, Pakistan, Genotype

Running Title: ACE gene polymorphism in RA


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Introduction

Rheumatoid arthritis (RA) is a systematic, inflammatory joints disease, occurs as a chronic inflammatory disorder that is characterized by cartilage destruction. Worldwide, its prevalence is about 0.3 -to 1.2% [1]. The disease is mainly characterized by angiogenesis of the synovium, growth of new blood vessels and infiltration of leukocytes. These events ultimately lead to invasion of tissue and destruction of joints [2]. Cytokines are thought to play an important role in the progression of Rheumatoid arthritis [3].

Exact etiology of RA is not so far known, however its development depends upon interaction between genetics elements in individual and non-genetic factors. Considerable amount of data have suggested that autoimmunity markers and genetic factors are very good indicators [4]. The contributions of genetic factors are estimated to be 50-60% [5]. Human leukocyte antigen (HLA) has the most prominent genetic association, which is estimated to be 30% of the total genetic contributions. It is also suggested that a number of non-HLA genes also play a very important role in the progression and pathogenesis of RA [6].

The angiotensin-converting enzyme (ACE) is a membrane-bound, zinc metalloendopeptidase. It is also known as peptidyl dipeptidase A or kininase II), encoded by the ACE gene. It is mapped on chromosome 17q23 and plays a central role in a number of diseases. This is a 21 kilo bases (kb) long gene having 26 exons and 25 introns [7]. ACE gene encodes two isoenzymes, the germinal and somatic. The germinal isoenzyme is expressed only in sperm, whereas somatic isoenzyme is expressed in numerous tissues, including vascular endothelial cells, testicular Leydig cells, lung and epithelial kidney [8].

In the National Center for Biotechnology Information (NCBI) records, more than 160 ACE gene polymorphisms have been reported so far, most of which are single nucleotide polymorphisms (SNPs). Only 34 of those polymorphisms are located in coding regions and 18 of them are missense mutations [7]. ACE gene contains a poly-
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morphism that is based on the presence (insertion=I) or absence (deletion=D) of a 287 base pair ALU repeat sequence in intron 16; that results in 3 genotypes: DD and II homozygous and ID heterozygous [9]. The DD genotype serum level is found to be the highest in normal individuals that is followed by the heterozygous ID genotype, while the level of II genotype is lowest [10,11].

Correlation between ACE gene level and I/D polymorphism raised question whether I/D polymorphism by itself has a functional significance or it is linked to a yet unidentified molecular variant in the ACE gene or in another locus on the chromosome 17 region where ACE is mapped. It has been proposed that regulatory elements that are present in intron 16 may affect gene transcription, thus control ACE gene level in tissues [7,12].

ACE gene ID polymorphism have been reported in different ethnic groups in different diseases including rheumatoid arthritis [Table-1]. ACE gene is a key regulator in inflammatory signal transduction pathways and is involved in RA pathogenesis [13]. Level of ACE gene in serum and tissue is regulated at transcriptional level and it can be hypothesized that that ACE genotype may be a contributing factor in the onset, pathogenesis and progression of RA [6].

Table 1- Study of ACE gene polymorphism in different diseases

<table>
<thead>
<tr>
<th>SN</th>
<th>Disease</th>
<th>Population</th>
<th>Year</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Psoriatic arthritis</td>
<td>Kuwait</td>
<td>2007</td>
<td>Not Associated</td>
</tr>
<tr>
<td>2</td>
<td>Cardiovascular disease</td>
<td>Italy</td>
<td>2012</td>
<td>Associated</td>
</tr>
<tr>
<td>3</td>
<td>Type 2 diabetes</td>
<td>Indonesia</td>
<td>2010</td>
<td>Not Associated</td>
</tr>
<tr>
<td>4</td>
<td>Rheumatoid arthritis</td>
<td>Kuwait</td>
<td>2007</td>
<td>Associated</td>
</tr>
<tr>
<td>5</td>
<td>Alzheimer Disease</td>
<td>Israeli Arab Community</td>
<td>2006</td>
<td>Associated</td>
</tr>
<tr>
<td>6</td>
<td>Systemic Lupus Erythematosus</td>
<td>Pakistan</td>
<td>2010</td>
<td>Associated</td>
</tr>
<tr>
<td>7</td>
<td>Atherosclerosis</td>
<td>Asians</td>
<td>2003</td>
<td>Associated</td>
</tr>
<tr>
<td>8</td>
<td>Breast cancer</td>
<td>Caucasian women</td>
<td>2005</td>
<td>Associated</td>
</tr>
<tr>
<td>9</td>
<td>Rheumatoid Arthritis (This Work)</td>
<td>Pakistan</td>
<td>2013</td>
<td>Associated</td>
</tr>
</tbody>
</table>

To the best of our knowledge, the possible role of this polymorphism to control risk to develop RA has not been studied in the Pakistani population. Therefore, to study the functional allelic differences in the ACE I/D polymorphism may be a determining factor in the pathogenesis of RA.

Materials and Methods

Materials

dNTP's (Invitrogen), Taq polymerase (Invitrogen), Mgcl2 (Fermentas), DNA ladder (Fermentas) and Agrose (Invitrogen) were used in this study. PCR Primers were designed using Primer 3 input software version 0.40 and were BLAST using NCBI PRIMER BLAST. Primer sequences were obtained from Eurofins Scientific.

Blood Collection and Storage of Samples

Blood samples were collected from October 2011 to June 2012 with a prior approval from Ethical Committees and Institutional Review Boards (IRBs) of National University of Sciences and Technology and the hospitals. All the patients fulfilled the American College of Rheumatology classification criteria. Informed written consent was obtained from each patient. The subjects were classified into different groups on the basis of age, gender and severity of disease (mild, moderate and severe). Two Hundred RA Patients (mean age 41±13) & two hundred healthy control individuals (mean age 42±12) were recruited in the present study. Each blood sample was collected in EDTA tube and was kept at 4°C.

DNA isolation

DNA was extracted from blood of RA patients and healthy individuals using standard Phenol-Chloroform method and the DNA was quantified through nanodrop.

Polymerase Chain Reaction

The intron 16 of ACE gene was amplified in a total reaction volume of 20µL. Each reaction volume contained 10 pmol of each forward and reverse primer: ACE-F: 5'CTGGAGACCACCTCCATCTTTTCT3' and ACE-R: 5'GATGTGGCCATCACATTCTGCAAT3', 25mM MgCl2, 100mM of 10x Taq buffer, 10mM dNTPs, 1U Taq polymerase and 2µL of DNA. Reaction conditions were 96°C for 5 minutes; 94°C for 30 seconds; 58°C for 1 minute, 72°C for 1 minute and a final extension at 72°C for 10 minutes. The PCR products which were amplified further run on 2% agarose gel using 100bp DNA ladder as a marker.

Hardy-Weinberg Equilibrium and Genotypes Distribution

Genotype frequencies in cases and controls were calculated according to Hardy-Weinberg equilibrium. This law states that: in population both allele and genotype frequencies remains constant, means they are in equilibrium from generation to generation unless specific disturbing influences are introduced.

Sequencing

PCR products were then sequenced directly in an ABI prism 310 Automated Sequencer, using the ABI Big dye Terminator V 3.1 cycle sequencing kit (PE Applied Biosystem), following purification by Rapid PCR Purification System (Marligen Biosciences USA).

Statistical Analysis

Statistical analysis was performed using SPSS v 16.0 using the level of p ≤ 0.05 as statistically significant. Alleles and genotype frequencies between patients and control subjects were compared by Z-test. Some clinical characteristics of patients and Hardy-Weinberg equilibrium were analyzed by Chi-square analysis.

Results

Association analysis of ACE gene I/D polymorphism was carried out in two hundred RA patients and two hundred healthy individuals using allele specific PCR as shown in [Fig-1] & [Fig-2]. Significant difference was found in frequencies of ACE genotypes and I/D alleles between patients and controls (p < 0.001).

Fig. 1- Detection of ACE gene I/D polymorphism by polymerase chain reaction (PCR). Lane 1: 100 bp DNA ladder, Lanes 2-9: PCR products from patients with ID genotype, Lane 10: PCR product from patient with DD genotype, Lanes 11-14: PCR products from patients with ID genotype, Lane 15: PCR product from patient with DD genotype, Lanes 16-18: PCR products from patients with ID genotype and Lane 19: PCR product from patients with DD. The products of PCR amplification were analyzed on 2% agarose gel and visualized under UV light.
We identified a fragment of 480bp which shows an insertion of Data regarding ACE gene I/D polymorphism genotypes frequencies in cases and controls are presented in [Fig-3]. In addition when a comparison was made between ACE genotype sub groups including migratory arthritis and familial type cases then significant association was observed (p < 0.001). However no significant association was found with gender, age at onset, duration of symptoms and disease severity (p > 0.05) [Table-2]. ACE gene distribution was in accordance with the Hardy-Weinberg expectations in the cases and control group.

289bp sequences when compared with wild type fragment of 190bp [Fig-4]. The 2% agarose gel result was confirmed by sequencing [Fig-5]. This sequence was aligned with the control using EMBOSS pair wise alignment tool, which showed 100% homology except the 289bp insertion. The mutant observed in this study is reported previously [14].

Fig. 2 - Detection of ACE gene I/D polymorphism by polymerase chain reaction (PCR). Lane 1: 100 bp DNA ladder, Lanes 2 - 17: PCR products from patients with ID genotype, Lane 18: PCR product from patient with II genotype, Lanes 19: PCR product from patients with DD genotype, Lane 20: PCR product from patient with ID genotype and Lane 21: PCR product from patients with II genotype. The products of PCR amplification were analyzed on 2% agarose gel and visualized under UV light.

Fig. 3- Distribution of the study subjects across different genotypes for the studied polymorphisms.

Table 2- ACE allele and genotype frequencies with respect to patient characteristics in the RA

<table>
<thead>
<tr>
<th>RA Patient Characteristics</th>
<th>n=200</th>
<th>Genotype Frequency</th>
<th>Allele Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>DD</td>
<td>II</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>87</td>
<td>18</td>
<td>13</td>
</tr>
<tr>
<td>Female</td>
<td>113</td>
<td>34</td>
<td>7</td>
</tr>
<tr>
<td>Age at onset</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤40</td>
<td>135</td>
<td>35</td>
<td>9</td>
</tr>
<tr>
<td>&gt;40</td>
<td>65</td>
<td>17</td>
<td>12</td>
</tr>
<tr>
<td>Family history</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>31</td>
<td>18</td>
<td>2</td>
</tr>
<tr>
<td>No</td>
<td>169</td>
<td>34</td>
<td>18</td>
</tr>
<tr>
<td>Duration of symptoms</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;6weeks</td>
<td>54</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>&gt;6months</td>
<td>90</td>
<td>24</td>
<td>6</td>
</tr>
<tr>
<td>&gt;1year</td>
<td>56</td>
<td>13</td>
<td>9</td>
</tr>
<tr>
<td>Migratory Arthritis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>104</td>
<td>26</td>
<td>10</td>
</tr>
<tr>
<td>No</td>
<td>96</td>
<td>26</td>
<td>10</td>
</tr>
<tr>
<td>Disease Severity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minor</td>
<td>32</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>Moderate</td>
<td>117</td>
<td>26</td>
<td>15</td>
</tr>
<tr>
<td>Severe</td>
<td>51</td>
<td>14</td>
<td>3</td>
</tr>
</tbody>
</table>

Fig. 4- The 289 bp Sequence in intron 16 of ACE gene. Forward & Reverse Primers (Blue) 289bp insertion (Red) with intronic sequence (Black).

Fig. 5- Sequencing Electropherogram showing analysis of ACE gene polymorphism (A) Sequence analysis of DD genotype of ACE gene in control sample with arrow indicating the point of insertion. (B) Insertion of 289bp in II genotype polymorphism of ACE gene in RA patients.

Discussion
Rheumatoid arthritis is an inflammatory disease and various cytokines are involved in the regulation of key pathways during disease pathogenesis. It is an autoimmune disease with complex etiology. Various hormonal, environmental and genetic factors have signifi-
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Systemic lupus erythematosus (SLE), is one more multisystem autoimmune disease, has also been revealed to be linked with ACE gene polymorphism. The DD genotype was more common in SLE patients than healthy controls [19]. A large difference has been reported in the usual circulation of ACE gene alleles in different populations. The frequency of D allele in normal Caucasians is 50-58%, but 35-39% in normal Chinese [20-22].

The renin-angiotensin system plays an key role in maintaining body fluid and sodium balance and has been involved in a many complex disorders [23-25]. ACE gene involves in the renin-angiotensin and kallikrein-kininogen systems by converting angiotensin I into pressor peptide angiotensin II by removing terminal His

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Conflict of Interest

This study has no conflict of interests. All authors have seen the original manuscript and they have approved it.

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