A STUDY ON ANGIOTENSIN CONVERTING ENZYME GENE INSERTION/DELETION POLYMORPHISM AND SERUM CORTISOL AMONG A SAMPLE OF EGYPTIAN PATIENTS WITH MAJOR DEPRESSION DISORDER

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ABSTRACT

Angiotensin-converting enzyme (ACE) is assumed to influence the activity of the hypothalamic-pituitary-adrenocortical (HPA) system, which shows hyperactivity in the majority of patients with major depression. The ACE gene, known to be associated with cardiovascular disorders, which in turn are accompanied with an increased susceptibility for depression, is therefore a promising candidate gene for effective disorders. However, the results are conflicting, with no reported studies on Egyptian depressed patients. This study aimed to assess ACE insertion / deletion (I/D) gene polymorphism among Egyptian depressed patients in order to clarify HPA system dysregulation, and to determine its possible association with severity of depression. This case/control study was conducted on 42 adult depressed patients, and 37 healthy controls were screened to detect genetic associations with unipolar major depression. Determination of ACE genotypes was performed for all subjects by real time PCR. Total serum cortisol levels were measured by ELISA. The frequencies of the DD, ID and II genotypes were 26.2%, 45.2%,

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and 28.2%, respectively among the cases, and 17.49%, 25.2%, and 56.41%, respectively among the controls. Significant differences in ACE genotype distribution were observed between cases and controls ($p = 0.0384$). Serum cortisol in patients show the highest value in the ID polymorphism while II polymorphism shows the lowest value of a.m. cortisol. Data illustrated a significant association of ID polymorphism with the more severity of illness. Our findings support that ACE gene I/D polymorphism and high D allele frequency are associated with depression, also hypercortisolimia is significantly higher in individuals with major depression compared to control among Egyptian adults. ACE gene polymorphism might provide a common link between developing depressive episode and dysfunctional HPA-axis.

**Keywords:** ACE gene –insertion\deletion polymorphism –cortisol – depression

**INTRODUCTION**

Depression is undoubtedly a common disabling disorder (Alonso *et al.*, 2004). The economic burden on society arises not only from the direct health and social care costs, but also from the indirect costs, due to the reduced work productivity of patients and premature death due to suicide (Baghai *et al.*, 2012). Experimental studies have investigated many different types of stress, and their effects on the hypothalamic-pituitary-adrenocortical (HPA) axis in many different circumstances (Sower *et al.*, 2009). Cortisol is released in response to stress and a low level of blood glucocorticoids acting to restore homeostasis (Holsboer, 2000). Changes in the activity of the renin-angiotensin-aldosterone system (RAAS) in depression have recently been reported (Murck *et al.*, 2006). Angiotensin I is converted to angiotensin II by angiotensin-converting enzyme (ACE) (Paul *et al.*, 2006). ACE is assumed to influence the activity of the HPA system, which shows hyperactivity in the majority of patients with major depression. The ACE gene, known to be associated with cardiovascular disorders, which in turn are accompanied with an increased susceptibility for depression, is therefore a promising candidate gene for affective disorders (Baghai *et al.*, 2006). Given the abundance of the renin–angiotensin system (RAS) components in the brain, their importance in behavior and cognition,
and the data that implicates them in the etiology and treatment of depression, it is possible that those RAS gene polymorphisms associated with increased RAS activity may also be associated with depression (Saab et al., 2007).

This study aimed to examine the ACE/ID polymorphism and estimation of serum cortisol level as a sign of the extent of HPA-axis system dysregulation in relation to this polymorphism in a sample of Egyptian patients with depression.

**MATERIALS AND METHODS**

1-**Study population**

The 42 cases of major depression disorder (MDD) patients were selected from the Institute of Psychiatry, Ain Shams University Hospitals. The control group consisted of 39 subjects of apparently healthy recruited from the visitors of Ain Shams University Hospitals and were selected to match the study group as much as possible.

The Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-I) has done by a clinician, semistructured interview with psychiatric patients or with non-patient community subjects who are undergoing evaluation for psychopathology for diagnosis of MDD. Both groups have permanently resided in the same region, with sex and age matched. The local committee for ethics of medical experiments on human subjects approved the study, and all participants gave their written informed consent. The Beck Depression Inventory (BDI) (Beck et al., 1961) was used to measure the behavioral manifestations of depression. On the other hand, Hamilton Rating Scale for Depression (Ham-D) (Appendix-VI) was designed to measure the severity of depressive symptoms in patients with primary depressive illness (Hamilton, 1960).

The reliability of the scale varies with conditions but is generally accepted, with internal consistency tends to be 0.92. It tends to be higher with the structured than unstructured interview (Potts et al., 1990). The inter rater reliability reached up to 0.9 (Rehm and O’Hara, 1985).

2-**Estimation of serum cortisol (a.m.):** Samples of patients and control had been estimated for the results of serum cortisol for in vitro diagnostic use with the IMMULITE 1000 Analysis –for quantitative measurement of cortisol (Siemens Healthcare, Diagnostics Products
The candidate gene selected in this study was Angiotensin converting enzyme gene (ACE gene).

3- Genotyping

Gene typing of genomic DNA samples obtained from 42 unrelated patients with MDD (20 women and 22 men, aged 34.79±9.1 years) and 39 unrelated healthy controls (20 women and 19 men, aged 35.56±10.8 years) was performed. The molecular part of this work was carried out at the Medical Research Center (MRC) Faculty of Medicine, Ain Shams University.

Genomic DNA was isolated from whole blood using the Qiagen-Kit. PCR (polymerase chain reaction) amplification of the ACE I/D polymorphism was carried out using the primers sequence:

Forward: 5'-GAT GTG GCC ATC ACA TTG GTC AGA T -3'.
Reverse: 5'-CTG GAG ACC ACT CCC ATC ATT TCT -3'.

The insertion (I-allele) resulted in a product of 478 bp, deletion (D-allele) in 191 bp. We report here the development of a single-tube real-time PCR assay to determine the ACE gene I/D polymorphism. This method takes advantages of the SYBR Green I fluorescent dye for real-time 45 ng of DNA is sufficient to use as a template for PCR. Amplification with 45 cycles only was optimal for ligation efficiencies. A mixture containing Taq polymerase and a proofreading polymerase, Taq must be in excess of a 10:1 ratio to ensure the presence of 3'A-overhangs on the PCR product.

The PCR in a 20 μl volume containing:

DNA Template 45 ng
10X PCR Buffer 2 μl
50 mM dNTPs 0.5 μl
Primers 1 μM each
Sterile water to a total volume of 19 μl
Taq Polymerase 1 unit
Total Volume 20 μl

Special care to avoid sources of nuclease contamination and long exposure to UV light to prevent degradation of the single A-overhangs.

Real-time PCR

Light cycler-based real-time PCR (LC-PCR) assay was used. Real-time PCR were performed with a Light Cycler 1.5 instrument (Roche
Molecular Diagnostics, Penzberg, Germany) by using a total reaction volume of 20µl in PCR capillaries. Optimization reactions were first performed until the best primer concentrations.

Melting curve analysis reveals only one peak at the characteristic melting temperature, when PCR results only in the specific amplicon as in insertion (490 bp) or Deletion (190 bp). Two peak at the characteristic melting temperature, when PCR results only in the two specific amplicon (as in heterozygot insertion and Deletion case 490 bp and 190 bp). Primer dimers and other nonspecific products would cause additional peaks.

According to the presence or absence of the insertion allele, the genotype of the subjects could be classified as II (homozygote for the insertion allele), DD (homozygote for the deletion allele), or ID (heterozygote).

The PCR products were separated on a 2% agarose gel and visualized by ethidium bromide staining under UV light. The insertion (I-allele) resulted in a product of 490 bp, the deletion (D-allele) in 191 bp (Bocchetta et al., 1999).

Statistical analyses: In some occasions Fisher-Exact probability was used if less than 5 observations are expected in a cell. Student-t test was used to detect difference between 2 groups’ means and ANOVA if more than 2 groups. All statistical manipulation and data analyses were performed using the 16th version of SPSS (Statistical Package for Social Sciences).
RESULTS

Table (1) shows Socio-demographic characteristics of cases compared to controls which show no significant differences between the two groups.

Table (1): Socio-demographic characteristics of cases compared to controls

<table>
<thead>
<tr>
<th></th>
<th>Cases n=</th>
<th>Controls n=</th>
<th>Test of Significance</th>
<th>df</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Education years</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>t= 1.89◊</td>
<td>79</td>
<td>0.062 (NS)</td>
</tr>
<tr>
<td>Min – Max</td>
<td>0 – 16</td>
<td>0 – 17</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Occupation</strong></td>
<td>N (%)</td>
<td>N (%)</td>
<td>X2=6.80</td>
<td>5</td>
<td>0.236 (NS)</td>
</tr>
<tr>
<td>Jobless</td>
<td>4 (9.5)</td>
<td>3 (7.7)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>House Wife</td>
<td>14 (33.3)</td>
<td>6 (15.4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-skilled</td>
<td>--</td>
<td>2 (5.1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Semi-skilled</td>
<td>6 (14.3)</td>
<td>5 (12.8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Semi-Professional</td>
<td>12 (28.6)</td>
<td>12 (30.8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Professional</td>
<td>6 (14.3)</td>
<td>11 (28.2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Social class</strong></td>
<td>N (%)</td>
<td>N (%)</td>
<td>X2= 5.95</td>
<td>3</td>
<td>0.114 (NS)</td>
</tr>
<tr>
<td>High-Mid</td>
<td>--</td>
<td>5 (12.8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low Mid</td>
<td>20 (47.6)</td>
<td>18 (46.2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>18 (42.9)</td>
<td>13 (33.3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Very Low</td>
<td>4 (9.5)</td>
<td>3 (7.7)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Marital Status</strong></td>
<td>N (%)</td>
<td>N (%)</td>
<td>X2= 1.161</td>
<td>3</td>
<td>0.762 (NS)</td>
</tr>
<tr>
<td>Single</td>
<td>10 (23.8)</td>
<td>7 (17.9)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Married</td>
<td>28 (66.7)</td>
<td>26 (66.7)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Divorced</td>
<td>2 (4.8)</td>
<td>4 (10.3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Widow</td>
<td>2 (4.8)</td>
<td>2 (5.1)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In this study, II polymorphism was encountered more frequently in the control group compared to only 28.6% in the cases. ID polymorphism and DD polymorphism were significantly and frequently found in the cases group than the controls (45.2% and 26.2% versus 25.6% and 17.94% respectively) (Figure 1).
Figure (1): ACE Genotype among the studied depression patients compared to controls

Table (2) shows significant difference between Insertion I and deletion D alleles frequencies among the studied patients with depression compared with matched controls by using the Fisher Exact Probability. Data revealed that the D allele was significantly prominent in the depressed group compared to controls (48.8% and 30.77% respectively).

**Table (2):** ACE Allele frequencies among the depression patients vs. controls

<table>
<thead>
<tr>
<th></th>
<th>Cases</th>
<th>Controls</th>
<th>$\chi^2$</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>43(51.19%)</td>
<td>54(69.23%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>41(48.81%)</td>
<td>24(30.77%)</td>
<td>4.754</td>
<td>0.029</td>
</tr>
</tbody>
</table>

"Fisher Exact probability has been used"

Table (3) illustrates a high significant level of serum cortisol in cases as compared to controls.
Table (3): Serum cortisol level in cases compared with control group

<table>
<thead>
<tr>
<th></th>
<th>Cases</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ±SD</td>
<td>Mean ±SD</td>
</tr>
<tr>
<td>Plasma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortisol</td>
<td>17.33±5.2</td>
<td>10.68±5.3</td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>15.7 – 18.9</td>
<td>8.9 – 12.4</td>
</tr>
<tr>
<td>Min – Max</td>
<td></td>
<td></td>
</tr>
<tr>
<td>t-value</td>
<td>5.69</td>
<td></td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.001*</td>
<td></td>
</tr>
</tbody>
</table>

Table (4) shows the distribution of different allele among depressed patients illustrating higher percentage of ID allele in comparison to II and DD alleles. We performed genotyping of the I/D polymorphism using polymerase chain reactions (PCR) amplification of the ACE I/D polymorphism in cases who were diagnosed according to DSM-VI criteria to have major depression compared to matched healthy controls.

Table (4): ACE polymorphism among the studied depression patients

<table>
<thead>
<tr>
<th>RAS Polymorphism</th>
<th>N (%)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>• II</td>
<td>12 (28.6)</td>
<td>16.2 – 44.8</td>
</tr>
<tr>
<td>• DD</td>
<td>11 (26.2)</td>
<td>14.4 – 42.3</td>
</tr>
<tr>
<td>• ID</td>
<td>19 (45.2)</td>
<td>30.2 – 61.2</td>
</tr>
</tbody>
</table>

Table (5): Serum cortisol levels among the studied depression patients in relation to ACE genotypes

<table>
<thead>
<tr>
<th>Serum Cortisol</th>
<th>II&lt;sup&gt;0&lt;/sup&gt;</th>
<th>DD&lt;sup&gt;0&lt;/sup&gt;</th>
<th>ID&lt;sup&gt;0&lt;/sup&gt;</th>
<th>f value&lt;sup&gt;0&lt;/sup&gt;</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (SD)</td>
<td>14.9 (2.1)</td>
<td>17.5 (6.6)</td>
<td>21.0 (5.3)</td>
<td>6.438</td>
<td>0.004*</td>
</tr>
<tr>
<td>95% CI</td>
<td>13.9 – 15.9</td>
<td>13.1 – 22.0</td>
<td>17.6 – 24.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>0</sup>f value (ANOVA test), df= 2 Between groups and within groups 39.
A STUDY ON ANGIOTENSIN CONVERTING ENZYME GENE ……

Figure (2): represents agarose gel electrophoresis illustrating 100 base pair (bp) loading marker in first and last lanes. In lanes 1 and 6 show homozygote insertion ACE gene polymorphism at 490 bp, in lanes 2 and 5, heterozygote insertion/deletion ACE gene polymorphism at 490 bp and 190 bp while lanes 3 and 4 represent homozygote deletion ACE gene polymorphism at 190 bp, and lane 7 represents control sample (no polymorphism).

DISCUSSION

Depression is a prevalent disorder and one of the leading disease causing disability and economic burden according to the World Bank reports (Kato and Serretti, 2010). Since epidemiological data denotes that depression may affect 10% of population, we except that we have about 8 million people with depression in Egypt (Ghanem et al., 2009).

That is why this study was designed to be the first step to find one important parameter for a biological marker which helps for the early detection of depression and reconfirmation of the diagnosis. This also may lead to early intervention hoping for recovery from this devastating disorder.

To fulfill our aim we investigated 42 patients suffering from major depression according to the DSM IV criteria for diagnosis of depression and we compared them with 39 matched healthy controls. Recent studies have shown that genetics variants of angiotensin converting enzyme (ACE) can contribute to disease susceptibility and
inter-individual differences in antidepressants response (Yanfeng et al., 2011). A genetic polymorphism is characterized by an insertion (I) or deletion (D). Both alleles have concomitant effects on the level of ACE. Thus individuals who are Homozygous for the I allele has the lowest level of the enzyme, while those who are homozygous for the D allele have the highest; while, heterozygous subjects have an intermediate level (Zintzaras et al., 2008).

The renin-angiotensin-aldosterone system (RAAS) has been implicated in mood disorders (Hallberg et al., 2011; Nasr et al., 2011). The contribution of the RAAS in controlling mood has been attributed to the angiotensin II that interacts with dopamine (DA) in specific brain regions and the ACE has an important role in the modulation of DA turnover. ACE is part of the renin-angiotensin system (RAS) and is involved in the conversion of angiotensin I to angiotensin II (Kobori et al., 2007), which is a peptide hormone acts as a stimulator of proinflammatory cytokines and interferes with hypothalamic pituitary adrenal (HPA) axis activation in response to stress (Ising and Jolsboer, 2006; Yanfeng et al., 2011).

There has been a growing interest in the study of ACE/ID polymorphism as a potential factor for developing depression (Baghai et al., 2006). However, despite the large number of studies with different designed, the role of the ACE gene I/D polymorphism on depression is still controversial. Previous association studies for ACE and depression conducted by genotyping ACE-I/D polymorphism have yielded inconsistent results (Heck et al., 2009). On the other hand, previous data pointed to the possibility that ACE/ID polymorphism could be a finger point or a biological marker of major depression and response to treatment (Baghai et al., 2003 and 2006; Murck et al., 2006).

Wu et al. (2012) provided a strong evidence of a positive association of the ACE/ID polymorphism as risk factor for depression in Caucasians but not in Asians. On the other hand, they failed to detect significant differences between cases and control groups as regards ACE/ID polymorphism in Chinese population.

In a similar culture to ours, Saab et al. (2007) in Lebanese population proved that the angiotensin receptor type I was significantly associated with depression, however he could not prove that the type of the ACE/ID polymorphism is associated with unipolar depression.
It seems that variation of results across ethnic groups could account for the differences in race specific genetics effects, sample selection diagnostic criteria, different methodology and different patient recruitment methods for patients and controls.

The dysregulation of the activity of the hypothalamic-pituitary-adrenocortical (HPA) system is one of the major neuroendocrine abnormalities in major depressive disorder. These include elevated circulating plasma levels of both corticotropin (adrenocorticotropic hormone, ACTH) and cortisol (Holsboer, 2000). RAAS is closely related to HPA-axis as ACTH is a common stimulus for both cortisol and aldosterone, moreover, cortisol release is suppressed by mineralocorticoid receptor agonists and finally angiotensin II releases corticotropin-releasing hormone (CRH) and vasopression from the hypothalamus (Murck et al., 2003).

In this study, serum cortisol in patients show the highest value in the ID polymorphism while II polymorphism shows the lowest value of a.m. cortisol and DD polymorphism shows the intermediate value (Table 5). The HPA-axis is suggested to be hyperactive in major depressive disorder (MDD) (Bao et al., 2008) and chronic depression is proved to be associated with increased level of cortisol (Lindquist et al., 2008). Not only hypercortisolism but also reduced feedback inhibition of the hypothalamus-pituitary adrenocortical (HPA) system has been reported in patients with depression (van den Bos et al., 2006).

The current research data are in agreement with previous reports which found that serum cortisol is significantly higher in patients with depression compared to controls (Murck et al., 2005; Baghi et al., 2006; Häfner et al., 2012).

In conclusion, our findings support that ACE gene I/D polymorphism and high D allele frequency are associated with depression, also hypercortisolimia is significantly higher in individuals with major depression compared to control. ACE gene polymorphism might provide a common link between developing depressive episode and dysfunctional HPA-axis.

REFERENCES


