Association of angiotensin-converting enzyme gene polymorphism and clinical characteristics in Egyptian patients with major depressive disorder

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Objectives
Depression is one of the leading causes of disability and suicide worldwide. It has strong genetic etiopathogenesis, especially that related to angiotensin-converting enzyme (ACE) gene polymorphism. Therefore, this research tackled the relation between genetic variants of ACE polymorphism and symptomatology profile of major depression.
Patients and methods
A total of 42 patients diagnosed with depression matched with 39 controls underwent Structured Clinical Interview for the DSM-IV Axis I diagnosis clinician version, Structured Clinical Interview for the DSM-IV Axis I diagnosis-nonpatient edition, Hamilton Depression Rating Scale, real-time PCR for genotyping, and serum cortisol level assay.
Results
ACE I/D gene polymorphism was significantly higher in patients with major depression (45.2%) compared with the control group (25.6%). Patients with I/D polymorphism showed longer duration of illness; greater severity; higher number of episodes and rate of hospitalization; higher tendency to be prescribed serotonin/norepinephrine reuptake inhibitors and to receive electroconvulsive therapy; higher scores of core depressive symptoms, such as guilt feeling, inability to work, and suicidal ideation; and higher serum cortisol level than the other genotype groups.
Conclusion
Our findings support the notion that ACE I/D polymorphism affects major depression severity and symptomatology imprint.

Keywords:
angiotensin-converting enzyme, cortisol, gene, major depressive disorders, polymorphism

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Background

Major depressive disorder is a severe, highly prevalent disorder associated with serious consequences such as suicide, interpersonal relationship problems, and vocational dysfunction (Baghai *et al.*, 2006; Kato and Serretti, 2010). Strong evidence declares that major depression is substantially influenced by genetic factors including the risk of susceptibility to the disorder, response to specific treatments, and the likelihood of adverse reactions to antidepressant therapies (Sullivan *et al.*, 2000).

Genetic variants of angiotensin-converting enzyme (ACE) can contribute to disease susceptibility (Baghai *et al.*, 2006). ACE is the key enzyme of the renin–angiotensin system (RAS) in the central nervous system that catalyzes the formation of a cascade of biologically active angiotensin peptides and angiotensin II hormone that contribute to the stress process in the body. It modulates the dopaminergic activity in the brain, which has been linked to mood regulation and depression picture. Additionally, the possible mechanism of action of ACE genotype in major depression may also come across substance P, which is involved in stress and affective regulation (Kobori *et al.*, 2007).

Brain angiotensin II was also observed to be one of the principal determinants in the regulation of the hypothalamic–pituitary–adrenal (HPA) axis, which has been reported to be dysregulated in patients with major depressive disorder (Saavedra *et al.*, 2004). Furthermore, another stress-related hormone, cortisol, can be a marker of trait vulnerability to depression, which may represent an illness endophenotype (Ising and Holsboer, 2006).

Accordingly, the RAS overactivity is assumed to predispose an individual to depression (Saab *et al.*, 2007).

A study's replication samples confirm that variants of the ACE gene such as SNP rs4291 are associated with unipolar major depression and HPA-axis overactivity (Baghai *et al.*, 2006). Other studies have investigated other polymorphisms of ACE gene, including those in linkage disequilibrium with I/D polymorphism (Zintzaras *et al.*, 2008), suggesting that ACE I/D polymorphism may be associated with suicide (Sparks *et al.*, 2009), major depression (Baghai *et al.*, 2006; Ising and Holsboer, 2006), and response to antidepressant therapies and electroconvulsive therapy (ECT) (Stewart *et al.*, 2009).

Lately, the RAS was proposed to be implicated in depression; moreover, blocking this system with ACE inhibitors or with angiotensin II type 1 (T_1) receptor blockers could offer clinical benefits for treatment of depression (Saavedra and Pavel, 2005; Saab *et al.*, 2007; Vian *et al.*, 2017) through enhancing neurogenesis and reducing oxidative inflammation effect of stress (Vian *et al.*, 2017), as an Iranian study found that RAS gene polymorphism was significantly linked to cognitive and behavioral symptoms in major depression and discovered a higher level of serum ACE activity (P=0.0001) in depressed patients compared with a healthy control group (Firouzabadi *et al.*, 2012).

The present work aimed at studying the relation between ACE gene polymorphism and major depression clinical history and symptomatology phenotyping, and also investigating its effect on the HPA-axis overactivity manifested in the form of hypercortisolemia associated with depression, as cortisol hormone plays an important role in the stress, mood changes, and other symptoms found in depression, in addition to the association between hypertension, coronary heart disease, and depression. Moreover, the study hypothesizes a significant relation between different ACE genotypes and major depression clinical history and symptom profile, with a probable estimated high level of serum cortisol, adding to the suggestion of the effect of ACE genotypes and HPA hyperactivity on susceptibility to depression.

Patients and methods

The study was approved by the Ethics and Research Committee of both the Medical Research Centers (MRC) in which the laboratory work was performed at Okasha Institute of Psychiatry, Ain Shams University Hospitals. Moreover, the study was approved by the local committee for ethics of medical experiments on human participants. All recruited participants signed a written informed consent after being fully explained of the procedures. Those who withdrew their consent after recruitment were not included in this research.

Study design

This is a cross-sectional comparative study carried out by convenient sampling during a 6-month period.

Participants

The study included 81 participants divided into two groups: case and control. The sample size was calculated by the measuring the point estimate of the mean average number of cases agreeing to undergo genetic and blood tests that can give eligible statistical results.

The case group included 42 inpatient participants diagnosed with major depression by convenient sampling recruited from Okasha Institute of Psychiatry, Ain Shams University Hospitals, during the first week of their admission. Inclusion criteria of the recruited patients were both sexes, aged from 20 to 50 years, Egyptians only, and with major depression diagnosis based on the Structured Clinical Interview for the DSM-IV Axis I Diagnosis (SCID-I) (First *et al.*, 1996). The diagnosis was done by consensus of two experienced psychiatrists to eliminate bias and to exclude other axis I diagnoses. Moreover, patients with current or past axis I comorbidity, substance abuse, or medical illness were excluded.

The control group comprised 39 apparently healthy participants among visitors of patients in the medical ward, matched with the study group. They had no current, past, or family history of psychiatric disorders.

Psychometric tools used

SCID-I clinician version (First *et al.*, 1996): the Arabic version was used (El Missiry *et al.*, 2004), and it was validated through its use in numerous studies conducted in research centers in Egypt. This scale was used to confirm the diagnosis of major depressive disorder for the patient group according to the Diagnostic and Statistical Manual, fourth edition (DSM-IV). It includes 10 diagnostic modules: mood episodes, psychotic and associated symptoms, psychotic disorders, mood disorders, substance use disorders, anxiety disorders, somatoform disorders, eating disorders, adjustment

disorders, and an optional module that allows psychiatric diagnoses that may be of the interviewer's interest, such as the module on acute stress disorder and on minor depressive disorder. It takes 36 min to be answered.

SCID-I nonpatient edition (First *et al.*, 2002): this form was used for the control group participants who were not identified as psychiatric patients to exclude the possibility of having any axis I psychiatric morbidity according to the DSM-IV.

Hamilton Depression Rating Scale (Hamilton, 1960): it is a clinician-rated questionnaire to assess the change and severity of depressive symptoms. The original version consists of 17 domains (depressed mood, feelings of guilt, suicide, insomnia: early in the night, insomnia: middle of the night, insomnia: early hours of the morning, work and activities, retardation, agitation, anxiety psychic, anxiety somatic, somatic symptoms: gastrointestinal, general somatic symptoms, genital symptoms, hypochondriasis, loss of weight, and insight). Scoring from 0 to 7 denotes normal range or clinical remission, and scoring from 20 and higher is considered at least moderate severity. The scale takes 20 to 30 to be answered. The internal consistency was estimated to be 0.92, being higher within the structured interviews (Potts *et al.*, 1990), and the inter-rater reliability is 0.9 (Rehm and O'Hara, 1985).

Laboratory procedures

Selection of candidate genes

The candidate gene selected in this study was ACE gene.

Selection of primers sequence

(1) Forward: 5′-GAT GTG GCC ATC ACA TTG GTC AGA T-3′.
(2) Reverse: 5′-CTG GAG ACC ACT CCC ATC ATT TCT-3′.
(3) ref|NM_001178057.1|.
(4) GENE ID: 1636 ACE.

Table 1 Descriptive analysis of the sociodemographic data among the whole sample

	Case group (*N*=42)	Control group (*N*=39)
Age (years) (mean ±SD)	34.79±9.1	35.56±10.8
Sex		
Males	22	20
Females	20	19

The laboratory procedures started by collecting 8–10 ml of peripheral blood by venipuncture. The generic DNA was isolated using Qiagen-kit. The PCR was used for amplification of the ACE/ID polymorphism utilizing the primer sequence. The light cycler-based real-time PCR protocol was performed with a light Cycler 1.5 instrument (Roche Molecular Diagnostic, Penzherg, Germany). The PCR products were analyzed by electrophoresis on 2% agarose gel. According to the presence or absence of the insertion allele, the genotype of the participant could be classified as D/D homozygote for the deletion allele, I/I homozygote for the insertion allele, or I/D for heterozygote (Gard, 2010).

Estimation of serum cortisol (A.M.) was assessed in vitro with the IMMULITE 1000 analysis for quantitative measurement of cortisol (Siemens Healthcare, Diagnosis Diagnostic Products Ltd Laberis, GwyneddLL55 4EL, United Kingdom).

Statistical analysis

Data were tabulated and analyzed using the Statistical Package for Social Sciences (SPSS), version 13 (IBM SPSS Statistics for Windows, 2004) (IBM SPSS Statistics for Windows, Version 13.0. Chicago, IL: IBM Corp; 2004). Categorical outcome data were analyzed with the Pearson χ^2 test. Student *t* test was used to detect differences between two groups, and the one-way analysis of variance model was used to compare the parametric data among the three genotypes in relation to the clinical characteristics of depression. The criterion for significance is set at *P* value less than 0.05 for all tests.

Results

Demographic data of the whole sample

The studied sample comprised 81 participants divided into the following: 42 patients with major depressive disorder (22 males and 20 females), with mean age of 34.79±9.1 years, and 39 matched healthy controls (20 females and 19 males), with mean age of 35.56±10.8 years (Table 1).

Serum cortisol

In the case group, mean±SD plasma cortisol level was 17.33±5.2 (minimum–maximum=15.7–18.9) and for the control group was 10.68±5.3 (minimum–maximum=8.9–12.4), with a statistically significant difference (t=5.69) (P<0.001). It is clear that the highest level of serum cortisol was encountered in I/D carriers followed by D/D and I/I carriers (Table 2 and Fig. 1).

Clinical data in relation to angiotensin-converting enzyme genotypes in patients with major depression

Clinical characteristics of each genotype presented in Table 2 illustrated that patients with I/I polymorphism showed the earliest 'age of onset of depression' followed by the I/D, and then D/D carriers (P=0.001). The mean duration of illness was the shortest in the II genotype group opposite to the I/D group, which recorded the longest term duration of the illness among the three groups (P=0.001).

The mean number of episodes was higher in I/D carriers (3.8±1.6) than in both I/I and D/D carries (2.3±1.4). Table 2 illustrates that whether having psychotic symptoms or not was not associated with any specific type of ACE gene I/D polymorphism.

Patients' condition necessitating previous hospitalization was the highest among I/D group compared with D/D and I/I group (P=0.009). Patients who had never been hospitalized were more frequently encountered in D/D and I/I carriers than I/D carriers.

The severity of depression according to Hamilton Depression Scale proved that I/D type was more likely to have severe degree of depression, the D/D carriers showed more tendency to moderate degree, whereas mild degree of depression was among the I/I genotype (P=0.014).

In Table 3, it is obvious that neither using combined antidepressants with other medications nor antidepressants alone showed any statistical differences among the studied groups; however, the type of antidepressants was significantly related to the ACE genotype. I/D carriers received serotonin norepinephrine reuptke inhibitors (SNRIs) more frequently (68.4%) than did the other carriers (P=0.003) who were prescribed selective serotonin reuptake inhibitors (SSRIs) more frequently.

The use of antipsychotics or mood stabilizers showed no significant differences among the three genotypes.

In comparison with D/D and I/I group, I/D group had the most highly significant ECT use (P=0.001), which may be explained by the severity of illness that necessitates this kind of intervention.

Depression symptomatology profile by the HAMD in relation to angiotensin-converting enzyme genotypes

The most significant depressive symptoms related to ACE I/D polymorphism were the inability to work, guilt feeling, and suicidality domains. In addition, absolute total score of depression was significantly higher in the I/D group (Table 4).

Discussion

Angiotensin-converting enzyme gene polymorphism and potential vulnerability to depression

Although the relation between genetic polymorphisms of RAS and depression as a potential factor for developing the disorder has been reported in literature studies (Baghai *et al.*, 2006; Saab *et al.*, 2007; Sparks *et al.*, 2009; Stewart *et al.*, 2009), the associations between clinical variables of depression and genetic polymorphisms of RAS components have not been studied in Egyptian population up to date.

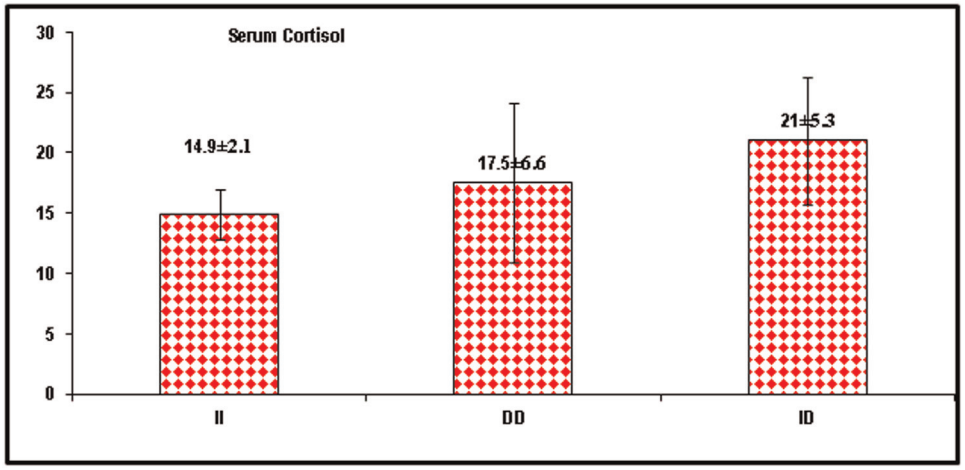
The present study found that the D allele was significantly prominent in the case group diagnosed with major depression compared with the healthy

Table 2 Clinical data of depression in relation to angiotensin-converting enzyme genotype

	II (n=12) (mean ±SD)	DD (n=11) (mean ±SD)	ID (n=19) (mean ±SD)	Test of significance ANOVA e	P value
Age of onset of depression (years)	23.7±5	34.6±8.1	29.7±6.1	f=8.57	0.001
Duration of depression (years)	3.2±1.1	4.8±3.0	8.2±4.0	f=9.98	<0.001
Number of episodes	2.3±1.4	2.9±1.2	3.8±1.6	f=4.32	0.02
Diagnosis [n (%)]				χ^2=1.60	0.448
Major depression with psychotic features	2 (16.7)	4 (36.4)	7 (36.8)		
Major depression without psychotic features	10 (83.3)	7 (63.6)	12 (63.2)		
Hamilton Depression Scale [n (%)]				χ^2=12.502	
Mild (8–13)	9 (75.0)	5 (45.5)	4 (21.1)		0.014
Moderate (14–18)	2 (16.7)	5 (45.5)	6 (31.6)		
Severe (19–1)	1 (8.3)	1 (9.1)	9 (47.4)		
Serum cortisol				f=6.438	0.004
	14.9±2.1	17.5±6.6	21.0±5.3		

ANOVA, analysis of variance.

Figure 1



Serum cortisol levels among the studied patients with major depression in relation to their ACE genotypes (f=6.438 and P=0.004*). ACE, angiotensin-converting enzyme.

Table 3 Medication and hospitalization variables in relation to angiotensin-converting enzyme genotypes among patients with depression

	II (*N*=12) [*n* (%)]	DD (*N*=11) [*n* (%)]	ID (*N*=19) [*n* (%)]	χ^2	*P* value
Medication				2.835	0.242
ADD+other medication	6 (50.0)	4 (36.4)	4 (21.1)		
ADD only	6 (50.0)	7 (63.6)	15 (78.9)		
Type of ADD				16.143	0.003
TCAs	3 (25.0)	0	2 (10.5)		
SSRIs	9 (75.0)	6 (54.5)	4 (21.1)		
SNRIs	0	5 (45.5)	13 (68.4)		
Type of APD				1.329	0.514
Oral typical antipsychotics	3 (100.0)	4 (80.0)	4 (66.7)		
Oral atypical antipsychotics	0	1 (20.0)	2 (33.3)		
Type of mood stabilizer				2.073	0.722
Lithium	1 (25.0)	1 (50.0)	3 (33.3)		
Antiepileptics	3 (75.0)	1 (50.0)	4 (44.4)		
Others	0	0	2 (22.2)		
Previous ECT courses	1 (8.3)	5 (45.5)	16 (84.2)	17.263	<0.001*
Previous hospitalization				17.2	0.009
Never	4 (33.3)	4 (36.4)	1 (5.3)		
Once	7 (58.3)	3 (27.3)	3 (15.8)		
Twice	1 (8.3)	2 (18.2)	6 (31.6)		
More than twice	0	2 (18.2)	9 (47.4)		

ECT, electroconvulsive therapy. *Means highly significant relation ($P<0.001$).

controls with significant frequency of both I/D and D/D polymorphism than I/I type. In agreement with our results, a number of investigators found that ACE gene I/D polymorphism was associated with vulnerability to depression (Arinami *et al.*, 1996; Bondy *et al.*, 2002; Baghai *et al.*, 2006; Ising and Holsboer, 2006; Saab *et al.*, 2007).

Similarly, a meta-analysis study provided a strong evidence of a positive association of the ACE I/D polymorphism and depression in white population but not in Asians, with a higher risk of depression among the D/D homozygote carriers compared with I/D and I/I carriers (Wu *et al.*, 2012; Mandelli and Serretti, 2013). In addition, a research revealed a significant relation between the depression domain of the positive and negative syndrome scale among the three ACE genotypes ($P<0.03$), with higher depression scores in patients with I/I than D/D genotypes ($P<0.05$) (Hui *et al.*, 2015).

On studying single nucleotide polymorphism of the ACE gene promoter in Thai patients, a research found that −240A allele was associated with decreased

Table 4 Depression symptomatology in relation to angiotensin-converting enzyme genotypes

	II	DD	ID	ANOVA	*P* value
DGS score: depression/guilt/suicidality				3.249	0.050
Mean (SD)	3.9 (2.2)	3.7 (3.2)	6.2 (3.3)		
95% CI	2.5–5.3	1.6–5.9	4.6–7.8		
I score: insomnia score				0.165	0.848
Mean (SD)	3.1 (0.3)	3.2 (0.7)	3.3 (1.1)		
95% CI	2.9–3.3	2.7–3.7	2.9–3.4		
W score: work				11.641	<0.001
Mean (SD)	1.6 (0.8)	2.8 (0.7)	3.0 (0.9)		
95% CI	1.1–2.1	2.3–3.3	2.6–3.4		
R score: retardation				3.490	0.04
Mean (SD)	2.3 (1.1)	1.3 (1.1)	1.7 (0.8)		
95% CI	1.6–3.0	0.5–2.0	1.3–2.1		
Ag score: agitation				0.230	0.795
Mean (SD)	0.3 (0.8)	0.5 (0.1)	0.4 (0.8)		
95% CI	0.16–0.8	0.19–0.9	0.02–0.8		
Anx score: anxiety				2.256	0.118
Mean (SD)	4.4 (2.2)	3.2 (1.3)	3.2 (1.5)		
95% CI	3.0–5.8	2.3–4.1	2.5–3.9		
S score: somatic				1.092	0.346
Mean (SD)	2.2 (0.6)	2.8 (1.1)	2.7 (1.1)		
95% CI	1.9–2.6	2.1–3.5	2.1–3.2		
Total HAMD score				5.745	0.007
Mean (SD)	18.4 (2.4)	19.4 (2.9)	22.1 (3.6)		
95% CI	16.9–19.9	17.4–21.3	20.3–23.9		
Depression severity	Moderate	Moderate to severe	Severe		

Severity of depression according to HAMD=(0–7 no depression, 8–13 mild depression, 14–18 moderate depression, 19–22 severe depression, >23 very severe depression). ANOVA, analysis of variance; CI, confidence interval; HAMD, hamilton depression rating scale; SNRIs: serotonin norepinephrine reuptke inhibitors; SSRIs, selective serotonin reuptake inhibitors.

vulnerability to major depression, whereas -240T/-93T allele was linked to increased susceptibility to contract major depression (Angunsri *et al.*, 2009). There was also an evident association between all seven single nucleotide polymorphisms of the ACE gene and late-onset depression in elderly persons having no past history of depression (P=0.005–0.0004), with an increase in serum cortisol in stressful conditions (Ancelin *et al.*, 2013). On the contrary, a study suggested that ACE I/D association with depression was different in Chinese adolescents, where D allele was associated with reduced depression prevalence and severity (Fan *et al.*, 2017), whereas other studies have failed to replicate the association between ACE genotype and depression (Furlong *et al.*, 2000; Pauls *et al.*, 2000; Hong *et al.*, 2002; Segman *et al.*, 2002; Baghai *et al.*, 2004; Bondy *et al.*, 2005; Mendlewicz *et al.*, 2005; InanIr *et al.*, 2016). Inconsistency in such findings can be attributed to differences in ethnic origins of the population, sample sizes, sample selection (e.g. age of onset, sex, or diagnostic criteria), and genotyping methodology (López-León *et al.*, 2006). Furthermore, epigenetic mechanisms appear to be important in altering ACE gene and its expression (Zill *et al.*, 2012).

Hypercortisolemia in patients with major depression disorder

The current study proved that hypercortisolemia was significantly higher in individuals with major depression than the control group, highlighting the link between depression and HPA-axis dysfunction in stress.

Moreover, the HPA-axis overactivation was reported in major depressive disorder (Bao *et al.*, 2008); this was explained by a review article on the risk factors of HPA-axis activation in depression like genetic polymorphisms, development problems, and environmental factors, where there are neurophysiological changes such as reduced GABAergic input, increased glutaminergic input from extra hypothalamic sites, and reduced inhibition by the suprachiasmatic nucleus) besides alterations in the monoamine or neuropeptide input, and a deficient cortisol feedback effect, leading to long-term increased CRH and cortisol levels (Bao and Swaab, 2019). Not only hypercortisolemia but also reduced feedback inhibition of the HPA system has been reported in patients with depression (van den Bos *et al.*, 2006). Previous studies supported the

interrelationship between ACE I/D polymorphism and the HPA-axis dysfunction and increased cortisol response to challenge in depressed patients (Murck *et al.*, 2005; Baghai *et al.*, 2006; Häfner *et al.*, 2012).

Sociodemographic variables and angiotensin-converting enzyme genotypes among patients with major depression

Similar to our findings, studies found no significant differences among the three groups of ACE gene polymorphism regarding the sociodemographic parameters (Bondy *et al.*, 2002; Hong *et al.*, 2002). However, a study proposed that ACE I/D gene polymorphism can differently affect the RAS function with both sexes (Hishimoto *et al.*, 2006). Some researchers have shown that ACE gene polymorphism found in major depression was expressed only in females (Baghai *et al.*, 2004). Thus, the possible mechanism of sex difference is that gonadal hormones affect ACE activity, estrogen by reducing and testosterone by enhancing ACE activity (Furlong *et al.*, 2000; Pauls *et al.*, 2000; Segman *et al.*, 2002). This point may need further investigation.

The relation between clinical history of depression and angiotensin-converting enzyme genotype

Regarding the age of onset of depression, in agreement with our results, a study reported that younger age of onset of depression was associated with I/D genotype in the whole patient group, but its finding was partly sex dependent. In male patients, I allele carried a higher risk of earlier age of onset depression, whereas in female patients, the higher risk was seen only in the heterozygous I/D allele carriers (Stewart *et al.*, 2009). On the contrary, no demonstrated association was found between the age of onset of major depression and the ACE gene I/D polymorphism (Hong *et al.*, 2002).

Regarding treatment response to depression, our results declared that the I/D genotype group had the highest rate of the SNRI use as well as the highest ECT use, which may be explained by the severity of illness and the increasing need for hospitalization in our sample, as the I/D genotype group was more likely to have severe degree of depression than the I/I and D/D carriers, who showed mild and moderate depression, respectively.

RAS polymorphisms were associated not only with the etiology of depression but also with the response to antidepressant medication, showing that the D allele of the ACE gene was associated with enhanced response to antidepressant drug. Furthermore, it seems reasonable to suggest that the association of ACE genetic variants and therapeutic antidepressant response may be limited to particular types of antidepressants, and this phenomenon explains why various studies found that patients with major depression with I/D genotype used and responded to SNRI medications, whereas the I/I and D/D used the SSRIs group of treatments more frequently (Baghai *et al.*, 2004; Bondy *et al.*, 2005; Baghai *et al.*, 2006). On the contrary, other studies suggested that ACE I/D polymorphism did not affect antidepressant responses of patients with major depressive disorder (Furlong *et al.*, 2000; Pauls *et al.*, 2000; Hong *et al.*, 2002) or treatment response to ECT (Stewart *et al.*, 2009).

Conclusion

ACE I/D gene polymorphism is significantly associated with severity of depressive symptoms, number of episodes and hospitalization, response to psychotropic medications, receiving ECT, and prevailing symptom parameters like inability to work, guilt feeling, and suicidality, suggesting the prominent role of angiotensin system in shaping the clinical profile of depression.

Strength and limitation

The strength of this study is being the second investigation of the RAS polymorphism in depression. Our study is the first Egyptian patient research pointing to the association between ACE gene polymorphism and the sociodemographic and symptomatology variables of major depression together with hypercortisolemia. Some limitations should be addressed. First, only one polymorphism was investigated in our study, which did not fully tag all the genetic variants in ACE gene. In the future, additional gene variants that influence ACE expression and activation should also be genotyped to assess other contributing factors. Second, the sample size of this study was relatively small owing to the sophistication of the genetic and laboratory investigations demanded from the participants, so future replication studies using a larger sample size are surely needed for the generalization of the results.

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