The Association of Angiotensin-1 Converting Enzyme (ACE) Serum Level and (I/D) Polymorphism in Egyptian Preeclamptic Women

Maha A. El Bassuoni MD1, Hanna Younes MD2, Randa M. Talaat MD3, Hanaa Amer MD2, Enas S. Eissa MD1 and Riham G. Mahfouz MD4

1Clinical Pathology Dept., Faculty of Medicine, Menoufiya University, 2 Obstetric and Gynecology Dept., Faculty of Medicine, Al Azhar University, 3 Microbiology Dept. Genetic Engineering and Biotechnology Sadat Institute, Menoufiya Universitym 4Biochemistry Dept. Faculty of Medicine, Menoufiya University

ABSTRACT

Preeclampsia (PE) a major cause of maternal and neonatal mortality and morbidity worldwide in which hypertensive disorders during pregnancy account for 25.7% of maternal deaths. Both maternal and fetal genetic factors may predispose towards pre- eclamptic pregnancy, especially severe forms. However, preeclampsia is thought to be the result of the interplay between important genetic components and environmental influences; still, factors and mechanisms that lead to preeclampsia remain mysterious. Insertion/deletion (I/D) polymorphism of ACE gene has attracted significant attention and has been extensively investigated with its serum activity in a spectrum of cardiovascular phenotypes. Aim to study the potential association of I/D polymorphism of ACE gene in PE Egyptian women that gets us closer to understanding the disease. Patients and Methods: One hundred hypertensive and age-matched normotensive primigravidae were recruited from Menoufiya university Hospital. Routine investigations were done for PE diagnosis. DNA was extracted from whole blood of patients and healthy controls. All samples were genotyped for ACE I/D polymorphism according to Rigat et al. using amplification and PCR of known allelic variants. ACE genotype was identified and followed by serum concentration of ACE activity for both groups. Results: ACE DD genotype was found in 60% of PE patients while 34% of normotensive subjects (P≤ 0.005). The odds ratio (95% CI) for developing hypertension in cases with DD genotype was 2.91 (1.29-6.57), while with II genotype was 0.18 (0.02-1.63). DD genotype revealed significantly more prevalent among cases than controls (P≤0.005), where it was 2.9 folds higher among cases than controls. The incidence of D allele of ACE gene was higher in hypertensive (0.98) than in normotensive (0.90, P > 0.05), although D allele was higher among cases than controls, but it did not show significance (P > 0.05). High significance was revealed when comparing the mean total ACE activity in the hypertensive patients (32.74 IU/l) and normotensive subjects (28.06 IU/l) (P <0.001). The ACE activity in cases and controls carrying DD allele differed significantly (P<0.001). In contrast the other ACE genotype ID and II did not show significance between cases and controls. Conclusion: These findings might bear implications for precise management of pregnancy in high-risk DD genotype women. Further large scale evaluation was required to provide added marker for risk assessment for PE patients.

INTRODUCTION

Preeclampsia (PE) is a maternal disease of pregnancy associated with increased blood pressure and proteinuria after 20 weeks of gestation (Diaz, 2006). It is a major cause of maternal and neonatal mortality and morbidity worldwide and has account for 25.7% of maternal deaths during pregnancy (WHO, 1988). The risk for an adverse pregnancy outcome in women who have previously had preeclampsia is markedly higher (from 20% to 40%) in comparison to women with history of normal pregnancy, but the mechanisms involved have not yet been identified (Mello, 2003). Previous proposals found that there are both maternal and fetal genetic factors that may predispose towards pre-eclamptic pregnancy, especially severe forms of preeclampsia. Maternal family history has the greatest influence on PE risk, but paternal family history of the condition can also contribute (Skjærven et al., 2005)

The causes of preeclampsia are not well understood, but several factors are known to contribute to the risk. Factors include diabetes, high blood pressure prior to pregnancy, obesity, and first pregnancy, also the possibility in part, a genetic basis. The condition is more likely among women whose relatives have also had it (Norma, 2006).
No definite genetic cause has yet been confirmed. However, PE is thought to be the result of the interplay between important genetic components and environmental influences; still, factors and mechanisms that lead to preeclampsia remain elusive (Ray et al., 2005). As a result, there is a lack of effective preventive interventions (et al, 2002).

Angiotensin-I Converting enzyme (ACE), which codes for the ACE gene, has been linked with preeclampsia in a number of different studies. The protein encoded by ACE is involved in controlling blood pressure and the balance of fluid and salts in blood (Rigat et al., 1990). Over the past decade, the insertion/deletion (I/D) polymorphism of a 287-bp in intron 16 of (ACE) gene has attracted significant attention and has been extensively investigated in a spectrum of cardiovascular phenotypes, because of its correlation with serum ACE activity (Niu et al, 2002).

Inappropriate activation of the renin–angiotensin system may play a part in the development of many cardiovascular disorders, including preeclampsia (Agerholm-Larsen et al., 1990). A common insertion/deletion polymorphism within the angiotensin-I converting enzyme gene (ACE-I/D) has been reliably associated with substantial differences in the plasma and tissue angiotensin-converting enzyme (ACE) activity in a codominant (additive) fashion not only in persons of European descent, but also in other populations such as Hispanics (Kammerer et al., 2005).

Previous experimental studies (Langer et al., 1998) suggested that the "physiological remodeling" of spiral arteries throughout pregnancy is mediated by the renin-angiotensin system (RAS), which is one of the main factors regulating blood pressure, fluid and electrolyte balance (Ito. et al., 1992). Throughout normal pregnancy, the RAS is stimulated; plasma renin activity, angiotensinogen, angiotensin II, and aldosterone levels are all increased (Ward. et al., 1993) At the same time, pregnancy induces refractoriness to the pressor effects of angiotensin II (Morgan et al., 1999). Moreover, recent data have shown that upregulation of angiotensin II type 1 (AT1) receptor subtype in the syncytiotrophoblasts could play a patho-physiological role patients with PE (Jackson et al., 2000). Moreover, PE is characterized by the loss of this physiological refractoriness to angiotensin II (Morgan et al., 1999 and Miller et al., 1988).

Plasma and tissue ACE activities are under genetic control. Increased ACE activity due to the deletion polymorphism of the ACE gene is associated with diseases that exhibit endothelial disturbance. Studies in various ethnic group have shown contradictory evidence on the association of ACE insertion/deletion (I/D) polymorphism with preeclampsia PE (El Shafei, 2007).

The researchers theorize that Ang II may restrict the fetal vessels that lie within the chorionic villi, which not only raises blood pressure, but also lowers oxygen and nutrient flow to the baby and may result in lower birth weight and other complications of preeclampsia (Anton, 2009).

ACE inhibitor drugs are currently used to lower Ang II in non-pregnant women with hypertension, but these drugs cannot be given to pregnant women.

Other therapies aimed at regulating blood pressure might be beneficial if they target the chorionic villi rather than the system as a whole. The current work is being done to determine if reductions in compatible factors that cause the placenta's blood supply to develop may also be regulated by the increase in Ang II (Anton, 2009).

Aim of the study

To investigate the hypothesis that preeclampsia is associated both ACE gene insertion-deletion polymorphism (I/D) and serum ACE activity and analyzing the relationship between preeclampsia and ACE genotype.

PATIENTS & METHODS

One hundred hypertensive and age-matched normotensive primigravidae were recruited from Menoufiya university Hospital. These subjects were followed up to delivery and perinatal outcome noted. The Inclusion Criteria were: Pregnant women with preeclampsia between 20-32 weeks gestation. The criteria used for the diagnosis of PE were in accordance with the guidelines of the American College of Obstetrics and Gynecology (ACOG). Preeclampsia is defined as a maternal blood pressure of >140/90 mmHg on two readings at least 6 hours apart with proteinuria of >300 mg/24 hours. The ACE genotype was identified and serum ACE activity was measured. Genotyping was performed for all the studied cases taking into account some well-known contributing factors in PE such as maternal age, primiparity, gestational age and proteinuria. All
these variables were significantly associated with PE. All the pregnancies with known maternal vascular diseases, e.g. essential hypertension, diabetes and systemic lupus erythematosis or congenital abnormalities were excluded. None of the women had vaginal bleeding or labor pains at the day of blood sampling. The control groups were gestational age-matched healthy pregnant women and had uncomplicated pregnancies.

2-Methods
A. Molecular Diagnosis for genotypes of ACE gene

Peripheral venous blood samples were collected from the antecubital vein in Vacutainer tubes containing 0.129 mol/L sodium citrate, the final blood/anticoagulant ratio being 9:1. Genomic DNA was extracted from whole blood leukocytes using a QIAmp Blood Kit (QIAGEN, Hilden, Germany). All samples were genotyped for ACE I/D polymorphisms using amplification after PCR of known allelic variants. I/D polymorphism in intron 16th of ACE gene was determined according to the method of Rigat et al. (Lindpaintner, et al., 1995). DNA was amplified at an annealing temperature of 60°C in the presence of 5% dimethylsulfoxide (DMSO) to reduce the incidence of mistyping ID as DD (Schimidt, et al., 1995). Moreover, each DD genotype was subjected to a second PCR amplification without 5% DMSO at an annealing temperature of 67°C and by using a primer pair that recognizes the insertion-specific sequence. These modifications were made to reduce underestimation of heterozygotes (Forgarty et al., 1996). Positive controls consisting of DNA samples from subjects of known genotypes (from previous work) and a negative control of complete PCR reaction mix without DNA were included in each PCR reaction. PCR amplification yielded a fragment with (I allele) or without the insertion allele (D allele) of ~490 and 190bp, respectively. All PCR products were resolved on an agarose gel by electrophoresis, these alleles were scored accordingly.

Lane 1: Molecular weight marker (50-bp ladder); lane 2: DD homozygote; lane 3: ID heterozygote with presence of both 190-bp D fragment and 490-bp I fragment; lane 4: II homozygote.

Determination of ACE genotypes by PCR amplification on ethidium bromide-stained agarose gel. Three patterns of PCR products (II, ID, and DD) for ACE gene I/D polymorphism are shown. Arrows on right indicate molecular weights of PCR products (allele I and D correspond to 490- and 190-bp products, respectively).

B. Serum concentration of ACE activity

SPECIMEN COLLECTION AND STORAGE

Since EDTA inhibits ACE activity, serum specimen should be used for the determination of ACE activity. Sufficient blood was collected (at least 0.5 ml) by venipuncture into an appropriate tube without anticoagulant. centrifuge at 4°C and 1000 x g, freeze the specimen at ~20°C if not assayed within 5 days. ACE activity in sterile serum is stable for up to 30 days at 2-8°C and 6 months at ~20°C. To avoid lipemic sera, blood samples should be taken from fasting patients. Due to interference with the photometric determination.
Icteric or hemolytic sera cannot be used for ACE activity determination.

**PRINCIPLE OF THE ASSAY**

ACE catalyses the conversion of angiotensin I to angiotensin II. The kinetic of this cleavage reaction is measured by recording the decrease in absorbance at 340 nm. The ACE kinetic method is standardized with the BÜHLMANN ACE colorimetric kit (order code: 01-KK-ACE) according to the reference method described (Ronca-Testoni, 1983 and Bénéteau and Baudin et al. 1986).

**CALCULATION**

Calculate the corresponding enzyme activity (Ex) of each unknown sample by dividing the difference in absorbance of the individual sample (ΔAx) through the mean of the absorbance difference of the calibrator vial (ΔAc) and multiply the results by the enzyme activity (Ec).

**RESULTS**

ACE DD genotype was found in 60% of subjects with PE but only in 34% of normotensive subjects (P ≤ 0.005). The odds ratio (95% CI) for developing hypertension in subjects with DD genotype was 2.91 (1.29-6.57), while the odds ratio for developing hypertension in subjects with II genotype was 0.18 (0.02-1.63). This shows that DD was significantly more prevalent among cases than controls (P ≤ 0.005), where it was 2.9 folds higher among cases than controls. The incidence of D allele of ACE gene was higher in hypertensive subjects (0.98) than in normotensive subjects (0.90, P > 0.05), and although D allele was higher among cases than controls, but there was no significant difference between ACE DD genotype in groups both (P > 0.05). However, the ACE genotype was similar in different categories of PIH (Pre-eclampsia induced hypertension) and did not affect the perinatal outcome.

The mean total ACE activity in the hypertensive subjects was (32.74 IU/l) and in normotensive subjects (28.06 IU/l) were of high significance (P <0.001). The ACE activity in cases and controls carrying DD allele differed significantly (P<0.001), in contrast of the other ACE genotype ID and II which did not show significant difference in between both cases and controls groups (P > 0.05).

| Table 1: The clinical data of PE patients and gestational age-matched controls |
|-----------------------------------------------|-----------------|---------------|--------|---------|
| Variables                                    | CASES (n=50)    | CONTROLS (n=50) | t      | P value |
| Maternal age                                 | 28.73±5.759     | 31.5±3.5144    | -1.26245 | >0.05   |
| Systolic blood pressure                      | 175.38±25.937   | 113.5±13.753   | 7.361628 | <0.001  |
| Diastolic blood pressure                     | 10.73±12.659    | 77.5±8.959     | 6.6338  | <0.001  |

| Table 2: ACE Genotype Frequencies in Patients and Controls |
|----------------------------------------------------------|----------------|----------------|------|----------|
| ACE genotype                                             | CASES (n=50)   | CONTROLS (n=50) | X²   | P value  | Odds ratio (95% CI) |
| DD                                                       | 30             | 17             | 6.78 | <0.005   | 2.91 (1.29-6.57)    |
| ID                                                       | 19             | 28             | 3.25 | >0.05    | 0.48 (0.22-1.06)    |
| II                                                       | 1              | 5              | 2.84 | >0.05    | 0.18 (0.02-1.63)    |

| Table 3: Incidence of D allele among cases and controls. |
|--------------------------------------------------------|----------------|----------------|--------|
| Group                                                  | CASES (n=50)   | CONTROLS (n=50) | Z test | P value |
| D allele                                               | 49             | 98             | 90     | >0.05   |

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Table 4: Total ACE activity Units/L (mean value) in different ACE Genotype Frequencies of Patients and Controls

<table>
<thead>
<tr>
<th>Studied Groups</th>
<th>Mean Total ACE activity Units/L</th>
<th>Mean ACE activity IU / L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypertensive</td>
<td>32.74 ± 8.19</td>
<td>21.3 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>36.41 ± 7.58</td>
<td>39.9 ± 3.14</td>
</tr>
<tr>
<td>Controls</td>
<td>28.06 ± 5.34</td>
<td>19.61 ± 3.97</td>
</tr>
<tr>
<td></td>
<td>34.27 ± 6.4</td>
<td>23.46 ± 4.28</td>
</tr>
<tr>
<td>P value`</td>
<td>&lt;0.001</td>
<td>&gt;0.05</td>
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</tbody>
</table>

Statistical Analyses

All data are presented as mean ± SD or percent. Mean \( \chi^2 \) tests were used for categoric variables. Gene polymorphisms ACE DD vs. ID/II; in addition, two-way interactions of these latter variables were also tested. All statistical analyses were performed using statistical packages (Instat v.2; Graph Software for Science, San Diego, CA USA).

DISCUSSION

Plasma and tissue ACE (angiotensin converting enzyme) activities are under genetic control. Increased ACE activity due to the deletion polymorphism of the ACE gene is associated with diseases that exhibit endothelial disturbance. Studies in various ethnic group have shown contradictory evidence on the association of ACE Insertion/deletion (I/D) polymorphism with preeclampsia (PE) (El Shafei, 2007).

In the present study, ACE DD genotype was found in 60% of subjects with PE but only in 34% of normotensive subjects (P=0.005). The odds ratio for developing hypertension in subjects with DD genotype was 2.91 (1.29-6.57 - 95% CI ), while the odds ratio for developing hypertension in subjects with II genotype was 0.18 (0.02-1.63).This shows that DD was significantly more prevalent among cases than controls (P=0.005), where it was 2.9 folds higher among cases than controls.

Carrying the DD genotype may have some influence on the pathogenesis of preeclampsia, perhaps through effects on placental hypoxia or the interaction of hypertensive disease and atherosclerosis, although this influence may not be strong. Additional studies using a larger number of patients and analyses that include other genetic and environmental factors will be necessary to confirm these results (Kobashi et al., 2005).

In line with these results were Mello et al., 2003 who found a difference in genotype distribution (P<0.0002) and allele frequency (P<0.0001) between women with and those without preeclampsia recurrence and fetal growth restriction as well as an association (P<0.0007) between DD genotype and risk of recurrent preeclampsia or fetal growth restriction. His study showed an association between ACE DD genotype and a recurrent pregnancy negative outcome after PE in a previous pregnancy indicating that DD genotype is not associated with risk of PE in the first pregnancy. Finding an association between the DD genotype and a negative outcome in the successive pregnancy suggests that the DD genotype can be related to PE and FGR (foetal growth retardation only).

Nevertheless, Mando et al, 2009 set up that the distribution of ACE genotypes was different in PE. This was confirmed in mild PE, whereas no significance was found in severe PE. This could suggest that different factors may lead to mild and severe PE, with ACE polymorphism playing a more important role in the mild form. Gürdöl et al., 2003 found an association between the genotype II and low ACE activity in preeclamptic women and an association between D allele frequency and preeclampsia in Turkish women. Pregnancy alone did not have an effect on the ACE activity.

They explained by that uterine artery resistance indexes were significantly lower in II, higher in DD, and intermediate in ID genotype carriers, whereas the Umbilical artery pulsatility index values were significantly higher in the DD group in comparison to ID and II genotypes, their study shows that the ACE I/D polymorphism affects uteroplacental and umbilical flows and the recurrence of an adverse pregnancy outcome in women with history of preeclampsia.

Choi et al. 2004 established that the frequency of DD genotype was significantly greater in preeclampsia (0.36) than in controls (0.14) (p<0.05). The frequency of D allele was 0.55 in preeclampsia and 0.40 in controls (p<0.05). However, there were no differences in
the onset of preeclampsia and pregnancy outcomes according to the ACE genotypes.

Previous studies have documented that the D/D polymorphism of the ACE gene is associated with increased plasma ACE concentrations, cardiac diseases such as myocardial infarction and left ventricular hypertrophy, and progression of diabetic nephropathy. These associations, however, have not been consistently replicated.

Norma et al., 2006 found that in an additive model (per-D-allele) revealed a null association between the ACE-I/D variant and preeclampsia risk (crude OR = 0.95 [95% CI, 0.81-1.10]) in a case-control study. They concluded that it is highly likely that the observed small nominal increase in risk of preeclampsia associated with the ACE D-allele is due to small-study bias, similar to that observed in cardiovascular disease. Reliable assessment of the origins of preeclampsia using a genetic approach may require the establishment of a collaborating consortium to generate a dataset of adequate size.

In addition, Zhang et al. 2006 study did not detect any association between the ACE D/D polymorphism and renal functional deterioration in univariate analysis. Several studies have now examined the association of the ACE D/D polymorphism with PE. We believe that the discrepancies in the above studies and the present work univariate and multivariate analyses may be in part related to sample size. Furthermore, Li et al., 2007 found no association of the ACE gene polymorphisms with preeclampsia. However, ACE gene I/D polymorphisms were associated with the severe proteinuria and the increase in serum uric acid, urea, and creatinine and renal dysfunction seen in preeclampsia. They inferred that preeclampsia patients carrying the D allele may be susceptible to renal dysfunction.

In contrast to the present finding, Young et al., 2004 indicated that the ACE polymorphism play no significant role in preeclampsia observed in Korean women. In addition, neither the I-D genotype distributions nor the allele frequencies differed significantly between pre-eclamptic and normotensive pregnancies in maternal or fetal samples in a study conducted by Morgan et al., 1999. In his study, the odds ratio for preeclampsia in women with the DD genotype, compared with the ID and II genotype, was 1.09 (95% confidence interval 0.55-2.16). The odds ratio associated with the DD genotype in the fetus was 1.14 (0.56-2.32) he concluded that his study has found no evidence that the insertion-deletion polymorphism in the angiotensin-converting enzyme gene is associated with preeclampsia.

In an Egyptian study, El Shafei et al., 2007 showed that the ACE II genotype may have a predisposing effects on preeclampsia especially in younger women and/or in women early pregnancy. The ACE DD genotype didn’t show any association with preeclampsia. They concluded that the genetic susceptibility in preeclampsia needs more studies about the role of other candidate genes in addition to the ACE gene.

CONCLUSIONS

ACE gene polymorphism in PE women included: insertion homozygote II, deletion homozygote (DD) and insertion/deletion heterozygote (ID) was higher than in controls. These findings suggested that ACE I/D polymorphism maybe involved in maternal uteroplacental modulation and fetal umbilical flows which might bear implications for more accurate management of pregnancy in high-risk DD genotype women. The clinical relevance of these results deserves to be further evaluated in larger studies to provide an additional marker for risk assessment of patients with PE history.

REFERENCES

5. Zaman MA, Oparil S, Callhoun DA (2002): Drugs targeting the renin-
angiotensinogen is associated with diabetic nephropathy in IDDM. *Diabetes*. 45: 1204-1208


29. Serrano, Norma C (NC); Díaz, Luis A (LA); Páez, Maria C (MC); Mesa, Clara M (CM); Cifuentes, Rodrigo (R); Monterrosa, Alvaro (A); González, Adriana (A); Smee, Liam (L); Hingorani, Aroon D (AD); Casas, Juan P (JP); (2006): Angiotensin-Converting Enzyme I/D Polymorphism and Preeclampsia Risk: Evidence of Small-Study Bias, PLoS Med, vol 3 (issue 12)

30. Li, Hua; Ma, Yuyan; Fu, Qingzhao; Wang, Leiyi (2007): Angiotensin-Converting Enzyme Insertion/Deletion (ACE I/D) and Angiotensin II Type 1 Receptor (AT1R) Gene Polymorphism and Its Association with Preeclampsia in Chinese Women. Hypertension in Pregnancy, Volume 26, Number 3, July , pp. 293-301(9)