Bulletin of High Institute of Public Health
Vol. 37 No. 3 [2007]

Angiotensinogen Gene (M235T) Variant and Pre-Eclampsia in Egyptian Pregnant Women

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Abstract: Association between the angiotensinogen gene (M235T) and pre-eclampsia has been confirmed in recent studies. Pre-eclampsia is a complication of pregnancy characterized by increased vascular resistance, higher blood pressure, proteinuria and oedema that appear in the second and third trimester of pregnancy. This study aimed at investigating the relationship between M235T gene polymorphism and pregnant women with different forms of pre-eclampsia. One hundred and fifteen pre-eclamptic women and 100 normal control group were recruited and evaluated for the frequency of M235T mutation using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). A positive association was found between maternal age over 35 years (OR= 6.67; CI: 2.09-23.59), previous family history of hypertension (OR= 3.01; CI: 1.18-7.66), previous pre-eclampsia (OR= 7.44; CI: 2.47-22.42), history of reproductive losses (OR = 53.98; CI: 3.23-90.88), fetal anomalies (OR= 8.4; CI: 1.06-180.33), and pre-eclampsia. The frequency of heterozygous carriers of M235T mutation in pre-eclampsia (19.1%) was higher than that in control (14%) but the difference was statistically non-significant. Also, the frequency of M235T mutation was higher in mild pre-eclampsia women (63.6%) compared to women with severe pre-eclampsia (36.4%), however this was statistically non-significant. This study revealed that the frequency of M235T mutation was higher within women with mild pre-eclampsia.

INTRODUCTION

Pre-eclampsia is a disorder associated with pregnancy that consists of hypertension and proteinuria and manifests often after 20th weeks of gestation.(1) It is a heterogenous disorder which complicates 5-7% of all pregnancy usually primigravida and in women with preexisting hypertension or vascular diseases.(2)

Although the etiology of pre-eclampsia remains unclear, it has been suggested that abnormal placentation and endothelial cell dysfunction are key features of pathogenesis of pre-

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eclampsia.[3] It was observed that pre-eclampsia is associated with increased risks of placental abruptia, acute renal failure, cerebrovascular and cardiovascular complications, disseminated intravascular coagulation, and maternal death.[4]

The fetus may be affected by growth retardation and about 20% cases die either in utero or as a result of prematurity.[5]

Pre-eclampsia can be classified as mild or severe. Severe pre-eclampsia is characterized by hypertension greater than (160/110 mmHg) and the presence of significant proteinuria. It is sometimes associated with oliguria, cerebral or visual disturbances, pulmonary oedema, or cyanosis. In mild pre-eclampsia, hypertension and proteinuria are present, but not to these extreme levels, and the patients has no evidence of other organ dysfunctions.[6]

Pre-eclampsia appears to have a genetic component and pedigree analysis suggests that it may be inherited as a single-gene disorder.[7]

Candidate genes that determine blood pressure variation include those whose products have a direct role in vascular biology, such as components of the renin-angiotensin system.[8] The importance of the rennin-angiotensin system for maintenance of normal cardiovascular homeostasis and its participation in the pathophysiology of pre-eclampsia is well established. The plasma-renin activity is high and the level of angiotensinogen may be also increased, suggesting a possible role of angiotensiogen in pre-eclampsia.[9]

The M235T angiotensinogen gene

mutation is a single base pair substitution of thymine (T) with cytosine (c) at nucleotide 704 (T704 → c) in exon 2 of the angiotensinogen gene (chromosome 1q42-43), leading the substitution of methionine with threonine at amino acid position 235 in the pre-proangiotensinogen molecule (M235T). T235 allele represents the mutant allele and M235 allele represents the wild type.[10]

The aim of this study was to investigate the relationship between M235T gene polymorphism and pregnant women with different forms of pre-eclampsia.

MATERIAL and METHODS

A total of 115 women with pre-eclampsia were recruited prospectively (at diagnosis) from El-Shatby Maternity University Hospital, over the preceding 24 months. All gave written consent to participate and met the following criteria of pre-eclampsia: they were hypertensive with a blood pressure greater than 140/90 mmHg with proteinuria (> 300 mg/24 hours) occurring after 20 weeks of gestation. All of these features were resolved by 3 months postpartum and no participants had a multiple birth pregnancy, concurrent diabetes, renal disease, or essential hypertension. These patients were divided on the basis of clinical criteria of pre-eclampsia into mild 81 (70.4%) and severe 34 (29.6%) phenotypes.

An age-matched control group of 100 normotensive (sealed blood pressure higher than 140/90 mmHg) pregnant women were recruited over the same period from El-Shatby Maternity University Hospital. These women had pregnancies uncomplicated by pre-eclampsia.

All the studied patients were to the following:

1. **Detailed history including:** medical history, obstetric history, family history of similar condition, reproductive losses, (repeated abortion, intra-uterine fetal deaths, and still births), congenital anomalies, and malignancy.
2. **General examination including:** blood pressure and lower limb oedema.
3. **Dip stick urine analysis testing for protein.**
4. **Laboratory investigations including:** CBC, urea, creatinine, fasting blood sugar, serum uric acid and liver enzymes.
5. **Ultrasonographic examination of the pelvis .**
6. **Molecular analysis:**

a- Genomic DNA was extracted from peripheral EDTA-treated blood cells by DNA salting out as described by Sambrook *et al.,* (1999)[11] with some modifications.

b- Detection of M235T angiotensinogen gene polymorphism:

The M235T polymorphism in exon 2 of the angiotensinogen was examined by using polymerase chain reaction, restriction fragment length polymorphism method (PCR-RFLP). The M235T allele creates a restriction site for *Tth 1111* enzyme that removes 165 base pairs of the PCR products. Fragments were finally size fractionated on agarose gel to allow allele assignment.

The detection for this mutation was done as follows:

1-DNA amplification

In order to amplify the 165 bp fragment that contains the M235T polymorphism, the first set of second exon primers described by Russ *et al*.,[12] and Jeunemaitre *et al.,*[13] were

used. The forward primer was:

$5'$CAGGGTGCTGTCCACACTGGACC CC-$3'$,

and the reverse primer was:

$5'$CCGTTTGTGCAGGGCCTGGCTCT CT-$3'$.

Genomic DNA (500 ng) was amplified in a reaction containing 0.2 µM of each primer, 50 mM KCl, 1.5 mM $MgCl_2$, 10m MTRIS-HCL (pH. 9.0 at 25°C), 200 µM of each deoxynucleotide triphosphate (dNTPs) and 2U of Taq polymerase (promega) in a volume of 100 µL. An initial denaturation for 10 minutes at 95°C was followed by 35 cycles of 1 minute at 94°C, 1 minute at 59°C, and 1 minute 30 seconds at 72°C, and a final elongation of 10 minutes at 72°C. The amplified fragment of 165 bp was visualized by electrophoresis on 3% agarose gel stained with 2 µL of 10 mg/ml ethiduim bromide solution.

2- Enzymatic Digestion

Enzymatic Digestion was carried out in 30 µL reaction volume containing 3 µL of PCR products, 1 µL of 10 × NE buffer 1(10 mM Bis Tris propane-HCl, 10 mM, $MgCl_2$, 1 mM dithithreitol, pH 7.0 at 25°C), 1 µL of restriction enzyme *Tth 1111* and 5 µL water. The enzymatic reaction was performed in a final volume of 10 µL at 65°C for 3 hours.

3- Electrophoresis

The digestion products were separated by electrophoresis on 3% agarose gel, (stained with 2 µL of 10 mg/ml ethidium bromide solution), for 30 minutes at 125V.

Interpretation of results:-

For a normal individual, (M235 homozygous) agarose gel electrophoresis allows visualization of 165 fragments, for T235T homozygous patient, a 141 bp fragment was

visualized, for a M235T heterogygous patient two bands of 165 and 141 bp were seen.

7. **Statistical Analysis:** Analysis was performed using Statistical Package for Social Sciences (SPSS), version 9.0 and Epi Info, version 6.04. For each of independent variable, odds ratio (OR) and 95% confidence interval (CI) associated with each category "referrence category". An adjusted OR with 95% CI that not include 1.0 was considered significant.

RESULTS

This study included 115 women with pre-eclampsia and 100 normal controls. The risk factors among patients and controls are shown in Table (1).

Maternal age was significantly higher in pre-eclamptic patients at age over 35 years when compared to controls. (OR = 6.67; CI: 2.09-23.59).

Multipara were higher in the patients group (OR = 1.23; CI: 0.69-2.2). Table (1) showed the family medical history for the patients and controls, where there was a significant difference between the two groups, concerning the presence of previous family history of hypertension (OR = 3.01; CI: 1.18-7.66) and pre-eclampsia (OR = 7.44; CI: 2.47-22.42).

There was a significant association between the reproductive losses in previous pregnancy and pre-eclampsia; (OR = 53.98; CI: 3.23-901.88).

Congenital fetal anomalies shown in 5 patients compared to one in the control group (OR = 12.63; CI: 0.69- 231.76).

This study showed that the M235T variant of the gene encoding angiotensinogen is present in pregnant

women with mild and severe pre-eclampsia. Thirty nine of the 115 pre-eclamptic women (33.9%) had M235T mutation; 22 (19.1%) were heterozygous (MT) and 17 (14.8%) were found to be homozygous (TT) for this mutation. Among the control group 22 (22%) had M235T mutation: 14 (14%) were heterozygous (MT) and 8 (8%) were homozygous, (TT) Figures (1 and 2).

Table (2) shows the distribution of cases and controls according to M235T mutation. The frequency of M235T mutation was higher in women with mild pre-eclampsia 25 (64.10%) than in women with severe pre-eclampsia, 14 (35.90%), although it is not statistically significant, Table (3)

DISCUSSION

Pre-eclampsia is a multifactorial complication of pregnancy and it remains one of the most important causes of maternal and fetal mortality, and morbidity in developed countries.[14] Sometimes, it progresses to eclampsia in which potentially life-threatening seizures result. Thus, there is critical need for strategies to predict, prevent, and improve management of this disorder that safely prolongs gestation and would be a major advance in prenatal care.[15]

Maternal age greater than 35 years is one of the maternal specific risk factors for pre-eclampsia.[16] In the present study, maternal age over 35 years was found to have a significant association with pre-eclampsia. This coincides with the values obtained by Tan and Tan, (1994)[17] who reported that 17% of pregnant women over the age of 35 years developed pre-eclampsia. On the contrary, Choi *et al.,* (2004)[2] found that most of the studied

women were below 35 years. These differences might be due to different ethnic background .

Women with familial history of hypertension and pre-eclampsia are at risk to develop different forms of pre-eclampsia during pregnancy.[10] Familial history of hypertension was observed in the current study in 17 (14.8%) women with different forms of hypertension. This is in agreement with other studies.[1,4] Myatt and Miodovnick, (1999)[18] reported that the risk of pre-eclampsia is 4 times higher the relative risk-being the daughter or a sister of a women who has had pre-eclampsia. In the present study, family history of pre-eclampsia had a significant higher risk for pre-eclampsia (OR = 3.01, CI: 2.47-22.42).

Pre-eclampsia is associated with higher risk of intra-uterine growth retardation (IUGR), abortion, intrauterine fetal deaths (IUFD), and congenital fetal anomalies.[5,19]

Significant frequencies of congenital fetal anomalies (4.3%) and reproductive losses (20%) (abortion, IUFD and still births) were observed in the present study among the pre-eclamptic women. This may be attributed to the fact that pre-eclampsia is associated with high perinatal morbidity and mortality rates as a result of iatrogenic prematurity.[20]

Although the etiology is unclear, a strong genetic component have been suggested.[2] Pre-eclampsia is associated with a common molecular variant of angiotensinogen (ATG) gene (Met 235 $\rightarrow$ Thr 235).[21] Several studies,[7,10,22,23] found that a molecular variant of angiotensinogen (M235T) in Japanese, Romenians, Caucasians, and Australians was significantly associated with pre-eclampsia and in part

influenced the development of pre-eclampsia.

Considering the total number of patients in this study, a high frequency (33.9%) of M235T mutation in angiotensinogen gene in pregnant women with different forms of hypertension as compared to the frequency of this mutation in women with normal pregnancies (22%). However, this frequency is statistically insignificant. This is in concordance with the results obtained by Hingorani *et al.*, (1996)[24] and Guo *et al.,* (1997).[25] However, different results were obtained by Ward *et al*., (1993)[22] and Moses *et al.,* (2000).[23] This discrepancy might be attributed to the criteria used to diagnose pre- eclampsia .

The present study revealed that 22 (19.1%) women with pre-eclampsia were heterozygous affected (MT) and 17 (14.8%) were homozygous affected (TT) for this mutation. Lucia *et al.*, (2002)[10] reported in their study that the frequency of heterozygous women for the mutation (64.28%) was higher than that of the homozygous (14.28%) ones.

CONCLUSION

The frequency of M235T mutation was higher in women with mild pre-eclampsia and there was no significant association between the mutation and pre-eclamptic patients.

This is a preliminary study and more hypertensive and normotensive pregnant women need to be comparatively studied in order to improve statistical significance and to confirm that the presence of M235T angiotensinogen gene polymorphism can be considered as a risk factor in pre-eclamptic women.

Table 1: Risk factors among patients and control

Risk factor	Patients		Control		OR	95%CI
	No.	%	No.	%		
• **Maternal age**						
below 35 years ®	90	78.3	96	96.0	1	- -
35 years or more	25	21.7	4	4.0	6.67	2.09-23.59 (S)
• **Parity**						
- Primigravida ®	47	40.9	46	46.0	1	- -
- Multipara	68	59.1	54	54.0	1.23	0.69-2.2 (NS)
• **Gestational age**						
<36 weeks ®	34	29.6	5	5.0	1	- -
36-<38 weeks	38	33.0	15	15.0	0.37	0.1-1.26 (NS)
38 weeks or more	43	37.4	80	80.0	0.08	0.03-0.23 (NS)
• **Medical Family History**						
- Irrelevant ®	71	61.7	88	88.0	1	- -
- Hypertension	17	14.8	7	7.0	3.01	1.18-7.66 (S)
- Previous pre-eclampsia	24	20.9	4	4.0	7.44	2.47-22.42 (S)
- Diabetes Mellitus	7	6.1	2	2.0	4.34	0.87-21.54 (NS)
- CV stroke	1	0.9	-	-	3.71	0.15-92.55 (NS)
- Gestational diabetes	1	0.9	-	-	3.71	0.15-92.55 (NS)
- Bronchial asthma	1	0.9	3	3.0	0.41	0.04-4.06 (NS)
• **Ultrasonographic Examination**						
- Normal ®	106	92.8	99	99.0	1	- -
- Abnormal US	9	7.8	1	1.0	8.41	1.06-180.33 (S)

Table 1: Continued

Risk factor	Patients		Control		OR	95%CI
	No.	%	No.	%		
• **Previous pregnancy and child congenital anomalies**						
- **No ®**	87	75.7	100	100	1	- -
- **Reproductive loss**	23	20.0	-	-	53.98	3.23-901.88 (S)
- **Congenital anomalies**	5	4.3	-	-	12.63	0.69-231.76 (NS)
• **Malignancy**						
- **No ®**	111	96.5	99	99.0	1	- -
- **Yes**	4	3.5	1	1.0	3.57	0.37-85.24 (NS)
• **Investigations**						
- **Normal ®**	112	92.2	100	100.0	1	- -
- **Abnormal liver enzymes**	1	0.9	-	-	2.68	0.11-66.54 (NS)
- **Abnormal kidney functions**	3	2.6	-	-	6.25	0.32-122.55 (NS)

® = Reference category.
S = Significant association.
NS = Non-significant association.

Table 2: Distribution of M235T variant in pre-eclamptic and control group

Genotype	Patients		Control		OR	95%CI
	No.	%	No.	%		
•**MM genotype Wild allele ®**	76	66.1	78	78.0	1	- -
•**MT genotype Heterozygous**	22	19.1	14	14.0	1.61	0.77-3.38 (NS)
•**TT genotype Homozygous**	17	14.8	8	8.0	2.18	0.89-5.35 (NS)

® = Reference category.
NS = Non-signifiant association.

Table 3: Distribution of M235T angiotensinogen variant in mild and severe pre-eclamptic women

Genotype	Degree of pre-eclampsia				OR	95%CI
	Severe		Mild			
	No.	%	No.	%		
•MM genotype Wild allele ®	20	26.3	56	73.7	1	- -
•MT genotype Heterozygous	8	36.4	14	63.6	1.6	0.52-4.88 (NS)
•TT genotype Homozygous	6	35.3	11	64.7	1.53	0.43-5.28 (NS)

® = Reference category.
NS = Non-signifiant association.

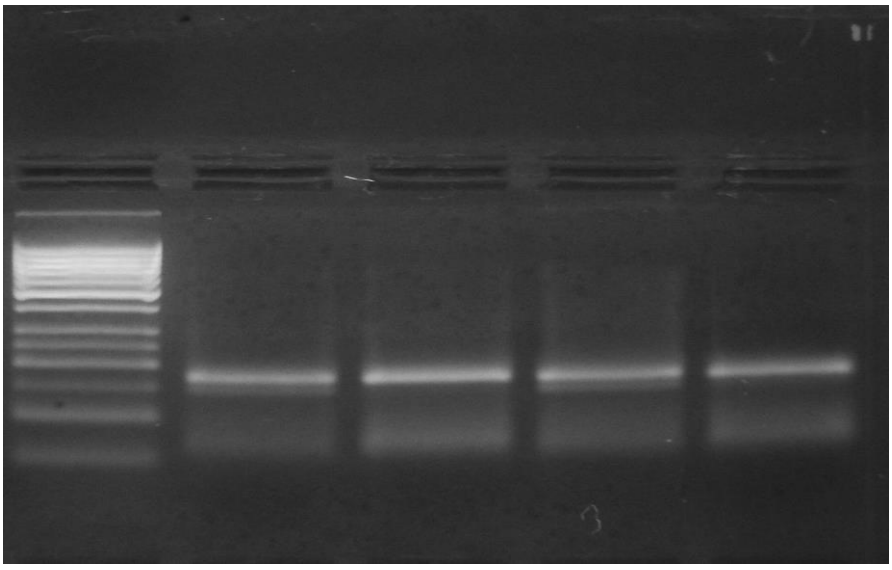

Figure 1:

Lane 1: 50 bp DNA marker
Lane 2: heterozygote (2 fragments one of 165 bp and the other of 141bp)
Lane 3: normal individual (undigested fragment of 165 bp)
Lane 4: heterozygote (2 fragments one of 165 bp and the other of 141 bp)
Lane 5: normal individual (undigested fragment of 165 bp)

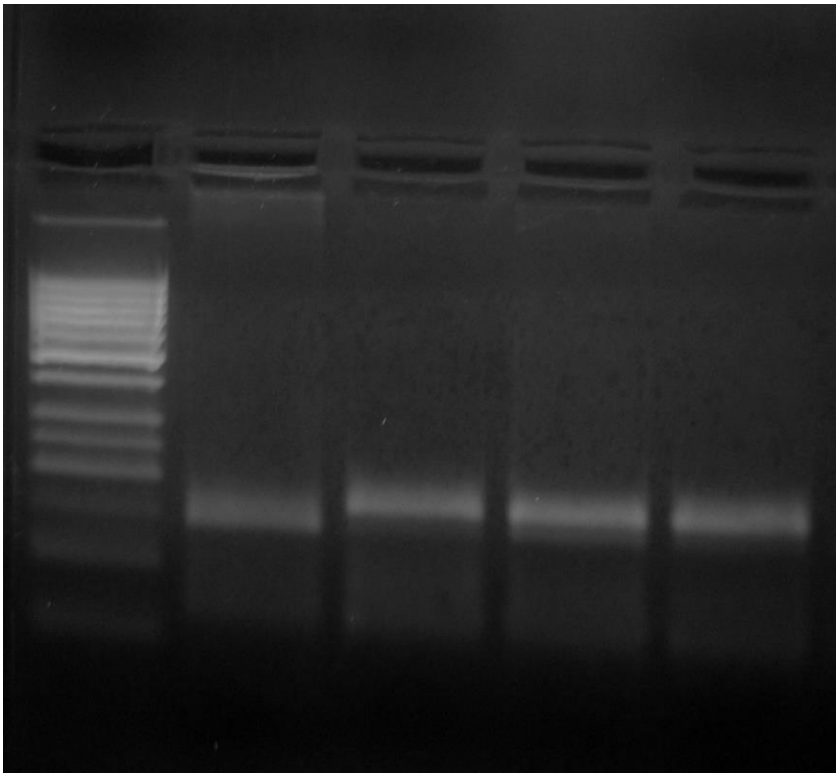

Figure 2 :
Lane 1: 50 bp DNA marker
Lane 2: homozygote (141 bp fragment)
Lanes 3, 4, and 5: heterozygote (2 fragments one of 165 bp and the other of 141 bp)

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