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Angiotensinogen gene M235T variant and pre-eclampsia in Romanian pregnant women

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Abstract

Background. Association between the human angiotensinogen gene and essential hypertension has been confirmed in recent studies. Pre-eclampsia is a complication of pregnancy characterised by increased vascular resistance, high blood pressure, proteinuria and oedema, that appears in the second and third trimester of pregnancy. The aim of our study was the analysis of M235T mutation in the gene encoding angiotensinogen in Romanian women with different forms of hypertension during pregnancy. *Methods.* Fourteen women with obstetric complications were tested for M235T angiotensinogen gene mutation. Indications for testing were: severe or mild pre-eclampsia and pre-eclampsia associated with chronic hypertension. We also tested for control 6 healthy women. The M235T angiotensinogen gene mutation was analysed by polymerase chain reaction followed by enzymatic digestion with *Tth 111I* restriction endonuclease enzyme and agarose gel electrophoresis of the products. *Results.* Eleven (78.57%) of the 14 women with complications of pregnancy had M235T mutation: 9 (64.28%) were found to be heterozygous carriers of the M235T variant of the angiotensinogen gene and 2 (14.28%) were found to be homozygous carriers. In the group of women with normal pregnancy, 3 (50%) of the 6 women had M235T mutation: 2 (33.33%) were found to be heterozygous carriers of the M235T variant of the angiotensinogen gene and 1 (16.66%) was found to be homozygous carrier. *Conclusions.* Our study shows that the M235T variant in the gene encoding angiotensinogen could be a risk factor in mild and severe pre-eclampsia.

Keywords: arterial hypertension - pre-eclampsia - pregnancy - angiotensinogen gene - M235T variant - polymerase chain reaction

Introduction

Blood pressure homeostasis is maintained by a balance of forces that affect blood flow, vascular resistance, electrolyte and water handling, and cell growth. An alteration of this equilibrum in favor of sodium and water retention, cardiovascular hypertrophy, or vasoconstriction can lead to sustained hypertension. Recently, an association between the human angiotensinogen gene and essential hypertension has been confirmed in some studies of 379 hypertensive subjects [1]. It was also demonstrated that plasma angiotensinogen level is

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increased in women who take oral contraceptives; these women can develop hypertension [2].

Pregnancy induces some adaptations in cardiovascular and renal physiology: decreased vascular resistance and mean blood pressure, increased plasma volume, the stimulation of renin-angiotensin system: increase of plasma renin activity and plasmatic levels of angiotensinogen, angiotensin II and aldosterone [3–5]. In contrast, pre-eclampsia is a complication of pregnancy, characterised by increased vascular resistance, high blood pressure, proteinuria and oedema, that appears in late pregnancy [6–9]. It affects 1-5% of all pregnancies [6,7]. A small proportion of women develop later eclampsia.

Pre-eclampsia and eclampsia are the most important causes of death in pregnancy in the UK, USA and Scandinavian countries. Five to 10% of women in their first trimesters develop pre-eclampsia. Even in developed countries, deaths still occur from eclampsia. In the United Kingdom, 80% of maternal deaths occur from eclampsia [10].

The initiation of the process begins soon after conception and the clinical signs can occur at any stage starting from late mid trimester. Pre-eclampsia may be caused by poor placental perfusion [11–13], possibly due to incomplete invasion of fetal trophoblast cells into the uterus and maternal resistance against such invasion [14]. Theoretically, both mother and fetus may contribute to the risk. Pre-eclampsia may reflect problems in the close biological interaction between the two subjects [15].

The importance of the renin- angiotensin system for maintenance of normal cardiovascular homeostasis and its participation in the pathophysiology of pre-eclampsia is well established. Plasma renin activity is higher and the level of angiotensinogen may be also increased [16]. This observation suggested a possible role of angiotensinogen in preeclampsia. Mothers with pre-eclampsia in their first pregnancy were nearly 12 times more likely than other mothers to have pre-eclampsia in their second pregnancy. The risk of developing pre-eclampsia is higher in the first pregnancy with a new partner, and in women with autoimmune diseases. The risk of pre-eclampsia in a second pregnancy increases with maternal age and with the time interval between pregnancies [14].

The M235T angiotensinogen gene mutation is a single base pair substitution of tymine (T) with cytosine (C) at nucleotide 704 (T704→C) in exon 2 of the angiotensinogen gene (chromosome 1q42-43), leading to the substitution of methionine with threonine at amino acid position 235 in the pre–proangiotensinogen molecule (M235T) [17,18]. T235 allele represents the mutant allele and M235 allele represents the wild type.

The association between preeclampsia and M235T variant was found significant in Caucasians as well as in Japanese [19] and in Australian populations [20].

The aim of our study was to analyse the occurence of M235T gene polymorphism in Romanian pregnant women with different forms of pre-eclampsia, compared to normotensive pregnant women.

Materials and methods

Patients

The characteristics of women with obstetrical complications and those with normal pregnancies are shown in Table 1.

Between September 2001 and January 2002, we studied 14 women who had pre-eclampsia during pregnancy. Indications for testing for the angiotensinogen gene M235T mutation were: severe pre-eclampsia, mild pre-eclampsia and pre-eclampsia associated with chronic hypertension. All patients were normotensive early in pregnancy except one who was hypertensive. The mean age of pregnant women with hypertension was 26±5 years. Of these 14 women with different types of pre-eclampsia, 8 had mild pre-eclampsia, 1 had pre-eclampsia superimposed on chronic hypertension and 5 had severe pre-eclampsia.

Familial history of hypertension was observed in 6 women. Two women were smokers and none of these pregnant women took oral contraceptives before pregnancy.

Control group

We also studied 6 women who had normal pregnancies. The patients from the control group were ascertained from the same department. None had a prior personal or familial history of hypertension. The mean age of these women was 29±5 years. One pregnant woman was a smoker and none of these pregnant women were taking oral contraceptives before pregnancy.

All patients agreed to take part in this study and were tested for the angiotensinogen gene mutation. The study had the agreement of the Ethical Board for Genetic Analysis.

Genotype analyses

Blood Collection and DNA Extraction

A 5mL sample of venous blood was obtained from subjects in each of the two groups. DNA extraction was performed using the method of Lahiry [21]. The concentration of DNA was determined by measuring the optical density at 260nm.

Identification of M235T angiotensinogen gene polymorphism

The M235T polymorphism in exon 2 of the angiotensinogen gene was examined by polymerase chain reaction (PCR) amplification of genomic DNA and subsequent restriction-endonuclease digestion.

DNA amplification

In order to amplify the 165bp fragment that contains the M235T polymorphism, we used the first set of second exon primers described by Russ [17] and Jeunemaitre *et al.* [18]. The forward primer was:

5'- CAGGGTGCTGTCCACACTGGACCCC-3',

and the reverse primer was:

5'- CCGTTTGTGCAGGGCCTGGCTCTCT-3'.

In brief, genomic DNA (500 ng) was amplified in a reaction containing 0.2 μM of each primer, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM TRIS-HCl (pH 9.0 at 25°C), 200 μM of each deoxynucleotide triphosphate (dNTPs), and 2 U of *Taq polymerase* (Promega) in a volume of 100μL. 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 165bp was visualized by electrophoresis on 3% agarose gel stained with 2μl of 10mg/ml ethidium bromide solution.

Enzymatic digestion

The specific mismatches incorporated into the antisense primer create a *Tth111*I site if the *T235* variant is present. The M235T polymorphism was genotyped as an *Tth 111I* (New England Biolabs) PCR restriction fragment length polymorphism (RFLP). The reagent supplied with the enzyme was: 10 X NE Buffer 1. The reaction conditions were: 1μl of 10 X NE Buffer 1 (1 X NE Buffer 1contains: 10mM Bis Tris Propane-HCl, 10mM $MgCl_2$, 1mM dithithreitol, pH 7.0 at 25°C), 3μl of polymerase

Table 1 The main clinical and anamnestic characteristics of pregnant women with pre-eclampsia compared to control.

	Mild pre-eclampsia (n= 8)	Severe pre-eclampsia (n=5)	Pre-eclampsia associated to chronic hypertension (n=1)	Controls (n=6)
Mean age *(years ± SD)*	22.88 ± 1.36	29.2 ± 5.35	34	28.83 ± 4.81
Systolic blood pressure *(mean value mmHg ± SD)*	147.5 ± 6.54	178 ± 12.54	210	110 ± 7.07
Diastolic blood pressure *(mean value mmHg ± SD)*	95 ± 5.34	98 ± 5.7	120	68.33 ± 4.08
Familial hypertension *No. of cases (%)*	3 (37.5%)	3 (60%)	–	–
Smoking *No. of cases (%)*	1 (12.5%)	1 (20%)	–	–
Alcohol consumption *No. of cases (%)*	–	1 (20%)	–	–
Oral contraceptives *No. of cases (%)*	–	–	–	–

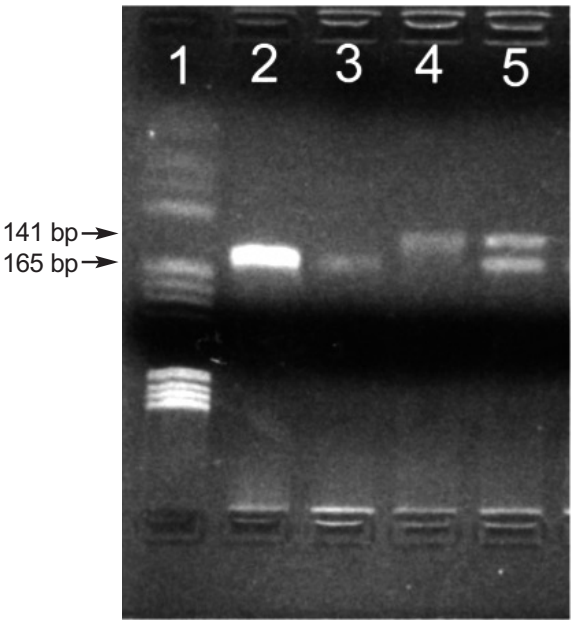

Fig. 1 Agarose gel electrophoresis illustrating the amplification of the 165bp fragment in the angiotensinogen gene and enzymatic digestion of this fragment with *Tth111I* restriction endonuclease enzyme.
Lane 1: pBR 322 Hae III digest-DNA molecular marker
Lane 2: amplified fragment of 165bp in the angiotensinogen gene
Lane 3: normal individual: undigested fragment of 165bp
Lane 4: homozygous individual: fragment of 141bp
Lane 5: heterozygous individual: fragments of 165bp and 141bp

chain reaction product, 1µl (4U/3µl PCR product) *Tth111I* restriction enzyme (the amount of restriction enzyme required for complete digestion) and 5µl water. The enzymatic digestion was performed in a final volume of 10µl at 65°C for 3 hours. The digestion products were separated by electrophoresis on 3 % agarose gel, stained with 2µl of 10mg/ml ethidium bromide solution, for 30 minutes at 125V. The normal allele 235M, gives an undigested fragment of 165bp, whereas the mutant allele 235T, gives two fragments of 141 and 24bp. For a normal individual, (M235M homozygous) agarose gel electrophoresis allows visualization of a 165bp fragment; for T235T homozygous patient agarose gel electrophoresis allows visualization of a 141bp fragment, for a M235T heterozygous patient we can see in agarose gel electrophoresis two bands of 165 and 141bp (Fig. 1).

Results

Our study shows that the M235T variant of the gene encoding angiotensinogen is present in pregnant women with mild pre-eclampsia and severe pre-eclampsia including one case with chronic hypertension (Table 2). Eleven of the 14 women investigated (78.57%) had M235T mutation: 9 (64.28%) were found to be heterozygous (MT) and 2 (14.28%) were found to be homozygous (TT) for this mutation. This polymorphism was also present in the group of women with normal pregnancies. Three of the 6 women (50%) had M235T mutation: 2 (33.33%) were found to be heterozygous and 1 (16.66%) was found to be homozygous for this polymorphism.

Systolic blood pressure was higher in hypertensive pregnant women who were homozygous or heterozygous for M235T mutation (164.54±23.28 mm Hg) than in the hypertensive pregnant women who did not carry this mutation (156.66±17.55 mm Hg). The same results for diastolic blood pressure: hypertensive pregnant women who were homozygous or heterozygous for M235T mutation had diastolic blood pressure 98.63±8.96 mm Hg compared to 95±5 mm Hg for the hypertensive pregnant women who did not carry this mutation.

Discussion

We analysed the following types of pre-eclampsia that can appear in pregnancy: mild pre-eclampsia, severe pre-eclampsia, and pre-eclampsia superimposed on chronic hypertension [7,8].

Pre-eclampsia is presented as the triad of hypertension (systolic blood pressure higher than 140 mm Hg and/or diastolic blood pressure higher than 90 mm Hg), proteinuria and oedema. Other modifications may appear: platelet count < 100,000/mm^3, elevated serum aminotransferase concentration [22].

Severe pre-eclampsia is characterized by modification of blood pressure, renal disease, hepatic abnormalities, neurological and haematological modifications [5]. Pregnant patients with severe pre-eclampsia have one or more of the following symptoms:

- systolic blood pressure > 160 mm Hg and/or diastolic blood pressure > 110 mm Hg;

Table 2 Distribution of M235T angiotensinogen variant in hypertensive and control groups.

	Hypertensive group			Control group
Genotype	**Mild pre-eclampsia (n=8)**	**Severe pre-eclampsia (n=5)**	**Pre-eclampsia with chronic hypertension (n=1)**	**(n=6)**
TT genotype *No. of cases (%)*	–	1 (20%)	1	1 (16.66%)
TM genotype *No. of cases (%)*	7 (87.5%)	2 (40%)	–	2 (33.33%)
MM genotype *No. of cases (%)*	1 (12.5%)	2 (40%)	–	3 (50%)
Total carriers of AGT variant M235T *No. of cases (%)*	7 (87.5%)	3 (60%)	1	3 (50%)

TT genotype- homozygous positive for M235T variant in the angiotensinogen gene;
TM genotype- heterozygous for M235T variant in the angiotensinogen gene;
MM genotype- homozygous negative for M235T variant in the angiotensinogen gene.

- proteinuria > 300 mg/day;
- elevated levels of serum aminotransferase;
- persistent headaches;
- thrombocytopenia.

Severe pre-eclampsia confers a serious risk of stroke in pregnant women because of the excessively high blood pressure.

In our study 6 (including one case with chronic hypertension) of 14 women with pre-eclampsia had proteinuria and elevated serum aminotransferase concentrations. Four (66%) of these 6 women were heterozygous or homozygous for M235T mutation.

Mild pre-eclampsia is defined as hypertension developing after 20 weeks of pregnancy, without other symptoms; blood pressure is returning to normal within a few months post partum.

Seven of the 8 patients with mild pre-eclampsia were positive for the angiotensinogen gene mutation: all were heterozygous for the M235T angiotensinogen gene polymorphism.

Familial history of essential hypertension was observed in 6 (42.85%) women with different forms of hypertension. This finding supports the hypothesis that women with familial history of hypertension may be at risk to develop different forms of pre-eclampsia during pregnancy.

Considering the total number of patients, we found a high frequency (78.57%) 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 (50%).

Our results are in agreement with those presented by Ward *et al.* In their study the proportion of subjects homozygous for the mutation M235T increased from 45% in controls to 77-80% in patients with pre-eclampsia [19].

However, this is a preliminary study and more hypertensive and normotensive pregnant women need to be comparatively studied in order to improve statistical significance.

In conclusion, our findings suggest that the presence of M235T angiotensinogen gene polymorphism should be considered and investigated as a risk factor in women with pre-eclampsia.

In these cases it is recommended monitoring of arterial blood pressure and testing all the possible causes of hypertension during pregnancy (including genetic analysis of M235T variant of the angiotensinogen gene).

Pre-eclampsia can be solved just after delivery, and in the case of pregnant women with severe pre-eclampsia the choice between operative and vaginal

delivery has to be considered. In severe cases (if pregnant women associate pre-eclampsia with one genetic cause such as M235T variant), the use of a combination of more antihypertensive drugs is recommended. Blood pressure should be controlled with the use of magnesium as a first line therapy, β blockers or nifedipine (oral or sublingual) but ACE inhibitors are recommended to be avoided because they do not contribute much to blood pressure control [23].

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