Influence of RAS Polymorphisms on the Development and Perinatal Outcome of Preeclampsia. Genetic RAS Evaluation

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Abstract

Objectives. *1. to determine the genotype distribution of Met235Thr, Thr174Met, insertion (I)/deletion (D) in the ACE gene, 18-83G/A in the REN gene, 1166A/C in the AGTR1 gene and 3123A/G in the AGTR2 gene in women with and without PE (preeclampsia); 2. to examine the possible influence of RAS gene polymorphisms in the development and perinatal outcome of PE.*
Methods. *Forty-five women with PE were included following inclusion and exclusion criteria. The control group comprised 51 normal pregnant women. PCR- RFLP methods were used.*
Results. *The frequency of the TT (M235T), MM (T174M) and D/D (ACE) genotypes was significantly higher (p=0.04; p=0.02; p=0.02) in female patients with PE than in female control subjects. When combined with the TT235 genotype, carriers of the MM174, D/D, AC + CC (AGTR1) or AC+ AA (AGTR2) genotypes had a 6.12; 12.5; 9.2; 5.29- fold increased risk for PE.*
Conclusions. *An increased risk of PE in women carrying the TT235, MM174 and D/D genotypes or combined TT235 and either MM174 or D/D genotypes was observed. There was a significant association of pregnancy outcome with the mutated Met235Thr (AGT), Thr174Met (AGT) and I/D (ACE) genotypes.*
Keywords: *RAS polymorphisms, preeclampsia, perinatal outcome, PCR-RFLP methods*

Introduction

Preeclampsia (PE) is a syndrome characterized by high blood pressure, proteinuria and/or swelling (edema) developing after the 20th week of gestation, that occurs in 5-6% of the pregnant women (Reynolds et al, 2003)[1]. It is a most common pregnancy complication, a major cause of maternal mortality, accounting for 8-36% of all deaths during the course of pregnancy or perinatal death in developed countries (Nilsson et al., 2004; Carr et al., 2005)[2,3].

Maternal phenotypic complications caused by PE include: HELLP (hemolysis, elevated liver enzyme levels, low platelet count) syndrome (Haukkamaa, 2004), premature placental detachment and eclampsia (involving the association of epileptiform seizures); foetal mortality is 13-30%[4]. Foetal complications include intrauterine growth retardation (in 10-25% of cases), low birth weight (important risk factors for the appearance of early atherosclerosis) (Davison, 2004), prematurity with a high risk of cardiac and pulmonary complications, hypoxia associated with neurological disorders and even intrauterine death (Sibai, 2003)[5,6].

In pregnancy complicated by preeclampsia, there was described an impaired placentation; one of the mechanisms of preeclampsia is utero-placental hypoperfusion because of a deficiency in the physiological remodeling of uterine spiral arteries (Morgan et al 1998)[7].

There are many hypotheses for the development of preeclampsia; according to most of them a maternal susceptibility gene results (van Dijk et al., 2005; Moses et al., 2006)[8,9]. The fact that the renin-angiotensin system (RAS) is one of the mediators of the process of spinal artery remodeling during pregnancy, the major site of conversion of angiotensin I to angiotensin II being the placenta, suggests the possible implication of mutant genes encoding angiotensinogen (AGT), renin (REN), angiotensin II converting enzyme (ACE), angiotensin II type 1 and 2 receptors (AGTR1 and AGTR2), in the pathogenesis of PE (Laskowska, 2004)[10]. There are studies which have confirmed the existence of local RAS and its role in uterine spiral artery remodeling (Robert et al 2002), and that the genetic variations in the genes encoding RAS components could be markers of the predisposition to preeclampsia[11].

Objectives

1. To determine the genotype distribution and allele frequency of 6 polymorphisms in 5 genes encoding the RAS components (Met235Thr and Thr174Met in the AGT gene, insertion (I)/deletion (D) in the ACE gene, 18-83G/A in the REN gene, 1166A/C in the AGTR1 gene and 3123A/G in the AGTR2 gene) in women with and without PE.

2. To examine the possible influence of RAS gene polymorphisms on the increase of systolic blood pressure (SBP)/ diastolic blood pressure (DBP) and in the development of PE.

3. To examine the synergic effect of RAS polymorphisms in the development of PE.

4. To evaluate the implications of the RAS polymorphisms in perinatal outcome during pregnancy complicated by PE.

Methods

In order to determine the status of RAS polymorphisms in Romanian women with PE, a case-control study was performed. Forty-five women with mild

Table 1

PCR- restriction fragment length polymorphism analysis of the 6 SNPs

SNPs	Gene	Forward (F) and reverse ® primer sequences	Annealing temperature (°)	PCR bp	Restriction enzyme	Fragment sizes, bp
Met235Thr	AGT	F:5'CCGTTTGTGCAGGGCCTGGC TCTCT- 3' R:5'CAGGGTGCTGTCCACACTGG ACCCC- 3'	64	165	*Tth111I*	Met: 165 Thr: 141 and 24
Thr174Met	AGT	F:5'GATGCGCACAAGGTCCTG- 3' R:5'CCAGGGTGCTGTCCACACTG GCTCGC- 3'	57	303	*NcoI*	T: 303 M: 211 and 92
I/D	ACE	F:5'CATCCTTTCTCCCATTTCTC 3' R:5'TGGGATTACAGGCGTGATA CAG 3'	69	390bp		I: 390 D: 290
$^{18\text{-}83}$A/G	REN	F:5'TGAGGTTCGAGTCGGCCCC CT- 3' R:5'TGCCCCAAACATGGCCACAC AT- 3'	66	250	*MboI*	G: 250 A: 171 and 79
1166A/C	AGTR1	F:5'ATAATGTAAGCTCATCCACC- 3' R:5'GAGATTGCATTTCTG TCAGT- 3'	57	350	*DdeI*	A: 350 C: 211 and 139
3123C/A	AGTR2	F:5'GGATTCAGA TTCTCTTTGAA- 3' R:5'GCATAGGAGTATGATTTAA TC-3'	53	340	*AluI*	C: 340 A: 227 and 113

and severe PE superimposed to chronic hypertension were included following inclusion and exclusion criteria.

Inclusion criteria:

1. gestational age between 20 and 33 weeks + 6 days;
2. blood pressure values of pregnant women ≥140/90 mmHg or increases in SBP values ≥30 mmHg and/or in DBP values ≥15 mmHg compared to values existing before 20 gestational weeks;
3. proteinuria ≥ 300mg/ l/ 24 h;
4. generalized edema.

Exclusion criteria: positive history of cardiovascular, renal, liver diseases, evidence of fetal anomalies.

The control group comprised 51 normal pregnant women; they were normotensive and had no proteinuria or signs of PE before delivery. Perinatal outcome including gestational age at delivery, birth gestational weight, neonatal morbidity and mortality were noted.

This study was approved by the Local Ethics Committee of our university. All patients gave their written informed consent.

DNA was isolated from peripheral blood leukocytes using the ZR genomic DNA II kit. Six polymorphisms in 5 genes- Met235Thr (AGT), Thr174Met (AGT); insertion(I)-deletion(D) (ACE); I8-83G/A (REN); 1166A/C (AGTR1) and 3123C/A (AGTR2) were examined using polymerase- chain reaction- restriction fragment length polymorphism (PCR-RFLP) (Table 1). In order to identify the genotypes for these 6 genetic variations, the methods of Russ (Met235Thr), Caulfield (Thr174Met), Evans (I/D), Frossard (I8-83G/A) and Takemoto (1166A/C and 3123C/A) with minor modifications were used[12-16].

Statistical analysis: SBP and DBP, gestational age at delivery and birth weight were expressed as mean ± SD. To evaluate statistical differences between the two groups, Student's test was used. In order to compare the genotype frequency between the two groups, statistical analysis was performed using the Fisher exact test. Odds ratio (OR) with 95%CI was calculated using the Fisher exact test. p less than 0.05 was considered statistically significant.

Results

In the present study we compared the genotype frequency in 45 pregnant women (mean age 27.84 ± 3.33 years) with PE and 51 healthy pregnant women (mean age 27.92 ± 5.32 years).

The clinical characteristics of the two studied groups are presented in table 2.

SBP and DBP were significantly higher in the group of women with PE compared to the group of women with normal pregnancies. There were significant differences in the gestational age at delivery and the mean birth weight, which were significantly lower in the preeclamptic cases ($p < 0.01$).

The genotype distribution and allele frequency are presented in Table 3.

In order to demonstrate that the risk for PE is increased in women carrying more than one RAS polymorphism, we checked the combination between TT235 and MM174, D/D- ACE, (AC +CC)- AGTR1 and (AC +AA)- AGTR2 genotypes in the two studied groups (Table 4).

The implications of RAS polymorphisms in perinatal outcome during pregnancy complicated by PE are presented in Table 5.

Discussion

Women with PE had a 3.59- fold increased risk to develop stroke and also a 1.2- fold increased risk of all causes of death compared to women without this pregnancy complication (Skjærven et al, 2005)[17]. The risk increased to 2.7 for women with PE and preterm birth. Moreover, women with PE who delivered a child of low birth weight had an 8- fold increased risk of death from cardiovascular diseases[17].

Increased SBP and/or DBP represent one of the causes of PE. The genes of the renin-angiotensin system (RAS) are

Table 2

Clinical characteristics of the studied groups

Clinical characteristics	Women with preeclampsia	Normal pregnant women	p value
Sample size	45	51	
Maternal age (years)	27.84 ± 3.33	27.92 ± 5.32	
SBP (mmHg)	155.13 ± 12.49	126.27 ± 4.56	<0.01
DBP (mmHg)	96.89 ± 4.46	74.7 ± 5.23	<0.01
Life style Smoking status, no (%) Alcohol use, no (%)	 7 (15.55%) 3 (6.66%)	 2 (3.92%) -	<0.05
Type of preeclampsia, no (%) Mild Severe Severe PE superimposed to chronic hypertension	 35 (77.77%) 9 (20%) 1 (2.22%)	 - - -	
Gestational age at delivery, weeks	33.18 ± 2.69	38.37 ± 1.34	<0.01
Birth weight (grams)	2764.28 ± 353.43	3316.66 ± 252.98	<0.01

p value was obtained with Student's test

Table 3

Genotype and allele frequency in the studied groups

Polymorphism/gene	Mutated genotypes	Preeclampsia no (%)	Control no (%)	OR, 95%CI	p
Met235Thr- AGT	TT TM T allele	13 (28.88) 23 (51.11) 49 (0.54)	6 (11.76) 21 (41.17) 33 (0.32)	3.04 [1.04-8.86] 1.49 [0.66-3.35] 2.49 [1.38-4.49]	0.04 0.41 < 0.01
Thr174Met- AGT	MM MT M allele	13 (28.88) 10 (19.6) 36 (0.4)	5 (9.8) 3 (5.88) 13 (0.127)	3.73 [1.21-11.52] 4.57[1.17-17.84] 4.56 [2.22-9.36]	0.02 0.03 < 0.01
I/D- ACE	DD I/D D allele	21(46.66) 20 (44.44) 62 (0.68)	12 (27.45) 24 (47.05) 48 (0.41)	2.84 [1.18-6.08] 0.9 [0.4-2.01] 2.49 [1.36-4.5]	0.02 0.83 <0.01
18-83G/A- REN	AA AG A allele	5 (11.11) 17 (37.77) 27 (0.3)	2 (3.92) 15 (29.41) 19 (0.18)	3.06 [0.56-16.63] 1.45 [1.62-3.41] 1.87 [0.95-3.66]	0.24 0.39 0.09
1166A/C- AGTR1	CC AC C allele	6 (13.33) 13 (28.88) 25 (0.27)	4 (7.84) 12 (23.52) 20 (0.196)	1.8 [0.47-6.86] 1.3 [0.52-3.29 1.57 [0.8-3.08]	0.5 0.64 0.23
3123C/A- AGTR2	AA AC A allele	9 (20) 18 (40) 36 (0.4)	8 (15.68) 21 (41.17) 37 (0.36)	1.34 [0.47-3.84] 0.95 [0.42-2.15] 1.17 [0.65-2.09]	0.6 1 0.65

Fisher exact test was used; OR- odds ratio (patients vs. controls); 95%CI- 95% confidence interval; p< 0.05 means statistically significant

Table 4

Frequency of combined mutated genotypes in the two studied groups: women with preeclampsia and control women

Combined RAS polymorphisms	Preeclampsia	Normal pregnant women	OR, 95%CI	p
Met235Thr + Thr174Met TT235 + MM174	9/45	2/51	6.12, [1.24-30.07]	0.02
Met235Thr + I/D- ACE TT235 + DD	9/45	1/51	12.5, [1.51-103.1]	<0.01
Met235Thr + 1166A/C TT235 + (AC + CC)	7/45	1/51	9.2, [1.08-78.07]	0.02
Met235Thr + 3123C/A TT235 + (AC + AA)	8/45	2/51	5.29, [1.06-26.42]	0.04

Fisher exact test was used; OR- odds ratio (patients vs. controls); 95%CI- 95% confidence interval; p<0.05 is statistically significant

an important group of candidate genes involved in blood pressure regulation. The RAS components involved angiotensinogen- AGT (the substrate for renin), renin- REN (the enzyme which converts AGT to angiotensin I- AngI), angiotensin converting enzyme- ACE (which converts AngI to angiotensin II- AngII and degrades bradykinin), AngII receptors (AGTR1 and AGTR2), which bind AngII and determine vasoconstriction and aldosterone release, water and salt reabsorbtion. Because RAS had an important role in PE, the genetic variations in the genes encoding the RAS components could be associated with the risk of PE.

Angiotensinogen has a role in the regulation of blood pressure, thus the gene encoding it represents a strong candidate gene for hypertension. Two variants, Met235Thr (a T to C substitution at position 704, exon 2, determines a change from methionine to threonine at position 235 in AGT) and Thr174Met (a C to T substitution at position 521 determines a change from threonine to methionine at position 174 in AGT) are associated with increased plasma AGT concentrations and an increased production of Ang II, which means an increased risk of hypertension (Jeunemaitre et al, 1992)[18]. The studies of Levesque et al (2004) confirm the association of the T235 allele and PE[19]. However, these results have not been confirmed by other researchers, Hyunah et al (2004)[20].

The DD genotype of the I/D polymorphism (insertion- deletion) in intron 16) has been associated with high ACE levels. Tiret et al in 1992 confirmed that ACE levels were twofold higher in hypertensive patients with the D/D genotype compared to the I/I genotype[21]. ACE activity in PE was significantly higher than in uncomplicated pregnancy (Choi et al 2004)[22]. More than 20 genetic studies have been performed on the association between the I/D polymorphism and PE in Asian,

Table 5

RAS polymorphisms and perinatal outcome in the preeclampsia group

RAS polymorphisms	Preeclampsia group			
	Gestational age (weeks)	p	Birth weight (grams)	p
Met235Thr- AGT				
MM	34 ± 2.08		2968.75 ± 153.38	
TT	30.09 ± 2.84	0.002	2554.16 ± 521.12	0.01
Thr174Met- AGT				
TT	33.83 ± 2.2		2829.54 ± 247.2	
MM	30.8 ± 2.61	0.01	2622.72 ± 364.25	0.01
I/D- ACE				
I/I	34 ± 1		2900 ± 100	
D/D	31.25 ± 3.08	<0.01	2695 ± 439.46	0.04
$^{18\text{-}83}$G/A- REN				
GG	32.46 ± 3.02		2741.17 ± 283.52	
AA	32.25 ± 3.59	0.43	2675 ± 386.22	0.23
1166A/C- AGTR1				
AA	32.94 ± 1.69		2770.45 ± 372.46	
CC	32.4 ± 1.51	0.24	2700 ± 333.16	0.36
3123A/C- AGTR2				
CC	33.12 ± 2.94		2753.12 ± 327.34	
AA	31.16 ± 3.06	0.14	2766.66 ± 304.13	0.155

p- mutated genotype vs. normal genotype; p <0.05 is statistically significant (Student's test)

European or African populations. The studies of Morgan et al (1999) and Choi et al (2004) showed a high risk of PE in women with the D/D genotype[22,23]. In a Korean study, Hyunah et al (2004) supported the fact that only the ACE mutated genotype represented a genetic risk factor for PE[20]. The frequency of the D allele was 0.55 in the group of patients with PE, compared to 0.4 (p<0.05) in the control group. Kaur et al (2005) showed that the odds ratio for developing hypertension in subjects with the D/D genotype was 3.5 and the odds ratio for developing hypertension in subjects with the D allele was 2.24[24]. A study performed by Roh et al (1997) showed an increased risk of PE in association with the I allele, but the study had some limitation because of the small number of cases[25].

In the present study, the analysis of the genotypes showed that the frequency of the TT (M235T), MM (T174M) and D/D (ACE) genotypes was significantly higher (p=0.04; p=0.02; p=0.02) in female patients with PE than in female control subjects. The frequency of the mutated T235, M174 and D alleles was significantly higher in preeclamptic women (0.54/0.4/0.68) than in normotensive pregnant women (0.32/0.12/0.41). Women positive for the T235, M174 or D allele had a 2.49, 4.56, 2.49- fold increased risk for developing hypertension during pregnancy. In all cases p value was <0.01 (table 3).

Because renin is a rate-limiting step in RAS, it could be a serious candidate gene in hypertension and cardiovascular disease (Kurtz et al, 1990)[26]. In this study, women with the AG genotype had a 1.45-fold (p=0.39) increased risk for PE and the risk increased to 3.06 (p=0.24) in women with the GG genotype. The risk for PE in women with at least one A allele was 1.87 (p=0.09) (table 3).

Ang II manifests its role by binding the two receptors, AGTR1 and AGTR2. For this reason, the two distinct genes encoding the receptors could be candidate genes for hypertension. Bonardeaux et al (1994) showed a high risk of hypertension in patients carrying the C allele of the 1166A/C polymorphism in the AGTR1 gene[27]. Our results do not support an association between 1166A/C- AGTR1 and PE. Women with the CC1166 genotype had 1.8-fold increased risk to develop PE, but the result was not statistically significant (p=0.5). The frequency of the C1166 allele was increased, but not significantly, in PE (0.27) compared to controls (0.196) (p=0.23) (table 3).

Although no significant difference was seen in patients with PE who carried the AA3123 genotype, patients with this genotype had a relative risk of 1.34 to develop PE (p=0.6). The frequency of the A3123 allele was slightly, but not significantly increased, in PE (0.4) compared to controls (0.36) (p=0.65) (table 3).

There is one study that analyzes the synergic efects of three genetic variations, Met235Thr in the AGT, I/D in the ACE and 1166A/C in the AGTR1 genes (Bouba et al, 2003)[28]. In our study, in order to confirm the risk of PE in patients carrying more than one RAS polymorphism, we studied the synergic effects of TT235 and MM174, D/D-ACE, (AC+ CC)- AGTR1 or (AC + AA)-AGTR2, respectively. When combined with the TT235 genotype, carriers of the MM174, D/D, AC + CC (AGTR1) or AC+ AA (AGTR2) genotypes had a higher and significantly increased risk for PE (Table 4). In the group of women with PE, 5 women (11.11%) simultaneously carried the TT, MM and DD genotypes and 6 (13.33%) carried the TT, MM and (AC +CC)-AGTR1 genotypes. None of the women from the control group were positive for three genetic variations. To our knowledge, this is the first study which analyzes the association of AGT with the ACE, AGTR1 and AGTR2 genes.

Children born from preeclamptic mothers frequently have low birth weights with a higher risk for premature cardiovascular diseases. The World Health Organization announced in 2002 that the rate of neonatal mortality was 12% and that children who survived had a higher risk to develop mental and/or cardiovascular-related diseases later in life[29]. In our study, gestational age at delivery and birth gestational weight were significantly lower ($p<0.01$) in preeclamptic women than in normotensive pregnant women (Table 2).

Mello et al (2003) found that the ACE genotype had an implication on the perinatal outcome of PE such as fetal growth restriction[30]. To our knowledge, this is the first study which confirms the implication of more than one RAS polymorphism, means Met235Thr, Thr174Met and I/D, in perinatal outcome during pregnancy complicated by PE. Differences in gestational age at delivery between the TT and MM genotypes of the Met235Thr polymorphism and between the MM and TT genotypes of the Thr174Met polymorphism were found. The same difference was found between the D/D and I/I genotypes of the ACE gene. Differences in birth gestational weight between the TT and MM genotypes of the Met235Thr polymorphism were observed. The same difference was observed between the MM and TT genotypes of the Thr174Met polymorphism and between the D/D and I/I genotypes of the I/D polymorphism (table 4).

Conclusions

An increased risk of PE in women carrying the T235, M174 and D alleles or combined TT235 and either MM174 or D/D genotypes was observed. AGTR1 and AGTR2 polymorphisms do not seem to be independently associated with PE. The results indicate that the effect of the AGTR1 and AGTR2 polymorphisms on PE may depend on the Met235Thr polymorphism.

In our research, there was a significant association of pregnancy outcome (gestational age at delivery and birth gestational weight) with the Met235Thr (AGT), Thr174Met (AGT) and I/D (ACE) genotypes. There were no differences in pregnancy outcomes (gestational age at delivery and birth weight) according to the REN, AGTR1 and AGTR2 genotypes. ■

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