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Association between 3'UTR polymorphisms in genes *ACVR2A*, *AGTR1* and *RGS2* and preeclampsia

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Abstract. Preeclampsia (PE) is a pregnancy specific disease with several risk factors such as genetic polymorphisms, environmental and social factors participating in its development. The aim of this study was to investigate whether distribution of three putative regulatory SNPs rs13430086, rs5186, rs4606 in 3'UTR of genes *ACVR2A*, *AGTR1* and *RGS2*, respectively, that have been associated with hypertension and regulation of trophoblast invasion differ between women with PE and control group. The associations of rs13430086, rs5186 and rs4606 with preeclampsia were tested in two groups – the group of 50 women with PE and the control group of 42 healthy pregnant women at term. DNA was isolated from blood samples and the determination of genotypes was performed using Real-Time PCR. Power analysis for the size of the cohort was performed and the results were analyzed using Fisher exact test. The AA genotype of ACVR2A rs13430086 was significantly associated with higher risk to preeclampsia compared with TT genotype ($p = 0.026$, OR: 5.39, 95%CI: 1.21–31.54). Results showed no association between genotypes and preeclampsia for polymorphisms rs5186, rs4606. Further studies are important in order to better understand the role of *ACVR2A* in the pathogenesis of PE.

Key words: Preeclampsia — 3'UTR polymorphisms — *ACVR2A* — *AGTR1* — *RGS2*

Introduction

Preeclampsia (PE) is a pregnancy specific disease with a worldwide prevalence of about 4-8% and occurs in all ethnic groups. It is characterized by a new onset of hypertension in the latter half of pregnancy. The disorder affects mothers, their infants and it is a leading cause of maternal and neonatal morbidity and mortality, leading to devastating effects to mother and their children (Roberts and Gammil 2005; Winn et al. 2009).

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PE is multisystemic syndrome, with genetic, environmental and social factors (Cnattingius et al. 2004) participating in its development and the only known treatment is delivery of the foetus and placenta (Romero and Chaiworapongsa 2013). Preeclampsia has a familial association, and this had led to the conclusion that genetic control and inheritance has a major function in the pathology of preeclampsia (Arngrimsson et al. 1990). The highest familial appearance was reported by linkage studies of *STOX1* and *ACVR2A* genes (van Dijk et al. 2005; Moses et al. 2006). In *ACVR2A*, single nucleotide polymorphisms (SNPs) were located in non-coding regions which led to conclusions that SNPs in regulatory sequences can be involved in the pathogenesis of preeclampsia (van Dijk and Oudejans 2013).

SNPs in regulatory sequences can influence the gene expression and it is generally accepted, that one of the genetic

contributors to the disease development is allele-specific gene expression identified by expression quantitative trait locus mapping (eQTL), and some SNPs have this specific effect (Westra and Franke 2014). The SNP can affect the promoter regions and influence the transcription binding site of a transcription factor. Different studies have determined the association of regulatory SNPs in promoter regions with preeclampsia (Stonek et al. 2008; Fenstad et al. 2010; Gurdol et al. 2012; Lasabova et al. 2014). In our previous study we have showed, that combination of common rs1800682 and rare rs34995925 of the *FAS* gene affecting the STAT transcription binding site is associated with preeclampsia and postulated a mechanism based on allele-specific gene expression (Lasabova et al. 2014).

In addition to the regulatory SNPs within the gene promoter regions, other mechanism causing allele-specific gene expression are allele-specific DNA methylation and regulation via the effect on miRNA binding site. The miRNA is a small non-coding RNAs involved in the posttranscriptional gene regulation *via* binding to specific seed sequence within the 3'untranslated region (3'UTR) of the mRNA and resulting in the degradation of the target mRNA molecule (Bohnsack et al. 2004; Czech and Hannon 2011). The rs13430086 within 3'UTR region of *ACVR2A* might affect the binding of microRNAs (miRNAs) and cause a change in the amount of *ACVR2A* mRNA available for translation into protein (van Dijk and Oudejans 2013).

The renin-angiotensin system (RAS) plays a key role in the maintaining of a normal pregnancy and is crucial in blood pressure regulation (Nalogowska-Glosnicka 2000; Vitoratos et al. 2012). Genetic association of rs5186 located within 3'UTR was reported for angiotensin II receptor type 1 (*AGTR1*) to be associated with severe hypertension in pregnancy (Kobashi et al. 2004) and possibly related to PE (Lee et al. 2012). 3'UTR C1114G polymorphism of *RGS2* (rs4606) is associated with low RGS2 levels and has been connected to hypertension and obesity (Semplicini et al. 2006).

The aim of our study is the analysis of the genetic association of the SNPs rs13430086, rs5186 and rs4606 localized within the 3'UTR of *ACVR2A*, *AGTR1* and *RGS2*, respectively, that have been identified as associated hypertension and regulation of trophoblast invasion differ between women with PE and control group of healthy pregnant women.

Materials and Methods

Subjects

The present study included a total of 92 cases aged between 20 and 51 years. The study group comprised 50 PE patients and 42 normal pregnant women, evaluated at the Department of Gynecology and Obstetrics, Jessenius Faculty of Medicine, Comenius University and University Hospital Martin. All patients were Slovak citizens, Caucasians and of Slavic origin. The study protocol was approved by the Regional Ethical Committee at the Jessenius Faculty of Medicine, Comenius University in Bratislava (Martin, Slovakia) and written informed patient consent was obtained before sample collection. The study was carried out in accordance with the Declaration of Helsinki for experiments involving humans. PE was defined as the onset of gestational hypertension and proteinuria after 20 weeks of gestation. Hypertension was defined as two or more recordings of a diastolic blood pressure of ≥ 90 mmHg taken ≥ 4 hours apart. Proteinuria was defined as the excretion of ≥ 300 mg of protein over 24 hours (Lasabova et al. 2014). The control group were healthy pregnant women delivering at term, without diseases and did not include premature rupture of membranes, placenta previa, threatened abortion, artificial insemination, twins, fetal macrosomia, and premature birth. We divided preeclamptic samples into subgroups with early-onset (< 34 weeks' gestation) and later-onset PE (≥ 34 weeks' gestation) and also based on their BMI (body mass index) status into subgroups of normal weight (BMI < 25kg/m^2), overweight (BMI ≥ 25kg/m^2 and < 30 kg/m^2) and obese (BMI ≥ 30kg/m^2).

Biological samples

10 ml of antecubital venous blood for genotyping was obtained from each subject in the study. The samples were collected in EDTA (ethylenediamine tetraacetic acid) tubes with added anticoagulant, immediately stored at 4°C and further processed within 6 hours. Samples were centrifuged at 3000 rpm for 10 minutes to separate the plasma and buffy coat containing leukocytes, and then frozen at –20°C for DNA extraction and genotyping.

Genotyping

Genomic DNA was isolated from buffy coats using DNeasy Blood and Tissue kit (Qiagen, Germany). All DNA samples were diluted to 20 ng *per* µl and were used as a template for genotyping.

The AB 7500 Fast Real-Time PCR system (Applied Biosystems, USA) was used to analyze polymorphisms rs4606 in *RGS2* (assay ID: C__2498717_10), rs5186 in *AGTR1* (assay ID: C__3187716_10) and rs13430086 in *ACVR2A* (assay ID: C__31219627_10). Each of these TaqMan genotyping assay mix contained forward and reverse primer, one probe with a perfect matching to the wild-type sequence variant labeled with VIC and the other probe labeled with FAM with a perfect matching to the mutant sequence variant. The TaqMan allelic discrimination real-time PCR was performed in a 20 µl volume, containing 0.5 µl TaqMan genotyping

assay mix, 10 µl TaqMan Genotyping Master Mix (Applied Biosystems, USA), 7.5 µl DNase-free water and 2 µl of diluted genomic DNA. The real-time PCR conditions were as follows: an initial step at 95°C for 10 min, followed by 50 cycles of denaturation at 92°C for 15 s and anneal/extend at 60°C for 1 min and 30 s. For each cycle, the detecting system detected the fluorescent signals from VIC/FAM labeled probes.

Statistical analysis

The calculation of sample size necessary for attaining the conventional power ($\beta = 0.80$) for the conventional significance level ($\alpha = 0.05$) was performed by a power analysis using the Genetic Power Calculator (Purcell et al. 2003). The frequencies were used form dbSNP (available from: https://www.ncbi.nlm.nih.gov/SNP/). The Genetic Relative Risk was estimated in the usual way, i.e., as the ratio of the frequency of disease for a genotype to the prevalence. The balanced design was assumed. All statistical analyses were performed in R (R Core Team 2015), using the libraries DescTools (Signorell 2016) to test the linear trend in the additive model, by the Cochran-Armitage test; HardyWeinberg (Graffelman and Camarena 2008) to test the Hardy-Weinberg equilibrium. Independence was tested by the Chisquared test with the simulated p-values, and the conditional Odds Ratio was computed inside the Fisher exact test. The standard evidential scale for *p*-values (Wasserman 2013) was used to assess the evidence against null hypothesis.

Results

Patients and clinical findings

In our study, 50 samples from women with preeclampsia and 42 healthy controls were included. The average age of patients with normal pregnancy and preeclampsia was 28.8 years (range, 20–51 years) and 28.6 years (range, 20–36 years), respectively. Table 1 provides a summary and evaluation of demographic parameters and their average values in the group of healthy women and in patients with preeclampsia. In Table 1, the risk of PE in the BMI group was compared with the control group, which indicated that PE was observed to be more prevalent among overweight and obese women compared to the control group. Using Fisher exact test, here was not significant association of BMI with any of tested SNPs (data not shown).

The input values for the power analysis were minor allele frequencies (MAFs) 0.28, 0.27, 0.35, for rs4606, rs5186, rs13430086, respectively. The sample size necessary for attaining the conventional power 0.80 for the conventional significance level 0.05 was 32, 29, 37 cases, respectively, therefor we concluded that the size of our cohort comprising 50 cases and 42 controls for all polymorphisms is sufficient for attaining the conventional power, to detect an association.

Association of the ACVR2A rs13430086 polymorphism with preeclampsia

The distribution of genotypes in controls was in agreement with Hardy-Weinberg equilibrium ($p = 0.535$) (Table 2). The minor allele was not associated with BMI (data not shown). Fisher exact test indicated that individuals with AA genotype have a significantly higher risk to preeclampsia than individuals with TT genotype ($p = 0.026$, OR: 5.39, 95%CI: 1.21–31.54) (Table 2). We further examined the potential associations between risk of preeclampsia and rs13430086 based on different genetic models (Table 2). In the recessive model, AA and AT genotype combination has almost five times higher risk of preeclampsia than TT genotype ($p = 0.018$, OR: 4.66, 95%CI: 1.28–23.24). Multiplicative model indicate weak significance that subjects carrying an A allele have higher risk for preeclampsia than subjects carrying a T allele ($p = 0.072$, OR: 1.71, 95%CI: 0.95–3.11). Then we also examined the potential associations between later onset of PE *versus* control group. When comparing the incidence of later-onset preeclampsia in carriers of the AA genotype and those with the TT genotype, we get a weak significance (AA *vs.* TT: $p = 0.075$, OR: 4.26, 95%CI: 0.92–25.43) (Table 3). Fisher exact test showed that in recessive model, when com-

Table 1. The clinical characteristics of study groups

	Preeclampsia ($n = 50$)		Controls ($n = 42$)		*p*-value
	Mean	SD	Mean	SD	
Age	28.6	4.4	28.8	6.5	0.839
Weight	83.2	18.2	76.5	12.9	0.095
Height	165.2	5.7	167.3	6.7	0.097
BMI	30.5	6.4	26.9	3.6	0.006
GA at delivery	36.8	3.6	39.8	2.9	<0.01
Birthweight	2473.5	926.9	3301.6	695.3	<0.01

GA at delivery, gestational age at delivery.

Table 2. Associations between SNPs and PE under different genetic models

SNP	Genotype/ Allele	PE	Controls	H-WE (*p*-value)	*p*-value
rs13430086 (*ACVR2A* gene)					
	AA	14	8	0.535	0.026
	AT	33	24		
	TT	3	10		
dominant	AA	14	8		0.339
	AT+TT	36	34		
recessive	AA+AT	47	32		0.018
	TT	3	10		
	A	61	40		0.072
	T	39	44		
rs5186 (*AGTR1* gene)					
	AA	29	20	0.726	0.556
	AC	17	19		
	CC	4	3		
dominant	AA	29	20		0.402
	AC+CC	21	22		
recessive	AA+AC	46	39		1
	CC	4	3		
	A	75	59		0.513
	C	25	25		
rs4606 (*RGS2* gene)					
	CC	29	25	0.670	0.410
	CG	20	14		
	GG	1	3		
dominant	CC	29	25		1
	CG+GG	21	17		
recessive	CC+CG	49	39		0.328
	GG	1	3		
	C	78	64		0.858
	G	22	20		

H-WE, Hardy-Weinberg equilibrium; PE, preeclampsia; SNP, single nucleotide polymorphism.

bination of genotypes AA and AT is used as the reference group, odds of having AA or AT genotype are significantly higher in group of later-onset preeclampsia than in controls ($p = 0.038$, OR: 3.97, 95%CI: 1.09–19.88) (Table 3).

Association of the AGTR1 rs5186 polymorphism with preeclampsia

The genotype distributions in control group were in the Hardy-Weinberg equilibrium for gene polymorphism rs5186 ($p = 0.726$) (Table 2). No statistically significant differences in the SNP rs5186 genotype distribution frequencies were observed between patients with PE and control group in dominant, recessive or additive model. In addition, no significant differences were observed between the preeclampsia and control groups based on their BMI status (data not shown).

Association of the RGS2 rs4606 polymorphism with preeclampsia

Rs4606 was successfully genotyped in all PE and control samples. The allele frequencies in controls were found to be in Hardy-Weinberg equilibrium ($p = 0.670$) (Table 2). There were no significant differences in the allele or genotype frequencies between the PE group and controls in dominant, recessive or additive model. Also there were no significant differences between the preeclampsia and control groups based on their BMI status (data not shown).

Discussion

Preeclampsia is a heterogeneous disease in which both fetal, i.e., placental and maternal factors are contributing. It seems that the mechanism of development of PE is a combination of several factors such as genetic, epigenetic and environmental factors (Fu et al. 2013). Obesity is a global health problem even among pregnant women. In line with previous study (Roberts et al. 2011; Jeyabalan 2013; Persson et al. 2016), we found that the risk of PE increases with increasing BMI ($p = 0.006$). However, BMI was not associated with any of the tested SNPs, therefore we conclude that the determined association discussed above are related with preeclampsia. To identify factors involved in PE, multiple genetic screens have been performed in multiple patient populations. Recent studies indicate that miRNAs might have a regulatory effect on several processes such as proliferation, apoptosis, migration and invasion of trophoblast cells as well as on the cell cycle of placental cells (Fu et al. 2013). Because polymorphisms in 3'UTR can affect the binding of miRNAs and might therefore cause low levels of mRNAs available for translation into protein (Semplicini et al. 2006; van Dijk and Oudejans 2013).

In the present study, we analyzed 3 SNPs located on 3'UTR of genes *RGS2*, *AGTR1* and *ACVR2A*.

Semplicini et al. (2006) suggested an association between the G allele of rs4606 polymorphism and essential hypertension. In addition, Kvehaugen et al. (2013) reported that women with a pregnancy complicated with preeclampsia differed in the genotype distribution of the rs4606 in *RGS2*, with a slightly higher proportion of women having either the CG or GG genotype, compared to women who never had preeclampsia. Later Kvehaugen et al. (2014) observed that women carrying the genotype CG or GG have a higher risk for developing hypertension after delivery. In our study rs4606,

an *RGS2* 3'UTR polymorphism connected to reduced *RGS2* expression (Semplicini et al. 2006), was not significantly associated with PE. In addition, overweight and obese pregnant women might represent a subgroup associated with the risk of PE (Karppanen et al. 2016). Development of preeclampsia depends on many factors, both fetal and maternal. One of the maternal risk factors is overweight and its contribution to preeclampsia susceptibility might be increased in presence of certain genetic factors like polymorphisms. Karppanen et al. demonstrated small effect of rs4606 polymorphism to preeclampsia susceptibility in obese women. Interestingly, the rs4606 CG or GG genotypes have been found to predict weight gain in young hypertensitive men (Sartori et al. 2008). In the present study we did not observe statistically significant differences between subgroups of women with normal weight, overweight or obese ($p = 0.2466$).

It is known that the RAS plays a key role in blood pressure regulation. The coding and regulatory regions of the genes encoding for renin, ACE, angiotensinogen and AGTR1 receptor have been partially characterized. This provides a basis for definition of specific polymorphisms within these genes and it seems that they might also play a key role in the susceptibility to pregnancy induced hypertension (Nałogowska-Głośnicka et al. 2000). The association between rs5186 polymorphism in *AGTR1* and PE has been studied extensively, but the results have been inconclusive. A meta-analysis (Staines-Urias et al. 2012) covering 10 studies found no significant association (OR = 1.22, 99% CI: 0.96–1.56), similarly the study of Li et al. (2016) found no association between this polymorphism and PE or pregnancy-induced hypertension (PIH). Also in our study we did not find any statistically significant differencies between rs5186 and PE. However, Nałogowska-Głośnicka et al. in their study suggest that rs5186 polymorphism of the *AGTR1* gene may increase the risk of development of PIH (Nałogowska-Głośnicka et al. 2000). *AGTR1* gene CC genotype was significantly more frequent in women with PIH than in control group. The frequency of the C allele was also significantly higher in PIH patients than in controls. Statistically significant increase in allelic frequency of rs5186 in hypertensive subjects in comparison with normotensive subjects was reported also in other studies (Bonnardeaux et al. 1994; Morgan et al. 1998).

Activin A receptors are important in decidualization (Jones et al. 2002), implantation and trophoblast invasion (Caniggia et al. 1997) during pregnancy. The genetic association between polymorphisms in *ACVR2A* and preeclampsia is not clear, some studies identified genetic associations and others failed to identify an association. Roten et al. (2009) suggest an involvement of the *ACVR2A* gene in preeclampsia pathogenesis. They found an association with preeclampsia in the SNPs: rs1424941, rs1014064, rs2161983, and rs3768687 in Norwegian population. However, the study of Fitzpatrick et al. (2009) has suggested a significant association between *ACVR2A* polymorphisms rs10497025 and rs13430086 and preeclampsia ($p = 0.025$; $p = 0.010$, respectively). Lokki et al. (2011) didn't confirm the association between rs1424954 and PE in Finnish population. Recently, Zeybek et al. (2013) studied three SNPs (rs10497025, rs1128919, rs13430086) in *ACVR2A* in Turkish population, in their study no statistically significant differences were found between women with PE and controls in terms of genotype and allele frequencies. They reported that for the rs13430086, the frequency of the AA genotype was significantly lower in patients with mild and severe preeclampsia, while the frequency of TT genotype

Table 3. Associations between SNPs and later onset of PE under different genetic models

SNP	Genotype	Later-onset of PE	Controls	*p*-value (later onset/ controls)
rs13430086 (*ACVR2A* gene)				
	AA	11	8	0.075
	AT	29	24	
	TT	3	10	
dominant	AA	11	8	0.604
	AT+TT	32	34	
recessive	AA+AT	40	32	0.038
	TT	3	10	
	A	51	40	0.166
	T	35	44	
rs5186 (*AGTR1* gene)				
	AA	25	20	0.521
	AC	14	19	
	CC	4	3	
dominant	AA	25	20	0.388
	AC+CC	18	22	
recessive	AA+AC	39	39	1
	CC	4	3	
	A		59	0.608
	C		25	
rs4606 (*RGS2* gene)				
	CC	27	25	0.706
	CG	15	14	
	GG	1	3	
dominant	CC	27	25	0.825
	CG+GG	16	17	
recessive	CC+CG	42	39	0.360
	GG	1	3	
	C	69	64	0.579
	G	17	20	

PE, preeclampsia; SNP, single nucleotide polymorphism.

was significantly higher in a group of patients with severe preeclampsia. These results suggest that polymorphism in the *ACVR2A* gene might play a role in the development of preeclampsia. More recently, Ferreira et al. (2015) reported that SNPs rs1014064, rs1424954 and rs2161983 in *ACVR2A* are associated with the early onset preeclampsia. In the present study, in relation to rs13430086, the AA genotype was significantly associated with higher risk of preeclampsia compared with the TT genotype (p = 0.026, OR: 5.39, 95%CI: 1.21–31.54). Although the carriers with the A allele had higher risk of PE than those with the T allele (p = 0.072, OR: 1.71, 95%CI: 0.95–3.11), the results showed only weak significance. We also studied potential association between early, later onset of preeclampsia and controls, and we got a significant result in recessive model (p = 0.037). We equally observed that the AA or AT genotype is significantly higher in patient's group with later onset of PE compared with controls (p = 0.038, OR: 3.97, 95%CI: 1.09–19.88).

The main limitation of our study is the relatively small sample size; in the future it is necessary to extend the number of study group to confirm or refute the results. On the other hand, we have performed a power analysis to estimate the sample size necessary for attaining the conventional power ($\beta = 0.80$) for the conventional significance level ($\alpha = 0.05$) and that analysis showed that the size of our cohort is sufficient.

Preeclampsia can not reliably be predicted. There are currently no tests available in early pregnancy that can accurately detect women who will go on to develop preeclampsia and therefore it is necessary to detect new reliable biomarkers in screening and diagnosing preeclampsia.

In conclusion, we have not confirmed association between polymorphism in *AGTR1* gene and PE. In *RGS2* gene the polymorphism's association is weakly significant. We showed that *ACVR2A* gene is associated with preeclampsia, especially AA genotype. Based on our data, it remains unclear what role the rs13430086 polymorphism in *ACVR2A* gene plays in the risk of development of preeclampsia. Further studies are necessary to increase the understanding of the role of *ACVR2A* in the pathogenesis of PE.

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Conflict of interest. The authors report no conflicts of interest.

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