

CLINICAL STUDY

Analysis of HLA-G 5'URR regulatory polymorphisms in Slovak women with pre-eclampsia

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ABSTRACT

OBJECTIVES: The objective of this study was to identify the impact of the selected HLA-G gene polymorphisms in the 5'URR region on the risk to develop pre-eclampsia.

BACKGROUND: Pre-eclampsia (PE) is a serious multisystem disorder that affects women during pregnancy. Despite many research studies, the pathology of PE is not fully understood. Human leukocyte antigen G (HLA-G) belongs to the molecules that induce immune tolerance at the fetal-maternal interface. HLA-G expression was found to be impaired in the women suffering from PE suggesting its involvement in the development of PE.

METHODS: 116 women with pre-eclampsia and 130 women with normotensive pregnancy were included in the study. The 16 gene polymorphisms in the HLA-G 5'URR region (promoter) affecting HLA-G expression were typed by direct sequencing.

RESULTS: The -201AA genotypes in recessive model were significantly more frequent in women with pre-eclampsia than in the controls ($p = 0.047$). Furthermore, the analysis of HLA-G 5'URR variants with clinical findings of PE showed a significant association of the -533delA/+ genotype with a higher mean level of diastolic blood pressure ($p = 0.02$).

CONCLUSION: Our results suggest a genetic association of selected HLA-G 5'URR variants with a risk of developing pre-eclampsia and possible contribution of HLA-G to disease pathology (Tab. 4, Ref. 51). Text in PDF www.elis.sk

KEY WORDS: HLA-G, pre-eclampsia, gene polymorphism, sequencing, diagnostics.

Introduction

Pre-eclampsia (PE) is a serious multisystem disorder observed in approximately 2 to 8% of all pregnancies. It accounts for the major cause of maternal morbidity and mortality worldwide (1). It is clinically characterized by hypertension (systolic and diastolic blood pressure of $\geq 140/90$ mm Hg) and proteinuria (protein excretion of ≥ 300 mg in a 24 hours urine collection) after 20 weeks of gestational age. The symptoms vary from mild to severe forms including the end organ damage such as HELLP (Hemolysis, Elevated liver enzymes and Low platelets) syndrome. Severe maternal complications may occur including: seizures, stroke, and liver and renal failure due to an acute tubular necrosis. Fetal complications include prematurity and low birth weight (2). Despite its clinical relevance and frequency, the etiology and pathogenic mechanisms

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Tab. 1. Clinical characteristics of women with PE (n = 116).

Parameter	Mean \pm SD/Number
Age (years)	32.00 \pm 5.16
Onset of PE*	Early – 31, Late – 85
Disease severity **	Mild – 27 Moderate – 54 Severe – 35
Edema (yes/no)	88/28
Primiparous women (yes/no)	69/47
Preterm birth (yes/no)	26/90
The highest systolic BP (mmHg)	154.00 \pm 15.88
The highest diastolic BP (mmHg)	98.53 \pm 8.96
Leukocytes (x10 ⁹ /L)	10.55 \pm 2.65
Thrombocytes (x10 ⁹ /L)	215.89 \pm 75.24
Serum creatinine (μ mol/L)	59.47 \pm 12.88
Uric acid (μ mol/L)	317.86 \pm 73.21
ALT (μ kat/L)	0.43 \pm 0.61
AST (μ kat/L)	0.49 \pm 0.34
Total serum proteins (g/L)	58.85 \pm 6.63
14bp Ins positivity (yes/no)	78/38

BP – blood pressure, PE – pre-eclampsia, SD – standard deviation, *Early onset: prior to 37th week of gestation, Late onset: after 37th week of gestation, **Mild: BP is 140 to 149 mmHg systolic and/ or 90 to 99 mmHg diastolic, Moderate: BP is 150 to 159 mmHg systolic and/ or 100 to 109 mmHg diastolic, Severe: BP is ≥ 160 mmHg systolic and/or ≥ 110 mmHg diastolic

Tab. 2. HLA-G 5'URR allele and genotype frequencies in patients with PE and controls.

Allele/ genotype	PE (n=116)	Control group (n=130)	Univariate analysis		Multivariate analysis	
			p	OR (95%CI)	p	OR (95%CI)
Haploblock -762C/T, -716T/G, -689A/G, -666G/T, -633G/A						
-762C	109 (46.98%)	141 (54.23%)	0.13	1.34 (0.94–1.91)	–	–
-762T	123 (53.02%)	119 (45.77%)				
CC	28 (24.14%)	39 (30.00%)	0.26	1.17 (0.64–2.15)	0.24	1.78 (0.61–5.20)
CT	53 (45.69%)	63 (48.46%)				
TT	35 (30.17%)	28 (21.54%)				
HWE (χ^2/P)	0.80/0.37	0.07/0.79				
-725C/G/T						
C	196 (84.48%)	218 (83.85%)	0.94	0.96 (0.32–2.90)	–	–
G	30 (12.93%)	35 (13.46%)				
T	6 (2.59%)	7 (2.69%)				
CC + CG + GG	110 (94.83%)	123 (94.62%)				
CT + GT + TT	6 (5.17%)	7 (5.38%)	0.94	0.96 (0.31–2.94)	–	–
HWE (χ^2/P)	–	–				
-646A/G						
A	228 (98.28%)	257 (98.85%)	0.88	1.50 (0.33–6.79)	–	–
G	4 (1.72%)	3 (1.15%)				
AA	112 (96.55%)	127 (97.69%)				
AG	4 (3.45%)	3 (2.31%)				
GG	0 (0.00%)	0 (0.00%)	0.59	1.51 (0.33–6.90)	0.32	2.30 (0.44–11.93)
HWE (χ^2/P)	0.04/0.85	0.02/0.89				
-540insG						
insG-	231 (99.57%)	256 (98.46%)	0.44	0.28 (0.03–2.50)	–	–
insG+	1 (0.43)	4 (1.54%)				
insG-/-	115 (99.14%)	126 (96.92%)	0.20	0.27 (0.03–2.48)	0.37	0.37 (0.04–3.69)
insG-/+	1 (0.86%)	4 (3.08%)				
insG+/+	0 (0.00%)	0 (0.00%)				
HWE (χ^2/P)	0.002/0.96	0.03/0.86				
-533delA						
delA-	230 (99.14%)	256 (98.46%)	0.79	0.56 (0.10–3.07)	–	–
delA+	2 (0.86%)	4 (1.54%)				
delA-/-	114 (98.28%)	126 (96.92%)	0.49	0.55 (0.10–3.07)	0.63	0.64 (0.10–4.03)
delA-/+	2 (1.72%)	4 (3.08%)				
delA+/+	0 (0.00%)	0 (0.00%)				
HWE (χ^2/P)	0.009/0.93	0.03/0.86				
-509C/G						
C	229 (98.71%)	256 (98.46%)	0.82	0.84 (0.19–3.79)	–	–
G	3 (1.29%)	4 (1.54%)				
CC	113 (97.41%)	126 (96.92%)				
CG	3 (2.59%)	4 (3.08%)				
GG	0 (0.00%)	0 (0.00%)	0.82	0.84 (0.18–3.82)	0.98	1.02 (0.19–5.42)
HWE (χ^2/P)	0.02/0.89	0.03/0.86				
-486A/C						
A	108 (46.55%)	140 (53.85%)	0.13	1.34 (0.94–1.91)	–	–
C	124 (53.45%)	120 (46.15%)				
AA	28 (24.14%)	39 (30.00%)	0.27	1.17 (0.64–2.15)	0.23	1.77 (0.61–5.19)
AC	52 (44.83%)	62 (47.69%)				
CC	36 (31.03%)	29 (22.31%)				
HWE (χ^2/P)	1.14/0.29	0.21/0.64				
-477G/C						
C	106 (45.69%)	135 (51.92%)	0.20	1.28 (0.90–1.83)	–	–
G	126 (54.31%)	125 (48.08%)				
GG	38 (32.76%)	34 (26.15%)	0.43	0.78 (0.43–1.43)	0.32	0.74 (0.36–1.53)
GC	50 (43.10%)	57 (43.85%)				
CC	28 (24.14%)	39 (30.00%)				
HWE (χ^2/P)	2.01/0.16	1.93/0.16				

Allele/ genotype	PE (n=116)	Control group (n=130)	Univariate analysis		Multivariate analysis	
			p	OR (95%CI)	p	OR (95%CI)
-400G/A						
G	228 (98.28%)	255 (98.08%)				
A	4 (1.72%)	5 (1.92%)	0.87	0.89 (0.24–3.37)	–	–
GG	112 (96.55%)	125 (96.15%)		1.00		1.00
GA	4 (3.45%)	5 (3.85%)	0.87	0.89 (0.23–3.41)	0.82	1.19 (0.28–5.12)
AA	0 (0.00%)	0 (0.00%)		–		–
HWE (χ^2/P)	0.04/0.85	0.05/0.82				
-391G/A						
G	228 (98.28%)	254 (97.69%)				
A	4 (1.72%)	6 (2.31%)	0.89	0.74 (0.21–2.67)	–	–
GG	112 (96.55%)	124 (95.38%)		1.00		1.00
GA	4 (3.45%)	6 (4.62%)	0.64	0.74 (0.20–2.68)	0.82	1.19 (0.28–5.11)
AA	0 (0.00%)	0 (0.00%)		–		–
HWE (χ^2/P)	0.04/0.85	0.07/0.79				
-369A/C						
C	105 (45.26%)	135 (51.92%)				
A	127 (54.74%)	125 (48.08%)	0.17	1.30 (0.92–1.86)	–	–
AA	38 (32.76%)	34 (26.15%)		1.00		1.00
AC	51 (43.97%)	57 (43.85%)	0.38	0.80 (0.44–1.45)	0.18	0.76 (0.37–1.56)
CC	27 (23.27%)	39 (30.00%)		0.62 (0.32–1.22)		0.32 (0.09–1.11)
HWE (χ^2/P)	1.47/0.22	1.93/0.16				
-201G/A						
G	106 (45.69%)	141 (54.23%)				
A	126 (54.31%)	119 (45.77%)	0.07	1.41 (0.99–2.01)	–	–
GG	28 (24.14%)	39 (30.00%)		1.00		1.00
GA	50 (43.10%)	63 (48.46%)	0.13	1.11 (0.60–2.04)	0.11	1.70 (0.58–4.99)
AA	38 (32.76%)	28 (21.54%)		1.89 (0.95–3.76)		3.15 (0.94–10.62)
GG	28 (24.14%)	39 (30.00%)		1.00		1.00
GA+AA	88 (75.86%)	91 (70.00%)	0.30	1.35 (0.76–2.37)	0.2	1.97 (0.69–5.62)
GG+GA	78 (67.24%)	102 (78.46%)		1.00		1.00
AA	38 (32.76%)	28 (21.54%)	0.047	1.77 (1.00–3.14)	0.07	1.97 (0.95–4.07)
HWE (χ^2/P)	2.01/0.16	0.07/0.79				

Allele and genotype frequencies are presented as absolute numbers with percentages in parentheses. Univariate analysis is based on χ^2 test. Multivariate analysis is adjusted by age and 14bp Ins positivity. $p < 0.05$ was considered as statistically significant. CI – confidence interval, HWE – Hardy–Weinberg equilibrium, OR – odds ratio, n – number

of PE remain still unclear. One cause may originate in an insufficiently developed placenta, referred to as poor placentation. It is characterized by impaired re-modelling of spiral arteries of the uterus caused by an imbalance of circulating angiogenic factors (3). Other key mechanisms leading to PE besides poor placentation are inflammation and aberrant immune interactions at the fetal–maternal interface (4).

Suitable biomarkers that could predict the risk of PE are still subject of investigation (5, 6). One of the candidate molecules that plays a beneficial role in the pregnancy is the human leukocyte antigen G (HLA-G). HLA-G belongs to the non-classical HLA-class I molecules and is involved in the extravillous suppression of immune response. It is mainly expressed in extra villous cytotrophoblast that helps to protect the fetus from maternal immune response (7). There are four membrane – bound isoforms HLA-G1, -G2, -G3, and -G4, and three soluble isoforms HLA-G5, -G6, and -G7 (8).

HLA-G mediates an inhibition of cytotoxic activity of uterine and peripheral blood natural killers (NK) cells and CD8⁺ T cells, the alloproliferative response of CD4⁺ T cells, inhibition of dendritic cell maturation and the differentiation of CD4⁺ T-cells into regulatory T cells (9). Immunosuppressive activity of the HLA-G

molecule is mediated mainly through its interaction with inhibitory receptors of immune cells, i.e. ILT-2 receptor present on B cells, antigen-presenting cells (APC) and some T, and NK cells, ILT-4 receptor present on APC and KIR2DL4 expressed by NK and some T cells (10). HLA-G can also induce the generation of immunosuppressive HLA-G⁺ APCs, such as DC-10 and HLA-G⁺ macrophages via trogocytosis (11). Finally, sHLA-G interacts with CD8 and triggers apoptosis of CD8⁺ cells by Fas/Fas-ligand interaction (12). In addition to its immunosuppressive activity, HLA-G is involved in ensuring an successful invasion of placental tissues into the maternal decidua (13).

The HLA-G gene is 4170 bp long and consists of 8 exons. It is characterized by low polymorphism, namely 82 HLA-G alleles, 22 HLA-G proteins, and 4 null alleles have been identified to date (IMGT/HLA release 3.42.0, September 2020). The most polymorphic sites that influence HLA-G expression were identified in two noncoding regions: the upstream regulatory region (5'URR) and untranslated region (3'UTR). In the 3'UTR region, a 14 bp insertion polymorphism (5'-ATTTGTTTCATGCCT-3') has been described, which affects the stability of mRNA causing decreased production of most membrane and soluble isoforms (14). HLA-G

5'URR region consists of the 1.4 kb upstream the ATG and shows at least 35 SNPs defining 68 haplotypes, of which 9–10 have been frequently observed in human populations (15). Several enhancer and suppressor elements have been described within the HLA-G 5'URR region to regulate HLA-G expression (16–18).

In women suffering from PE, the decreased HLA-G level was determined (19–23). As the expression of soluble HLA-G may be influenced by the above-mentioned HLA-G gene polymorphisms, the possible association between these variants and the risk of PE has been investigated. The most studies analyzed the impact of 14 bp Ins/Del polymorphism on the risk of PE development (23–31). However, no study evaluated the association between HLA-G 5'URR polymorphisms and the risk of developing PE so far. Thus, the aim of the study was to analyze the association between 16 polymorphic variants in HLA-G 5'URR region and clinical findings in women with PE.

Materials and methods

Study subjects

We investigated 116 women suffering from pre-eclampsia and 130 women with physiological pregnancies. PE was defined as the presence of blood pressure of at least 140/90 mmHg with readings at least 6 hours apart and proteinuria (300 mg/24 h or ≥ 30 mg/dl by urine analysis (32). The clinical characteristics of the women with PE are shown in the Table 1. The mean age of women with PE was 32.00 ± 5.16 years; the mean age of controls was 40.22 ± 9.28 . All the study subjects provided a written informed consent for enrolling in the study and for personal data management. The study was approved by the Ethics Committee of the University Hospital Bratislava. All the investigations were carried out in accordance with the International Ethical Guidelines and the Declaration of Helsinki.

Sequencing of the HLA-G 5'URR region

Genomic DNA was isolated from EDTA-treated peripheral PE and control blood samples (2 mL) by a modified salting-out procedure (33). To quantify the amount and purity of DNA, the Nanodrop spectrophotometer was used (Thermo Fisher Scientific). HLA-G 5'URR region from – 828 bp to – 5 bp was amplified by forward primer 5'-CACGGAACTTAGGGCTACGG-3 and reverse primer 5'-GCGTCTGGGGAGAATGAGTCC-3' as described by Catamo et al (34). A PCR reaction mixture with a total volume of 25 μ L contained 50 ng of template DNA, 0.2 mM of each dNTP (Thermo Fisher Scientific, USA), 1 U of Taq DNA polymerase (Thermo Fisher Scientific, USA), 1.5 mmol $MgCl_2$ (Thermo Fisher Scientific, USA), and 10 pmol of each specific primer. The PCR conditions were 95 °C for 3 min, followed by 30 cycles (denaturation at 95 °C for 1 min, annealing at 65 °C for 30 s, and elongation at 72 °C for 1 min) and final elongation at 72 °C for 10 min. The PCR products were run in 1.0 % agarose gel for 20 min and then visualized under UV light. Fragment size of 823 bp was confirmed using the 100 bp DNA ladder (Solis BioDyne, EU). For a direct sequence analysis, the PCR products were purified using an EXO SAP-IT kit according to the manufacturer's recommendations (USB, USA) and then sequenced (approxim-

mately 100 ng) using BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, USA). The PCR products were sequenced individually on both strands using forward and reverse PCR primers. The sequencing reaction was run on a 3130 ABI PRISM Genetic analyzer (Applied Biosystems) and the sequence data were analyzed by SeqScape software (Applied Biosystems) using the reference sequence reported on website <http://www.ebi.ac.uk/imgt/hla/align.html>.

Statistical analysis

Allele and genotype frequencies were evaluated by a direct counting. Genotypes were tested for their fit to Hardy-Weinberg equilibrium using the chi-square test. Haplotype frequency estimation was defined using PHASE 2.1 software (35). Statistical significance of differences in allele, genotype and haplotype frequencies between the two studied groups (women with pre-eclampsia vs control group) was evaluated by the standard chi square test using the InStat statistical software (GraphPad Software, Inc., San Diego, USA). The p values, odds ratios (OR) and 95% confidence intervals (95% CI) were calculated in co-dominant, dominant and recessive inheritance models. The multivariate logistic regression analysis adjusted for age and HLA-G +14 bp Ins positivity as possible influencing factors was performed by the SNPstats web software available at <https://snapstat.net/snpstats/>. Finally, linear regression analysis and Student t-test with Welch correction was used to investigate the correlation between HLA-G 5'URR polymorphisms and clinical variables as the onset of PE, the highest systolic blood pressure (BP), the highest diastolic BP, serum creatinine, uric acid and total serum proteins level. The p value of <0.05 was considered statistically significant.

Results

Characteristics of the women with pre-eclampsia

The clinical characteristics of the women with PE are shown in the Table 1. The mean age of women with PE was 32.00 ± 5.16 years. Out of 116 women with PE, 69 were primiparous. Early disease onset (before 37th week of gestation) was observed in 31 women. The mean gestation week at PE onset was 34.62 ± 4.42 . Regarding the disease severity, mild PE ($BP \leq 140/90$ mmHg) was classified in 27 women, moderate PE (BP from 140/90 to 150/100 mmHg) was classified in 54 women and severe PE ($BP \geq 160/110$ mmHg) was observed in 35 women. The mean highest systolic blood pressure of the women with PE was 154.00 ± 15.88 mmHg, the mean highest diastolic blood pressure was 98.53 ± 8.96 mmHg. Oedemas were observed in 88 women suffering from PE.

HLA-G 5'URR allele and genotype frequencies among women with PE and the control group

In 116 women with PE and 130 controls, 16 polymorphisms in the HLA-G 5'URR region have been analyzed: -762C/T (rs1632946), -725C/G/T (rs1233334), -716T/G (rs2249863), -689A/G (rs2735022), -666G/T (rs35674592), -646A>G (rs17875391), -633G/A (rs1632944), -540insG (rs17875392), -533delA (rs370236002), -509C/G (rs17875393), -486A/C

Tab. 3. HLA-G 5'URR haplotypes/3'UTR 14bp Ins/Del among women with pre-eclampsia and control group.

Haplotypes	Pre-eclampsia 2N (%)	Control group 2N (%)	p
5'URR-1 (TCGGTAACCGGGAIns)	96 (41.38%)	94 (36.15%)	0.2732
5'URR-2 (CCTAGAGCACGGCGDel)	71 (30.60%)	96 (36.92%)	0.1668
5'URR-3 (CGTAGAGCACGGCGDel)	29 (12.50%)	36 (13.85%)	0.7590
5'URR-4 (TCGGTAACCGGGAADel)	27 (11.64%)	21 (8.08%)	0.2393
5'URR-5 (CTTAGGGGAGAAAAIns)	3 (1.29%)	0 (0.00%)	0.2080
5'URR-6 (CCTAGAGdelACACGGCGDel)	2 (0.86%)	1 (0.39%)	0.9211
5'URR-7 (CTTAGAGinsGCACGGCGIns)	1 (0.43%)	1 (0.39%)	0.9356
5'URR-8 (CGTAGAGCCCGGCGDel)	1 (0.43%)	0 (0.00%)	0.9545
5'URR-9 (CTTAGGGCACAAAGIns)	1 (0.43%)	0 (0.00%)	0.9545
5'URR-10 (CTTAGAGCACGGCGIns)	1 (0.43%)	0 (0.00%)	0.9545
5'URR- 11 (CCTAGAGCCGGGAAIns)	0 (0.00%)	4 (1.54%)	0.1633
5'URR- 12 (TTGGTGAGAGAAAGIns)	0 (0.00%)	3 (1.15%)	0.2887
Others*	0 (0.00%)	5 (1.92%)	0.0944

Sample size: Pre-eclampsia 2N = 232, Control group 2N = 260. p values were calculated using χ^2 test. $p < 0.01$ was considered as statistically significant. n – number, UTR – untranslated region, Ins – insertion, Del – deletion. *Group of all haplotypes with frequency lower than 1%. Haplotype consensus sequences are represented by: -762C/T, -725C/G/T, -716G/T, -689G/A, -666T/G, -646A/G, -633G/A, -540insG, -533delA, -509C/G, -486C/A, -477G/C, -400G/A, -391G/A, -369A/C, -201A/G, 14bp Ins/Del

(rs1736933), -477G/C (rs1736932), -400G/A (rs17875395), -391G/A (rs17875396), -369A/C (rs1632943), -201G/A (rs1233333). Distribution of HLA-G 14 bp insertion/deletion polymorphism in the HLA-G 3'UTR region was described previously (30). Allele and genotype frequencies of the HLA-G 5'URR gene polymorphisms observed in analyzed groups are shown in Table 2. Genotype frequencies fit the Hardy–Weinberg equilibrium in women with PE ($\chi^2 = 0.002-2.01$, $p = 0.16-0.96$) as well as in the controls ($\chi^2 = 0.02-1.93$, $p = 0.16-0.89$). In the HLA-G 5'URR region, 5 SNPs, namely -762C/T, -716T/G, -689A/G, -666G/T, -633G/A, were in strong linkage disequilibrium (LD) and constituted a haplblock ($D' > 0.99$). The analysis of 16 HLA-G 5'URR variants revealed that -201AA carriers in recessive model (AA vs GG+GA) in PE group had significantly higher frequencies as compared to the controls ($p = 0.047$, OR = 1.77, 95% CI = 1.00–3.14). However,

after adjustment for age and +14bp Ins, no statistically significant differences in allele and genotypes frequencies between women with PE and the controls were observed ($p > 0.05$) (Tab. 2).

The distribution of the HLA-G 5'URR haplotypes in women with PE and the control group was also performed (Tab. 3). Phase software estimated 10 HLA-G 5'URR haplotypes in women with PE and 12 HLA-G 5'URR haplotypes in the control group. The HLA-G 5'URR-1 was the most frequent haplotype in women with PE, and HLA-G 5'URR-2 was the most frequent in the control group. No statistically significant differences in haplotype frequencies between PE patients and the controls were observed ($p > 0.05$) (Tab. 3).

Association of HLA-G 5'URR variants with clinical variables in women with PE

The association between 16 HLA-G 5'URR polymorphisms and clinical variables as the onset of PE, the highest systolic BP, the highest diastolic BP, serum creatinine, uric acid and total serum proteins level was performed in PE patients (Tab. 4). The analysis revealed that heterozygous -533delA-/+ carriers tended to have a significantly higher mean level of the highest diastolic BP as compared to other genotype carriers (112.50 ± 10.61 vs 98.16 ± 8.69 , $p = 0.02$). After adjustment for age and +14bp Ins positivity, a statistically significant correlation between the above mentioned carriers and the higher mean level of the diastolic BP was also found ($p = 0.003$). Further findings revealed that after correction for age and +14bp Ins positivity, a statistically significant association of -533delA-/+ carriers with the higher mean level of the systolic BP was determined ($p = 0.049$) (Tab. 4).

Tab. 4. Association of HLA-G 5'URR polymorphisms with clinical signs of PE.

Parameter	HLA-G 5'URR genotypes					
	Haplblock -762C/T, -716T/G, -689A/G, -666G/T, -633G/A					
	-762CC (n=28)	-762CT (n=53)	-762TT (n=35)	P/P* CM	P/P* DM	P/P* RM
Onset of PE, mean±SD (weeks)	34.00±4.45	34.67±4.23	35.00±4.79	0.77/0.74	0.61/0.50	0.50/0.59
The highest systolic BP (mm Hg), mean±SD	150.39±17.37	156.12±11.68	153.72±19.51	0.47/0.81	0.92/0.59	0.27/0.85
The highest diastolic BP (mm Hg), mean±SD	98.89±10.53	98.03±8.35	98.96±8.91	0.91/0.28	0.77/0.88	0.85/0.11
Serum creatinine (µmol/l), mean±SD	61.11±13.56	57.84±11.28	60.63±14.69	0.59/0.35	0.59/0.51	0.54/0.28
Uric acid (µmol/l), mean±SD	323.65±82.65	328.38±68.87	298.40±71.60	0.29/0.35	0.12/0.15	0.71/0.61
Total serum proteins (g/l), mean±SD	57.63±6.57	59.32±7.04	59.16±5.99	0.74/0.45	0.84/0.64	0.44/0.21
	-533delA-/ (n=114)	-533delA-/+ (n=2)	-533delA+/+ (n=0)	P/P* CM	P/P* DM	P/P* RM
Onset of PE, mean ± SD (weeks)	34.55±4.45	37.50±0.71	0.00±0.00	0.35/0.22	–	–
The highest systolic BP (mm Hg), mean±SD	153.55±15.77	171.00±12.73	0.00±0.00	0.13/ 0.049	–	–
The highest diastolic BP (mm Hg), mean±SD	98.16±8.69	112.50±10.61	0.00±0.00	0.02/0.003	–	–
Serum creatinine (µmol/l), mean±SD	59.12±12.75	73.05±14.64	0.00±0.00	0.13/0.07	–	–
Uric acid (µmol/l), mean±SD	318.85±73.91	281.20±21.35	0.00±0.00	0.48/0.66	–	–
Total serum proteins (g/l), mean±SD	59.04±6.55	54.17±6.61	0.00±0.00	0.31/0.28	–	–

p values were calculated using Student t test with Welch correction. $p < 0.05$ was considered as statistically significant, p* – statistical analysis adjusted by age and 14bp insertion, BP – blood pressure, n – number, CM – co-dominant model, DM – dominant model, RM – recessive model, SD – standard deviation

Discussion

Pre-eclampsia (PE) is one of the serious pregnancy-specific disease. Despite treatment, this multisystem disorder remains a major cause of maternal and neonatal mortality and morbidity worldwide. Biomarkers identifying the risk of PE were not available until now. According to clinical data, the potential markers of PE should relate to pathophysiology of the angiogenesis and inflammation (5, 6). Immunosuppressive molecule HLA-G belongs to candidate molecules with the potential to predict PE diagnosis. An important role of HLA-G in fetal development was described more than 20 years ago (7). The molecule is mainly expressed on cytotrophoblast cells of the placenta and induces the immune tolerance of fetus by maternal immune response (7, 9). It is accepted that expression of HLA-G may be influenced by the polymorphic variants in the 5' and 3' untranslated regions. In the 3'UTR region, a 14 bp insertion polymorphism (5'- ATTTGTTTCATGCCT-3'), which affects the stability of mRNA has been mostly analyzed (14). However, the meta-analyses of several smaller studies revealed no associations between the HLAG 14bp Ins/Del genotype and the development of PE (28, 36). The other polymorphisms in the 3'UTR that can influence soluble HLA-G level including SNPs at the +3142 (C/G), +3187 (A/G) and +3196 position (C/G). They can target certain miRNAs that are responsible for degrading HLA-G mRNA (+3142C/G) or are located just before and after an AUUUA motif associated with mRNA stability (+3187A/G, +3196C/G) (37). Polymorphisms in the HLA-G 5'URR region, i.e. in the promoter can also affect the regulation of gene expression (15, 38). However, there is no consensus, which HLA-G variants predispose to complications of pregnancy, such as recurrent miscarriages or pre-eclampsia.

In our study, we compared the allelic and genotypic frequencies of 16 HLA-G 5'URR polymorphisms (from -762 to -201bp) between the women suffering from PE and the controls. HLA-G 5'URR region (promoter) consists of the 1.4 kb upstream the ATG and includes several enhancer and suppressors elements to regulate HLA-G expression (16-18). Only few studies reported the association of HLA-G 5'URR region with a susceptibility to recurrent miscarriages to date (39–43). Ober et al (39) reported the highest fetal loss rates among couples, who carried -725G allele. Berger et al (40) found a significant association between -486A/C SNP and miscarriage. Agrawal and colleagues (42) reported the association of women carriers with mutant genotypes of -1179G/A, -725C/G/T and -486A/C with an increased risk of developing recurrent spontaneous abortion (RSA). They also have seen a decreased mRNA HLA-G expression for -1179AA and -725GG genotypes in cases of idiopathic RSA. Yazdani et al (43) found the impact of -1573T/C and -1746C/A SNPs in the promoter of the HLA-G gene on the risk to recurrent pregnancy loss. However, no study evaluated an association of HLA-G 5'URR region with a susceptibility to develop PE until now. Our results revealed that -201AA genotypes in a recessive model were significantly more frequent in women with pre-eclampsia than in the controls. As it is known that -201G/A polymorphism is located in the NF- κ B2 element (enhancer A), its possible involvement in affecting gene

expression can be hypothesized (38). It can be speculated that the minor allele at positions -201 may affect the binding of transcription factor leading to differences in HLA-G expression.

The distribution of the HLA-G 5'URR haplotypes including 14bp polymorphism at the 3'UTR in women with PE and the control group was also performed. The HLA-G 5'URR-1 was the most frequent haplotype in women with PE, and HLA-G 5'URR-2 was the most frequent in the control group. No statistically significant differences in haplotype frequencies between PE patients and the controls were observed. Hviid et al (44) reported that the HLA-G 5'URR-1 (composed by -762T, -725C, -716G, -689G, -666T, -646A, -633A, -509C, -486C, -477G, -400G, -391G, -369A, -201A, 14bp Ins) is the most frequent haplotype in human population.

In our study, the association between HLA-G 5'URR variants and the main clinical variables such as the onset of PE, the highest systolic BP, the highest diastolic BP, serum creatinine, uric acid and total serum proteins level was also evaluated. To our knowledge, such correlation analysis in PE cases wasn't reported until now. Our results revealed that heterozygous -533delA-/+ carriers tend to have the higher mean level of the highest diastolic BP as compared to other genotype carriers. After adjustment for age and +14bp Ins positivity, a statistically significant correlation between the above mentioned carriers and the higher mean level of the diastolic BP was also found. Endothelial dysfunction is the one of the main causes involved in the pathophysiology of PE (45). The impairment of the vascular endothelium leads to a disturbed balance between vasoconstrictive and vasodilatation substances. It was shown that sHLA-G inhibits endothelial cell proliferation, providing an evidence that HLA-G may play an essential role in vascular remodeling (46). It should be hypothesized that a decreased level of HLA-G resulting from -533delA-/+ polymorphism is one of the mechanisms to cause endothelial dysfunction leading to hypertension in PE patients.

Many studies showed that the decreased HLA-G level is associated with complications of pregnancy including pre-eclampsia (19-23, 47-49). Some studies reported the impact of HLA-G 5'URR polymorphisms on HLA-G expression. Higher HLA-G transcription rate in JEG-3 choriocarcinoma cell was described for the miscarriage-associated HLA-G -725G allele (50). It is in accordance with study of Jassem et al (41), who reported lower levels of sHLA-G in RPL women carriers for -725CC genotypes. Among other SNPs at 5'URR, the -716TT and -201GG genotypes have been associated with high soluble HLA-G levels (51). Concerning our results, the -201AA genotypes should be associated with a decreased HLA-G expression causing an impaired HLA-G function during pregnancy. Taken together, our observations suggest that HLA-G 5'URR variants may create an immunological microenvironment, which may affect the fetal-maternal tolerance. HLA-G gene variants seem to be promising biomarkers to further investigate their role in pathogenesis of PE.

Learning points

- This is the first study performing a comprehensive association analysis between 16 HLA-G 5'URR variants (promoter) and the risk of developing pre-eclampsia in Slovak women.

- The -201AA genotypes in recessive model were significantly more frequent in women with pre-eclampsia than in the controls.
- The -533delA/+ women carriers tend to have a significantly higher mean level of the diastolic blood pressure as compared to other genotype carriers.
- Our results suggest that HLA-G 5'URR variants may create an immunological microenvironment, which could affect the fetal-maternal tolerance.

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