

CLINICAL STUDY

Analysis of HLA-G gene polymorphisms in Slovak women with pre-eclampsia

Durmanova V¹, Drobny J², Shawkatova I¹, Dlhopolcek J², Bucova M¹*Institute of Immunology, Faculty of Medicine, Comenius University in Bratislava, Slovakia.*

vladimira.durmanova@fmed.uniba.sk

ABSTRACT

OBJECTIVES: To identify possible association between the selected HLA-G gene polymorphisms and risk of pre-eclampsia.

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

METHODS: 123 women with pre-eclampsia and 102 women with normotensive pregnancy were included in the study. HLA-G gene polymorphisms affecting its expression was determined, namely the HLA-G 14 bp insertion/deletion polymorphism in the 3'UTR and HLA-G 1597ΔC polymorphism tagging the HLA-G*01:05N null allele. Genotyping was performed by PCR and PCR-RFLP.

RESULTS: No statistically significant differences in either allele or genotype frequencies between pre-eclampsia cases and control group have been observed ($p > 0.05$).

CONCLUSION: Genetic predisposition of HLA-G to pre-eclampsia in Slovak women was examined for the first time. No association between analysed HLA-G gene polymorphisms and susceptibility to pre-eclampsia was observed. Further investigations are needed to determine the role of immunosuppressive molecule HLA-G in pre-eclampsia development (*Tab. 5, Fig. 2, Ref. 37*). Text in PDF www.elis.sk.

KEY WORDS: HLA-G, pre-eclampsia, gene polymorphism, genotyping, diagnostic.

Introduction

Pre-eclampsia (PE) is a serious pregnancy-specific disease affecting 2–5 % of pregnancies worldwide. This multisystem disorder is manifested by hypertension (systolic and diastolic blood pressure of $\geq 140/90$ mm Hg) and proteinuria (protein excretion of ≥ 300 mg in a 24 hrs urine collection). Advanced clinical symptoms include seizures, renal failure, IUGR (Intrauterine Growth Restriction) and/or HELLP (Hemolysis, Elevated Liver Enzymes and Low Platelets) syndrome. Finally, the generalized damage of the maternal endothelium, kidneys and liver can develop, leading to increased mortality of mother as well as fetus. The clinical symptoms of pre-eclampsia can be observed in the second or the third trimester of pregnancy and are most common in primiparas (1). Only symptomatic treatment is available to prevent hypertension or convulsive seizures. Radical resolution of pre-eclampsia includes the removal of the placenta and pre-term birth (2).

Despite many research studies, the pathology of pre-eclampsia is not fully understood. One cause may originate in an insufficiently developed placenta, referred to as poor placentation. It is characterized by impaired re-modeling of spiral arteries of the uterus caused by an imbalance of circulating angiogenic factors (3). Other key mechanisms leading to pre-eclampsia 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. 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 mediating immunosuppression. It is mainly expressed in extravillous cytotrophoblasts, which help to protect the fetus from maternal immune response (5). HLA-G is involved in inhibition of cytotoxic activity of uterine and peripheral blood NK cells and CD8+ T cells, the alloproliferative response of CD4+ T cells, inhibition of dendritic cell maturation and activation of regulatory T cells (6). Immunosuppressive activity of the HLA-G molecule is mediated mainly through its interaction with inhibitory receptors of immune cells: ILT-2 receptor present on B cells, APC and some T, and NK cells, ILT-4 receptor present on APC and KIR2DL4 expressed by NK and some T cells (6, 7). In addition to its immunosuppressive activity, HLA-G is involved in ensuring the successful invasion of placental tissues into the maternal decidua (8).

¹Institute of Immunology, Faculty of Medicine, Comenius University in Bratislava, Slovakia, and ²1st Department of Obstetrics and Gynecology, Faculty of Medicine and University Hospital Bratislava, Comenius University in Bratislava, Slovakia

Address for correspondence: V. Durmanova, Assoc. Prof., Institute of Immunology, Faculty of Medicine, Comenius University in Bratislava, Odborarske namestie 14, SK-813 72, Bratislava, Slovakia. Phone: +421.2.59357582

The HLA-G gene is 4170 bp long and consists of 8 exons. By an alternative splicing of the primary transcript, 7 HLA-G isoforms can be generated. Four isoforms HLA-G1, -G2, -G3 and -G4 are membrane bound, whereas three isoforms HLA-G5, -G6 and -G7 are soluble. Only the structure of HLA-G1 molecule resembles the structure of other full-length HLA-I molecules (9). HLA-G gene is characterized by low polymorphism, namely 53 HLA-G alleles, 18 HLA-G proteins and 2 null alleles have been identified until now (IMGT/HLA database, January 2017). The most polymorphic sites that influence HLA-G expression were identified in the 5' and 3' non-coding regions (10, 11). In the 3' UTR, the 14 bp insertion polymorphism (5'-ATTTGTTTCATGCCT-3') was described, that affects stability of mRNA causing a lower production of most membrane and soluble isoforms (10). The HLA-G*01:05N null allele characterized by a single base-pair deletion of cytosine (1597ΔC) in exon 3 of HLA-G is associated with an abolished expression of the full-length HLA-G protein isoforms HLA-G1 and HLA-G5 in women who are homozygous for the 1597ΔC mutation (12).

The role of HLA-G in pre-eclampsia was determined through observations of a decreased HLA-G expression in mothers suffering from PE (13, 14, 15). 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 risk of PE was investigated with controversial results. Some studies found a significant increase of the 14 bp insertion polymorphism and the HLA-G 01:05N null allele in women with PE in comparison to women with physiological pregnancies (16, 17, 18). However, other studies did not confirm the association of these polymorphisms with PE development (19, 20, 21). Our study investigated the possible genetic association of selected HLA-G gene polymorphisms and the risk of pre-eclampsia in Slovak pregnant women.

Materials and methods

Study subjects

We investigated 123 women suffering from pre-eclampsia and 102 women with physiological pregnancies. PE was defined as the presence of blood pressure of at least 140/90 mmHg with readings at least 6 hrs apart and proteinuria (300 mg/24 h or \geq 30 mg/dl by urine analysis (22). The clinical characteristics of the women with PE are shown in the Table 1. The mean age of women with PE was 32 ± 5.07 years; the mean age of controls was 36.32 ± 5.08 . All 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.

Genotyping of HLA-G gene polymorphisms

Both patient and control DNA was extracted from EDTA-treated peripheral blood samples by a modified salting out procedure (23). To quantify the amount and purity of DNA, the Nanodrop spectrophotometer was used (Thermo Fisher Scientific). Two

HLA-G gene polymorphisms were studied: the 14 bp insertion/deletion polymorphism in the 3' UTR (*rs66554220*) and the cytosine deletion at codon 130 in exon 3 (1597ΔC, *rs41557518*) tagging HLA-G*01:05N null allele. Genotyping of HLA-G 14-bp insertion/deletion polymorphism was performed as described by Hviid et al (24). Briefly, DNA was amplified by forward primer 5'GTGATGGGCTGTTTAAAGTGTACC-3' and reverse primer 5'GGAAGGAATGCAGTTCAGCATGA-3' using a PCR cycler (Biometra). 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), 1 unit of Taq DNA polymerase (Thermo Fisher Scientific), 1.5 mmol MgCl₂ (Thermo Fisher Scientific) and 10 pmol of each specific primer. PCR conditions were 95 °C for 3 min, followed by 30 cycles (denaturation at 95 °C for 1 min, annealing at 64 °C for 1 min and elongation at 72 °C for 1 min) and final elongation at 72 °C for 10 min. The PCR products were run in 3 % agarose gel for 20 min. and then visualized under UV-light. Fragment size was confirmed using the 100 bp DNA ladder (SBS). PCR fragments of 224 bp (14 bp insertion) and PCR fragments of 210 bp (14 bp deletion) were identified (Fig. 1). The presence of the HLA-G*01:05N null allele was performed by PCR-RFLP as described by Alizadeh et al (25). Genomic DNA was amplified by forward primer 5'CAGGTTCTCACACCCTCCAG3' and reverse primer 5'CCTCCACTCCCTCAGAGACTTCATC3'. PCR conditions were the same as by HLA-G 14 bp genotyping except for the annealing at 63 °C for 30 sec. Amplification of PCR products of 504 bp was confirmed in 1.5 % agarose gel. PCR products were digested with FastDigest pPPUMI restriction enzyme at 37 °C for 3 hrs, according to manufacturer's instructions (Thermo Fisher Scientific). The restriction products were run in 2 % agarose gel for 20 min, either producing an intact PCR fragment (presence of

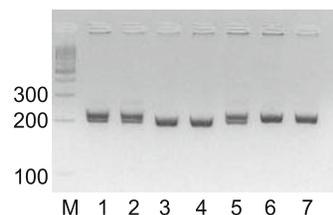


Fig. 1. HLA-G 14 bp ins/del genotyping by PCR. M – the 100 bp DNA ladder, Lane 1, 2, 5 – heterozygous 14 bp ins/del genotype (224 bp and 210 bp), Lane 3, 4 – homozygous 14 bp del genotype (210 bp), Lane 6, 7 – homozygous 14 bp ins genotype (224 bp).

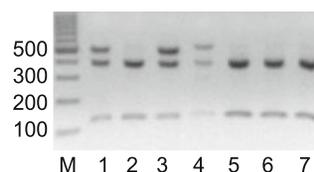


Fig. 2. HLA-G 1597ΔC genotyping by PCR-RFLP. M – the 100 bp DNA ladder, Lane 1, 3, 4 – heterozygous HLA-G 1597ΔC genotype tagging HLA-G*01:05N allele (504bp, 389 bp and 115 bp), Lane 2, 5, 6, 7 – homozygous HLA-G 1597C genotype (389 bp and 115 bp).

Tab. 1. Clinical characteristics of women with PE (n = 123).

Parameter	Mean±SD/Number
Age (years)	32±5.07
Onset of pre-eclampsia*	early – 47, late – 76
	mild – 27
Disease severity**	moderate – 59
	severe – 37
Edema	yes – 96, no – 27
Primiparous women	yes – 75, no – 48
Preterm birth	yes – 26, no – 97
The highest systolic blood pressure (mmHg)	154.36±16.01
The highest diastolic blood pressure (mmHg)	98.99±9.63
Leukocytes (x10 ⁹ /l)	10.58±2.61
Thrombocytes (x10 ⁹ /l)	214.91±73.17
Serum creatinine (µmol/l)	59.71±12.61
Uric acid (µmol/l)	312.08±78.06
ALT (µkat/l)	0.42±0.59
AST (µkat/l)	0.48±0.33
Total serum proteins (g/l)	59.03±6.51

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

the HLA-G*01:05N allele) or two fragments of 389 bp and 115 bp (absence of the HLA-G*01:05N allele) (Fig. 2).

Statistical analysis

Allele and genotype frequencies were calculated by a direct counting. The data were tested for the Hardy–Weinberg equilibrium. The statistical analysis was performed by chi-square test using SNPstats web software available at <http://bioinfo.iconcolgia.net/snpstats/start.htm>. The odds ratios (OR) and 95 % confidence intervals (95% CI) were calculated in co-dominant, dominant and recessive inheritance models. Multivariate logistic-regression analysis adjusted for age as a possible influencing factor was also performed. Linear regression analysis was used to investigate the correlation between observed HLA-G genotypes and the main clinical features 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 $p < 0.05$ was considered as 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 ± 5.07 years. Out of 123 women with PE, 75 were primiparous. Early disease onset (before 37th week of gestation) was developed in 47 women. The mean gestation week at PE onset was 34.76 ± 4.29 . Regarding the disease severity, mild PE (BP ≤ 140/90 mmHg) was classified in 27 women, moderate PE (BP from 140/90 to 150/100 mmHg) was classified in 59 women and severe PE (BP ≥ 160/110 mmHg) was observed in 37 women. The mean highest systolic blood pressure of the women with PE was 154.36 ± 16.01 mmHg, the mean highest diastolic blood pressure was 98.99 ± 9.63 mmHg. Oedemas were observed in 96 women suffering from PE.

HLA-G allele and genotypes frequencies in women with PE and controls

Genotyping was used to determine HLA-G 14 bp insertion/deletion polymorphism and HLA-G 1597ΔC polymorphism tagging the HLA-G*01:05N null allele in two studied groups: women with PE (n = 123) and controls (n = 102). Allele and genotype frequencies of HLA-G gene polymorphisms are shown in Tables 2 and 3. Frequencies of genotypes fit the Hardy–Weinberg equilibrium in controls (HLA-G 14 bp ins/del: $\chi^2 = 0.16939$, $p = 0.68065$, HLA-G*01:05N null allele: $\chi^2 = 0.33134$, $p = 0.56487$) as well as in women with PE (HLA-G 14 bp: $\chi^2 = 0.40268$, $p = 0.52571$, HLA-G*01:05N null allele: $\chi^2 = 0.76662$, $p = 0.38127$). No statistically significant differences in allele and genotypes frequencies for HLA-G 14 bp deletion/insertion polymorphism between women with PE and women with physiological pregnancy were observed (Tab. 2). In the group with PE, 20.33 % of women carried the homozygous +14/+14 bp genotype vs 19.61 % of women in the control group ($p = 0.89$, OR = 1.05, 95% CI = 0.54–2.02). The allele and genotype frequencies for the 1597ΔC polymorphism

Tab. 2. HLA-G 14 bp allele and genotypes frequencies in women with PE and control group.

Allele/genotype	Women with PE (n=123)	Control group (n=102)	Univariate analysis		Multivariate analysis*	
			p	OR (95%CI)	p	OR (95%CI)
-14	139 (56.50%)	116 (56.86%)				
+14	107 (43.50%)	88 (43.14%)	0.94	1.02 (0.70–1.48)	–	–
-14/-14	41 (33.33%)	34 (33.33%)		1.00		1.00
-14/+14	57 (46.34%)	48 (47.06%)	0.99	0.98 (0.54–1.79)	0.6	0.79 (0.40–1.54)
+14/+14	25 (20.33%)	20 (19.61%)		1.04 (0.49–2.18)		1.14 (0.50–2.60)
-14/-14	41 (33.33%)	34 (33.33%)		1.00		1.00
-14/+14 and +14/+14	82 (66.67%)	68 (66.67%)	1	1.00 (0.57–1.75)	0.7	0.89 (0.48–1.65)
-14/-14 and -14/+14	98 (79.67%)	82 (80.39%)		1.00		1.00
+14/+14	25 (20.33%)	20 (19.61%)	0.89	1.05 (0.54–2.02)	0.47	1.31 (0.63–2.71)
-14/-14 and +14/+14	66 (53.66%)	54 (52.94%)		1.00		1.00
-14/+14	57 (46.34%)	48 (47.06%)	0.91	0.97 (0.57–1.64)	0.34	0.75 (0.41–1.36)

Allele and genotype frequencies are presented as absolute numbers with percentages in parentheses. OR – odds ratio; CI – confidence interval. Univariate analysis is based on χ^2 test. *Multivariate analysis is adjusted by age. n – number, -14 – HLA-G 14 bp deletion, +14 – HLA-G 14 bp insertion.

Tab. 3. HLA-G*01:05N allele and genotypes frequencies in women with PE and control group.

Allele/ genotype	Women with PE (n=123)	Control group (n=102)	Univariate analysis		Multivariate analysis	
			p	OR (95%CI)	p	OR (95%CI)
C	228 (92.68%)	193 (94.61%)				
–C	18 (7.32%)	11 (5.39%)	0.41	1.39 (0.64–3.01)		–
C/C	105 (85.37%)	91 (89.22%)		1.00		1.00
–C/C	18 (14.63%)	11 (10.78%)	0.39	1.42 (0.64–3.16)	0.21	1.78 (0.72–4.37)
–C/–C	0 (0.0%)	0 (0.0%)	–	–	–	–
C/C	105 (85.37%)	91 (89.22%)		1.00		1.00
–C/C and –C/–C	18 (14.63%)	11 (10.78%)	0.39	1.42 (0.64–3.16)	0.21	1.78 (0.72–4.37)

Allele and genotype frequencies are presented as absolute numbers with percentages in parentheses. OR – odds ratio; CI – confidence interval. Univariate analysis is based on χ^2 test. Multivariate analysis is adjusted by age. C – absence of HLA-G*01:05N, minus C – presence of HLA-G*01:05N

Tab. 4. Association of HLA-G 14 bp polymorphism with clinical signs of PE.

Parameter	–14/–14	–14/+14	+14/+14	P/P*	P/P*	P/P*
	(n=41)	(n=57)	(n=25)	CM	DM	RM
Onset of PE, mean±SD (weeks)	34.57±0.72	35.1±1.22	36.37±1.6	0.73/0.96	0.59/0.79	0.45/0.96
The highest systolic BP (mmHg), mean±SD	140.89±6.06	147.64±4.45	136.04±10.7	0.56/0.46	0.77/0.34	0.4/0.75
The highest diastolic BP (mmHg), mean±SD	96.36±1.53	96.18±2.73	96.28±4.55	1.0/0.33	0.97/0.14	1.0/0.43
Serum creatinine ($\mu\text{mol/l}$), mean±SD	120.5±15.86	173.39±19.47	108.54±18.71	0.12/0.65	0.28/0.62	0.23/0.58
Uric acid ($\mu\text{mol/l}$), mean±SD	333.84±12.03	321.3±8	298.32±12.02	0.35/0.56	0.27/0.98	0.18/0.33
Total serum proteins (g/l), mean±SD	59.81±1.36	59.79±1.65	78.47±18.21	0.28/0.53	0.54/0.94	0.11/0.29

BP – blood pressure, –14 – HLA-G 14 bp deletion, +14 – HLA-G 14 bp insertion, CM – codominant model, DM – dominant model, RM – recessive model, * statistical analysis adjusted by age

Tab. 5. Association of HLA-G*01:05N allele with clinical signs of PE.

Parameter	C/C	C/–C	–C/–C	P/P*	P/P*	P/P*
	(n=105)	(n=18)	(n=0)	CM	DM	RM
Onset of PE (weeks), mean±SD	35.41±0.76	33.56±1.35	–	0.49/0.74	–	–
The highest systolic BP (mm Hg), mean±SD	144.53±3.59	129.17±14.57	–	0.24/0.42	–	–
The highest diastolic BP (mm Hg), mean±SD	95.41±1.79	103.33±3.58	–	0.22/0.29	–	–
Serum creatinine ($\mu\text{mol/l}$), mean±SD	144.89±12.48	106.97±19.71	–	0.42/0.15	–	–
Uric acid ($\mu\text{mol/l}$), mean±SD	319.31±6.9	326.13±7.58	–	0.82/0.21	–	–
Total serum proteins (g/l), mean±SD	64.86±4.57	57.6±3.19	–	0.65/0.97	–	–

* statistical analysis adjusted by age, BP – blood pressure, C – absence of HLA-G*01:05N, minus C – presence of HLA-G*01:05N, CM – codominant model, DM – dominant model, RM – recessive model

tagging the HLA-G*01:05N null allele showed no differences between the PE cases and controls as well (Tab. 3). In the group with PE, 14.63 % of women carried one copy of the 1597 Δ C allele vs 10.78 % of normotensive women ($p = 0.34$, OR = 1.42, 95% CI = 0.64–3.16). Women carrying two copies of the 1597 Δ C allele were not identified in any group. Multivariate logistic-regression analysis adjusted for age showed slightly decreased p values for both HLA-G gene polymorphisms in comparison with univariate analysis, however no statistically significant difference was found. The effect of HLA-G genotypes on clinical findings like the onset of PE, the highest systolic BP, the highest diastolic BP, level of serum creatinine, uric acid and total serum proteins level have been analyzed as well. As given in Tables 4 and 5, comparison of investigated clinical findings with HLA-G genotypes did not reveal any significant differences ($p > 0.05$).

Discussion

Pre-eclampsia is one of very serious complications during pregnancy. Despite treatment, this multisystem disorder still remains a major cause of maternal and neonatal mortality and mor-

bidity worldwide. Several promising markers identifying the risk of PE have been described; however, no screening test is available up to date. According to clinical data, the potential biomarkers of PE should be connected with pathophysiology of the angiogenesis and inflammation (26). 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 (5). The molecule is mainly expressed on trophoblast cells in the placenta and induces the immune tolerance of fetus by maternal immune response (5, 6). In relation to pregnancy disorders, an association between HLA-G polymorphism, HLA-G level and HLA-G function was described (6). 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 have analyzed two HLA-G gene polymorphisms affecting its expression: HLA-G 14 bp insertion/deletion polymorphism in the 3' UTR and HLA-G 1597 Δ C polymorphism tagging HLA-G*01:05N null allele.

The 14-bp ins/del (rs 66554220) polymorphism in the 3' UTR is the most studied HLA-G gene polymorphism. This polymor-

phism has been shown to influence HLA-G mRNA transcript size and stability (10). The 14 bp insertion polymorphism has been associated with lower levels of soluble HLA-G suggesting its role in PE development (13, 27). We did not find any statistically significant differences in HLA-G 14 bp ins/del allele and genotype distribution between women with PE and controls as given by others (19, 20, 28, 29). The newest meta-analysis of several smaller studies also revealed no associations between the HLA-G 14 bp ins/del genotype and development of PE (30). However, some studies found a significant increase of the +14/+14 genotype in women with PE in comparison to women with physiological pregnancies (16, 17).

Within HLA-G gene polymorphisms, two null alleles, HLA-G*01:03N and HLA-G*01:05N, have been described. The HLA-G*01:05N null allele is characterized by a single base pair deletion cytosine in exon 3 (1597ΔC), which inhibits production of the full-length HLA-G protein isoform HLA-G1 and soluble HLA-G5. However, this null allele retains its ability to translate membrane – bound HLA-G2 and HLA-G3 and soluble HLA-G6 and HLA-G7 isoforms (12). Our study did not reveal any association of heterozygotes carrying HLA-G*01:05N null allele with an increased risk of PE as given by others (21). This finding suggests that heterozygotes could compensate the lack of full length HLA-G1 isoform that contributes to proper invasion of maternal spiral arteries by extravillous cytotrophoblast. Our observations did not confirm the results published by Loisel et al (18), who found a significantly increased frequency of the maternal 1597ΔC allele in African-American women with PE compared to normal pregnancies ($p = 0.00027$). Controversial results could be explained by different HLA-G*01:05N null allele distributions in various ethnic populations. The HLA-G*01:05N allele is relatively frequent allele in African-Americans (7.4 to 8 %). The lower frequency was found in Middle Europeans (2.3 %) and Northern Europeans (0.6 %) (31, 32, 33). Above all, the frequency of HLA-G*01:05N allele was the highest in Iranian populations (20 %) (34). Our study found out that in healthy Slovak women the frequency of HLA-G*01:05N null allele was 5.39 %, in Slovak women with PE the frequency of HLA-G*01:05N null allele has increased to 7.32 %. Homozygous women carrying the HLA-G*01:05N null allele were not found in our study.

Many studies have shown that the HLA-G level was associated with complications of pregnancy like recurrent miscarriages and preeclampsia (14, 15, 29, 35, 36, 37). Our results did not reveal any association between the HLA-G 14 bp polymorphism and the 1597ΔC polymorphism tagging the HLA-G*01:05N null allele and the risk of pre-eclampsia in Slovak women. However, other HLA-G polymorphisms affecting its expression should also be investigated in order to determine the risk factor for PE. The polymorphisms in the 3'UTR that can influence soluble HLA-G level include SNPs at the +3142 position (C/G), at the +3187 (A/G) and +3196 (C/G). The +3142 polymorphism is a target for certain miRNAs that are responsible for degrading HLA-G mRNA. Furthermore, the +3187 and the +3196 SNPs are located just before and after an AUUUA motif associated with mRNA stability (11).

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Received April 26, 2017.

Accepted May 15, 2017.