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ORIGINAL RESEARCH

The maternal *HLA-G* 1597ΔC null mutation is associated with increased risk of pre-eclampsia and reduced HLA-G expression during pregnancy in African-American women

Dagan A. Loisel[1,5], Christine Billstrand[1], Kathleen Murray[1], Kristen Patterson[1], Tinnakorn Chaiworapongsa[3,4], Roberto Romero[4], and Carole Ober[1,2,*]

[1]Department of Human Genetics, The University of Chicago, 920 E 58th St., Chicago, IL 60637, USA [2]Department of Obstetrics and Gynecology, The University of Chicago, 920 E 58th St.,Chicago, IL 60637, USA [3]Department of Obstetrics and Gynecology, Wayne State University School of Medicine, Detroit, MI, USA [4]Perinatology Research Branch, Program for Perinatal Research and Obstetrics, Division of Intramural Research, NICHD/NIH/DHHS, Bethesda, MD, USA

*Correspondence address. Tel: +1-773-834-0753; Fax: +1-773-834-0505; E-mail: c-ober@genetics.uchicago.edu

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ABSTRACT: The non-classical major histocompatibility complex molecule, human leukocyte antigen (HLA)-G, is thought to contribute to maternal immune tolerance and successful placentation during pregnancy. Genetic polymorphisms in *HLA-G* are known to influence expression levels as well as the relative expression of individual protein isoforms. As diminished or aberrant HLA-G expression patterns may contribute to the development of certain pregnancy complications, we sought to investigate the association between functional *HLA-G* polymorphisms and the risk of pre-eclampsia (PE) in African-American women. The association between maternal and fetal genotype at six *HLA-G* polymorphisms and risk of PE was assessed in 372 pregnancies (314 normotensive; 58 pre-eclamptic). We observed an elevated risk of PE ($P = 0.00027$) in pregnancies where the mother carried the 1597ΔC allele, a null allele that abolishes expression of full-length HLA-G isoforms. Furthermore, the frequency of the maternal 1597ΔC allele was highest in the subset of pre-eclamptic pregnancies that were delivered preterm, suggesting an association between the null allele and the severity of PE. We then replicated the association between higher maternal 1597ΔC allele frequency and increased severity of PE ($P = 0.038$) in an independent sample of 533 African-American women. Finally, to investigate the mechanistic basis of this association, we measured circulating soluble HLA-G (sHLA-G) concentrations in maternal serum collected during pregnancy in 51 healthy, normotensive African-American control women and found significantly lower levels in women carrying the 1597ΔC allele ($P = 0.012$). These results demonstrate that maternal *HLA-G* genotype is significantly associated with risk of PE in African-American women and is predictive of circulating sHLA-G levels during pregnancy.

Key words: genetic predisposition / DNA variants / soluble HLA-G / toxemia of pregnancy / mutation

Introduction

Pre-eclampsia (PE) is a multisystem disorder characterized by the onset of gestational hypertension and proteinuria in the second half of pregnancy (Sibai, 2003; Steegers *et al.*, 2010). PE occurs in 2–5% of all pregnancies in the USA (Wallis *et al.*, 2008; Berg *et al.*, 2009) and is a major contributor to maternal and fetal mortality and morbidity (Sibai *et al.*, 2005). The precise etiology and pathogenesis of PE remains incompletely understood; however, it is generally accepted that the syndrome emerges from the action of multiple genetic, environmental and behavioral factors in the mother and the fetus (Roberts and Gammill, 2005; Redman and Sargent, 2005; Sibai *et al.*, 2005). Furthermore, abnormal interactions between the maternal immune response and fetal-specific factors may contribute to the incidence and severity of PE (Roberts and Gammill, 2005; Steegers *et al.*, 2010).

[5] Present address: Department of Biology, Saint Michael's College, Burlington, VT, USA.

Familial inheritance of PE has been long recognized (Chesley *et al.*, 1968), suggesting a genetic contribution to the syndrome. Indeed, both maternal and fetal (i.e. paternally derived) genetic factors have been associated with a predisposition to PE (Lie *et al.*, 1998; Esplin *et al.*, 2001). Candidate gene association studies have linked PE risk to genes involved in specific pathophysiological pathways, including the renin–angiotensin system, thrombophilia and hypofibrinolysis, oxidative stress and lipid metabolism, angiogenesis/anti-angiogenesis, endothelial dysfunction, intravascular inflammation and immune function (Redman and Sargent, 2005; Sibai *et al.*, 2005; Mutze *et al.*, 2008; Romero *et al.*, 2008; Williams and Morgan, 2012). Among the genes involved in immunity, the human leukocyte antigen (HLA) gene, *HLA-G*, has been identified as a potential contributor to PE risk.

HLA-G is a non-classical HLA class Ib molecule with immunomodulatory properties that is thought to be involved in maternal tolerance of the semi-allogenic fetus. During pregnancy, HLA-G is highly expressed at the maternal–fetal interface by fetal extravillous cytotrophoblast cells (Kovats *et al.*, 1990). Elevated levels of soluble HLA-G (sHLA-G) have been observed in the maternal peripheral blood during pregnancy (Hunt *et al.*, 2000; Pfeiffer *et al.*, 2000; Steinborn *et al.*, 2007; Rizzo *et al.*, 2009), and those levels likely reflect HLA-G expression from both fetal placental cells and maternal immune cells, such as monocytes (Alegre *et al.*, 2007; Feger *et al.*, 2007). A role for HLA-G in the pathogenesis of PE is supported by several lines of evidence. First, circulating sHLA-G levels during pregnancy were significantly lower in women who subsequently experienced PE compared with women with uncomplicated, successful pregnancies (Yie *et al.*, 2005; Hackmon *et al.*, 2007; Steinborn *et al.*, 2007; Rizzo *et al.*, 2009). Second, genetic variation in the *HLA-G* gene has been associated with variation in circulating sHLA-G levels during pregnancy (Hviid *et al.*, 2004; Chen *et al.*, 2008). Third, HLA-G polymorphisms, including those associated with circulating levels, have been associated with increased risk of PE in some studies (O'Brien *et al.*, 2001; Moreau *et al.*, 2008; Tan *et al.*, 2008; Yie *et al.*, 2008; Larsen *et al.*, 2010), although not in others (Aldrich *et al.*, 2000; Lin *et al.*, 2006; Vianna *et al.*, 2007; Iversen *et al.*, 2008).

Despite evidence for HLA-G playing an important role during pregnancy, the precise relationship between genetic variation in *HLA-G*, levels of circulating sHLA-G and PE remains unresolved. To date, few studies have examined all the three in the same population. In addition, studies showing an association between *HLA-G* polymorphisms and sHLA-G levels have focused primarily on women of European ancestry. Consequently, they do not provide insight into the functional consequences of polymorphisms, such as the 1597ΔC null mutation, that are rare in Europeans but more common in other global populations (Matte *et al.*, 2000; Aldrich *et al.*, 2002). The focus on PE in women of European ancestry also fails to provide insight into the factors contributing to the disorder in other, more vulnerable populations. For example, few studies have focused on the genetic basis of PE in African-American women, despite rates of PE being consistently higher in that group (Samadi *et al.*, 1996; Caughey *et al.*, 2005; Tanaka *et al.*, 2007; Gong *et al.*, 2012).

To better understand the functional and clinical effects of *HLA-G* polymorphisms in an at-risk population, we examined *HLA-G* genetic variation in African-American mothers and infants enrolled in the Chicago Lying-In Pregnancy Program (CLIPP; Joseph *et al.*, 2008; Neidich *et al.*, 2008), and tested for an association between the observed genetic variation and risk and severity of PE. Next, we assessed the reproducibility of the observed genetic association in an independent population of African-American women with PE. Finally, we examined the functional consequences of the 1597ΔC allele, which abolishes expression of full-length transmembrane G1 and soluble G5 protein isoforms, on circulating sHLA-G levels in uncomplicated, term pregnancies. This study represents one of the most comprehensive studies of an important *HLA-G* polymorphism in an underrepresented minority population at high risk for PE.

Materials and Methods

Study subjects

Self-reported African-American women were identified through retrospective surveys of the CLIPP biobank (Joseph *et al.*, 2008; Neidich *et al.*, 2008). An initial sample of 69 African-American pre-eclamptic pregnancies was identified in the CLIPP delivery records, excluding women with chronic hypertension, pre-conceptual or gestational diabetes mellitus, multiple gestations or fetal anomalies. PE was defined as blood pressure of at least 140/90 mmHg with readings at least 6 h apart and proteinuria (300 mg/24 h or $\geq$30 mg/dl on random urine analysis; Gifford *et al.*, 2000; Lindheimer *et al.*, 2010). We then selected four to five women with normotensive term pregnancies (same exclusion criteria) for each woman with PE from the CLIPP database, matching for age ($\pm$3 years), race (African-American) and parity (0 versus $\geq$1). After confirming diagnoses and exclusions by medical record reviews and on the basis of availability of DNA samples, a total of 372 women remained: 58 with PE and 314 with a normotensive term delivery. In this final sample, there was a non-significant excess of nulliparous women among the pre-eclamptics compared with the controls (46.6 versus 33.0%, $P = 0.068$) (Table I). Among the pre-eclamptic pregnancies, 23 women delivered preterm and 35 women delivered at term. This study was approved by the University of Chicago Institutional Review Board and all participants gave written informed consent.

To replicate our findings in the CLIPP Chicago population, *HLA-G* variation was examined in a second population of African-American women (also by self-report) with preterm PE ($n = 98$), term PE ($n = 151$) and term normotensive, uncomplicated (i.e. control) pregnancies ($n = 284$). These pregnancies were identified by searching the clinical database and the bank of biological specimens of the Perinatology Research Branch and Wayne State University, Detroit, MI. All the patients provided written informed consent for the collection and use of samples for research purposes (including DNA) under the protocols approved by the Institutional Review Boards of Wayne State University and the Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Department of Health and Human Services (NICHD/NIH/DHHS).

Women were excluded from these studies if their pregnancies were associated with chronic hypertension, known major fetal or chromosomal anomaly or multiple gestations. All the women were enrolled at Hutzel Women's Hospital, Detroit, MI, and followed until delivery. PE was defined as a systolic blood pressure of at least 140 or a diastolic blood pressure of at least 90 mmHg on two separate readings at least 4 h apart and the presence of proteinuria (300 mg protein in 24 h or >30 mg/dl on two occasions at least 4 h apart; Sibai *et al.*, 1997; Gifford *et al.*, 2000). The control pregnancies were considered to be uncomplicated if there were no major medical, obstetrical or surgical complications and delivery occurred at term (>37 weeks) with an infant whose birthweight was appropriate for gestational age (10–90th percentile) according to the reference range for the USA (Alexander *et al.*, 1996).

Table I Clinical characteristics of the CLIPP study participants, with PE cases stratified by preterm versus term delivery.

	Pre-eclampsia			Controls (n = 314)
	All (n = 58)	Preterm (n = 23)	Term (n = 35)	
Mean age, years (SD)	25.1 (6.0)	24.4 (6.3)	25.6 (5.7)	25.6 (5.0)
% Nulliparous	46.6	39.1	51.4	33.0
Parity range	0–4	0–3	0–4	0–6
Mean gestational age at delivery, weeks (SD)	36.1 (0.2)	32.2 (4.1)	38.7 (1.2)	39.8 (1.1)
% Primigravida	32.8	26.1	37.1	21.8
Gravidity range	1–8	1–7	1–8	1–10
Infant sex, % male	45.6	36.4	51.4	52.1
Mean infant birthweight, g (SD)	2625.9 (983.2)	1731.9 (875.9)	3214.0 (474.7)	3277.9 (438.3)
Mean maximum systolic blood pressure in labor (SD), mmHg	164.3 (13.2)	170.3 (13.4)	160.3 (11.6)	<140
Mean maximum diastolic blood pressure in labor (SD), mmHg	103.8 (11.9)	108.8 (12.3)	100.5 (10.5)	<90

HLA-G genotyping

For the CLIPP subjects, maternal DNA was extracted from EDTA-anticoagulated whole blood using an isopropanol precipitation-based protocol using an Autogen AGF3000 DNA Extractor (Autogen, Inc., Holliston, MA). Infant DNA was extracted from either cord blood samples, using Gentra Puregene DNA isolation kits (Qiagen, Valencia, CA) or from placental villous tissue. In the latter case, ~100 mg of placental villous tissue was obtained from the manual dissection of placental tissue under a microscope at 40× power. After isolating the placental villous tissue from the maternally derived tissues, it was washed twice in sterile Hank's balanced salt solution and then used for whole genome DNA extraction (Gentra Puregene kit). DNA concentration and purity were assessed by UV spectrophotometry and automated fluorimetric quantification (PicoGreen dsDNA Quantification Kit; Molecular Probes, Eugene, OR).

For the Detroit subjects, genomic DNA was isolated from 150 μl of buffy coat with an EZ1 DNA Blood 350 μl kit (Qiagen, USA) using an EZ1 instrument (Qiagen, USA). A DropSense 96 spectrophotometer (Trinean NV, Belgium) was used to quantify the amount of DNA prior to amplification. The whole genome amplification from genomic DNA was performed with REPLI-g Midi Kit (Qiagen, USA) according to the manufacturer's instruction. Briefly, 3 μl genomic DNA (>10 ng) from each specimen was mixed with 2 μl of Buffer D3 in individual wells in a 96-well plate and incubated at room temperature for 5 min to denature the DNA. Master mix (44 μl) that contained stop solution, water, REPLI-g Midi reaction buffer and REPLI-g Midi DNA polymerase was added to the denatured DNA. The mixture was incubated at 30°C for 8–16 h. After the incubation, the REPLI-g DNA polymerase was inactivated by heating the sample at 65°C for 3 min. The amplified DNA was stored at −20°C until further use.

DNA samples were genotyped for six *HLA-G* polymorphisms using a modification of the SNaPshot (Applied Biosystems, Carlsbad, CA) protocol described in Tan *et al.* (2008) that also included the 3′UTR +3142 (G/C) polymorphism from Tan *et al.* (2007). Polymorphisms were chosen on the basis of previously published associations with functional or clinical phenotypes and to capture the major haplotype structure of the gene. The six polymorphisms studied were: (i) the promoter variant −725 (G/C/T; rs1233334) associated with sporadic miscarriage (Ober *et al.*, 2003) and variation in gene expression (Ober *et al.*, 2006; Jassem *et al.*, 2012); (ii) the +36 (G/A) polymorphism (rs1630185) in the untranslated first exon that differentiates the two major promoter clades (Tan *et al.*, 2005); (iii) the 1597ΔC single-base insertion/deletion polymorphism (rs41557518) that prevents expression of full-length HLA-G protein isoforms (Ober *et al.*, 1998) and has been associated with recurrent miscarriage (Aldrich *et al.*, 2001; Pfeiffer *et al.*, 2001); (iv) the non-conservative amino acid substitution (rs12722482) in exon 4 at codon 258 (Thr→Met) that has been associated with PE (Moreau *et al.*, 2008; Tan *et al.*, 2008); (v) the 14 bp insertion/deletion polymorphism (rs66554220) in the 3′UTR that has been associated with PE risk (Hylenius *et al.*, 2004; Moreau *et al.*, 2008; Larsen *et al.*, 2010), *HLA-G* transcript levels (Hviid *et al.*, 2003) and circulating sHLA-G levels in plasma (Chen *et al.*, 2008) and (vi) the +3142 (G/C) polymorphism (rs1063320) in the 3′UTR that disrupts a micro(mi)RNA target site and influences HLA-G expression in the presence of specific miRNAs (Tan *et al.*, 2007).

Detection of sHLA-G

sHLA-G protein (specifically, soluble G5 and shed transmembrane G1) was measured in serum from CLIPP participants using the EXBIO (Vestec, Czech Republic)/BioVendor (Brno, Czech Republic) ELISA kit, according to the manufacturer's instructions. For each well, absorbance at 450 nm was measured using a SpectraMax Plus^{384} Absorbance Microplate Reader (Molecular Devices, Sunnyvale, CA). sHLA-G concentrations of samples were determined using a calibration curve constructed from four-parameter logistic curve fitting of the mean absorbance of calibrators of known concentration. Each individual's serum sample was run in duplicate on the same ELISA plate; individuals who showed a coefficient of variation greater than 35% were excluded from further analyses. The mean sHLA-G concentration for each serum sample was used for subsequent analyses. The estimated mean sHLA-G concentration was below the limits of detection for eight women (five who did not carry the 1597ΔC allele and three who did carry it) and above the limits of detection for one individual. Because of this censoring, rank-based tests were used to subsequently assess the relationship between sHLA-G and clinical or genetic data. For the purposes of visualization and inclusion in the rank-based tests, the eight individuals with sHLA-G below the limit of detection were assigned a value of 0.00001 and the one above the limit of detection was assigned a value of 250 U/ml.

Statistical analyses

Two tests were used to assess *HLA-G* genetic associations with PE in CLIPP subjects. First, an allele test was used to compare allele counts in cases and controls using a 2×2 contingency table assessed by the Pearson chi-square test. Because the −725 polymorphism was tri-allelic (C/G/T), each of the two minor alleles was analyzed separately. Specifically, the *P*-value for the T allele corresponds to the difference in the number of T and non-T alleles in cases compared with controls, and the *P*-value for the G allele corresponds to the difference in the number of G and non-G alleles in cases versus controls. Second, a genotype test was used to compare genotype counts in PE cases and controls using a 2×3 contingency table assessed by the Pearson chi-square test or Fisher's exact probability text (one-tailed) when cell frequencies were equal to or less than five. At the tri-allelic −725 polymorphism, the six genotypes observed in mothers and five genotypes observed in infants were compared in 2×6 and 2×5 contingency tables, respectively.

The Cochran–Armitage linear trend test was used to test for an association between 1597ΔC genotype frequencies and increasing severity of PE. Genotype counts at 1597ΔC were compared across three groups ordered by severity (normotensive term pregnancy = 0, term pre-eclamptic pregnancy = 1, preterm pre-eclamptic pregnancy = 2). Because of the rarity of 1597ΔC allele homozygotes (only 6 homozygotes in 847 individuals), the trend test was performed in a 2×3 table comparing the CC genotype count with the pooled CT/TT genotype count. The trend test was implemented in the coin package (Hothorn *et al.*, 2008) in R (version 2.15.0) using a standardized scalar test statistic, an alternative (one-sided) hypothesis and a null distribution approximated by Monte-Carlo resampling (2 000 000 random permutations). The association signals (*P*-values) obtained from the trend tests in the CLIPP and Detroit samples were combined using Stouffer's weighted Z-method (Whitlock, 2005), as implemented in the survcomp package in R.

The relationship between serum sHLA-G concentration and estimated gestational age and trimester at collection was assessed using the Wilcoxon rank sum test. The association between the presence/absence of the 1597ΔC allele in the mother and estimated serum sHLA-G concentration was also assessed using the Wilcoxon rank sum test, as implemented in the JMP software (SAS institute Inc., Cary, NC), version 10.0.0.

Results

Clinical characteristics of the CLIPP pregnancies diagnosed with PE and normotensive term pregnancies (i.e. controls) are described in Table I. PE cases and controls did not differ significantly in percent nulliparity, percent primigravida, maternal age or infant sex ratio (Table I). Not unexpectedly, PE pregnancies were delivered earlier than those of controls and, as a result, their infants were on average of lower birthweight. However, birthweights between term infants of PE and control pregnancies were not significantly different ($P = 0.74$).

HLA-G allele frequencies in PE cases and controls

Allele frequencies in the mothers did not differ in the 44 PE cases compared with the 271 controls for five of the six *HLA-G* polymorphisms; only the 1597ΔC polymorphism showed a significant difference between the two groups ($P = 0.00027$; Table II). Specifically, the frequency of the 1597ΔC allele was nearly three times higher in the PE cases (15.9%) compared with the controls (5.4%). The 1597ΔC allele frequency in the controls was similar to frequencies observed in previous studies of African-American individuals (Ishitani *et al.*, 1999; Aldrich *et al.*, 2002). The genotype frequency distribution for the 1597ΔC polymorphism also differed between the cases and the controls ($P = 0.0029$; Table III): women carrying one or two copies of the 1597ΔC allele had a significantly increased risk of PE [odds ratio (OR) = 3.39; 95% confidence interval (CI): 1.56–7.34].

HLA-G allele frequencies in the infants of 47 pre-eclamptic pregnancies and 281 control pregnancies did not differ for any of the six polymorphisms (Table IV). Although the 1597ΔC allele occurred at a higher frequency in the infants from PE pregnancies (11.7%) compared with infants from control pregnancies (6.6%), this difference was not significant ($P = 0.078$; Table IV) and could just reflect the higher allele frequency in the mothers with pre-eclamptic pregnancies.

Table II Allele frequencies for six *HLA-G* polymorphisms in 44 women with PE and 271 healthy, normotensive control women from the CLIPP study.

Polymorphism	Allele	PE cases	Controls	*P*-value
−725C/G/T	C	0.90	0.83	
	G	0.049	0.065	0.58[a]
	T	0.049	0.10	0.11[a]
+36G/A	A	0.61	0.55	0.36
	G	0.39	0.45	
1597ΔC	C	0.84	0.95	0.00027
	Δ	0.16	0.054	
Thr258Met	Thr	0.99	0.99	0.53
	Met	0.012	0.0077	
14-bp indel	Del	0.54	0.58	0.42
	Ins	0.46	0.42	
+3142G/C	G	0.64	0.66	0.76
	C	0.36	0.34	

[a]Because the −725 polymorphism was tri-allelic, allele counts for each of the minor alleles were compared independently.

Table III *HLA-G* 1597ΔC genotype counts in 44 women with PE and 271 normotensive women with term deliveries (Controls) from the CLIPP study.

Group	Number of each genotype			*P*-value
	C/C	C/Δ	Δ/Δ	
PE cases (%)	32 (72.7%)	10 (22.7%)	2 (4.5%)	0.0029
Controls (%)	244 (90%)	25 (9.2%)	2 (0.74%)	

The 1597ΔC allele and severity of PE

Among the CLIPP women with PE, 39.7% delivered preterm (Table I). The women with preterm PE had significantly higher mean maximum systolic blood pressure ($P = 0.0040$) and mean maximum diastolic blood pressure during labor ($P = 0.0075$) compared with the women with PE who delivered at term (Table I).

We hypothesized that the frequency of the 1597ΔC allele would be further elevated in women with severe PE. As predicted, the 1597 deletion allele was present in six (37.5%) of the women with preterm PE (5 Δ/C, 1 Δ/Δ), six (21.4%) of the women with term PE (5 Δ/C, 1 Δ/Δ) and 27 (9.96%) of the women with normotensive pregnancies who delivered at term (25 Δ/C, 2 Δ/Δ) (Fig. 1). This association between an increasing frequency of the maternal 1597ΔC allele and increasing severity was statistically significant (Cochran–Armitage trend test, $Z = 3.57$, $P_{trend} = 0.0012$). No such relationship was observed when considering infants' 1597ΔC allele frequency (not shown).

Table IV Allele frequencies for six *HLA-G* polymorphisms in 47 infants from PE pregnancies and 281 infants from healthy, normotensive control pregnancies from the CLIPP study.

Polymorphism	Allele	PE cases	Controls	*P*-value
−725C/G/T	C	0.89	0.83	
	G	0.063	0.057	0.84[a]
	T	0.052	0.11	0.073[a]
+36G/A	A	0.63	0.54	0.086
	G	0.37	0.46	
1597ΔC	C	0.88	0.93	0.078
	Δ	0.12	0.066	
Thr258Met	Thr	1.0	0.98	0.27
	Met	0.00	0.018	
14-bp indel	Del	0.62	0.55	0.28
	Ins	0.38	0.45	
+3142G/C	G	0.68	0.65	0.51
	C	0.32	0.35	

[a]Because the −725 polymorphism was tri-allelic, allele counts for each of the minor alleles were compared independently.

Replication of the 1597ΔC association with PE

The association between higher maternal 1597ΔC allele frequency and increased severity of PE was evaluated in an independent sample of African-American women from Detroit, Michigan. The proportion of Detroit women carrying the 1597ΔC allele was 17.4% in the preterm PE group, 13.3% in the term PE group and 10.2% in the controls (Fig. 1); this trend was statistically significant (Cochran–Armitage trend test, $Z = 1.88$, $P_{trend} = 0.038$). No association was observed between the 1597ΔC allele frequency in the Detroit infants and severity of PE. The positive association between 1597ΔC allele frequency and increased severity of PE was significant in the combined sample of CLIPP and Detroit subjects (Fig. 1; weighted *Z*-transform method, $P = 0.0011$).

1597ΔC allele and sHLA-G levels during pregnancy

Serum samples collected during pregnancy were available for 56 of the healthy, normotensive control CLIPP pregnancies, but only for six of the women with PE. Therefore, associations between circulating sHLA-G levels and the 1597ΔC allele were assessed only in the controls. Five of the 56 control pregnancies were excluded from analysis because sHLA-G concentrations of their duplicate samples showed a coefficient of variation greater than 35%. For the remaining 51 samples, median sHLA-G concentrations were significantly lower in the 11 women who carried the 1597ΔC allele (10 heterozygotes, 1 homozygote) compared with the 40 women who did not carry this allele (Fig. 2; median sHLA-G = 12.5 U/ml for mothers carrying the 1597ΔC allele and 32.8 U/ml for mothers who did not carry the allele; Wilcoxon rank sum test, $\chi^2 = 6.31$, df = 1, $P = 0.012$). Consistent with the results of the aforementioned association studies, the 1597ΔC of the infant was not a significant predicator of serum sHLA-G levels in the mother (median sHLA-G = 15.1 U/ml for 12 infants carrying the 1597ΔC allele and 31.8 U/ml for 36 infants who did not carry the allele; Wilcoxon rank sum test, $\chi^2 = 1.72$, df = 1,

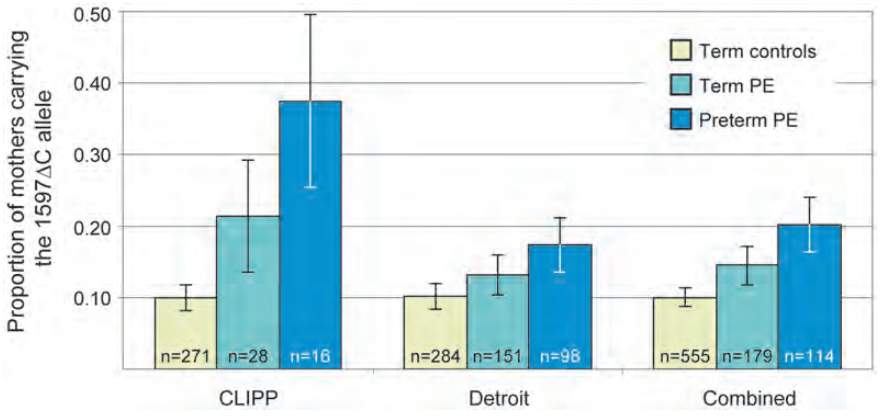

Figure 1 Proportion of women carrying the 1597ΔC allele in the CLIPP, Detroit and combined samples. The proportion of women carrying the 1597ΔC allele is shown separately for the term control, term PE and preterm PE pregnancies in each sample. The association between presence of the 1597ΔC allele and increased severity of PE was significant in the combined sample (Weighted Z-transform method, $P = 0.0011$). Error bars indicate the standard error of the proportion.

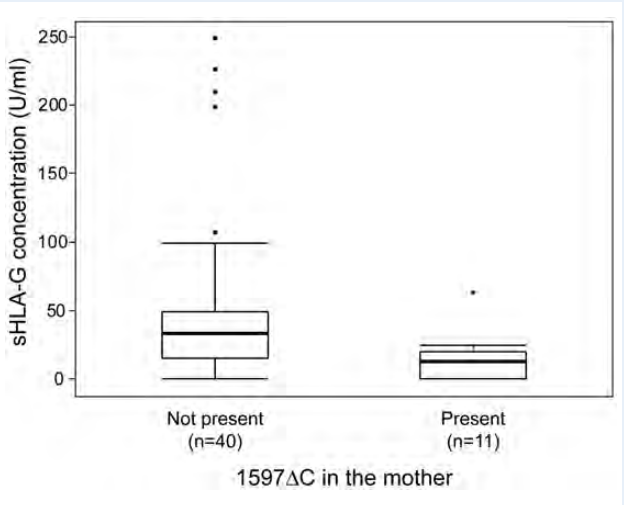

Figure 2 Concentrations of sHLA-G protein in maternal serum in uncomplicated, normotensive pregnancies as a function of maternal 1597ΔC. Box plots show the distribution of circulating sHLA-G in women who carried the 1597ΔC allele (Present) and those who did not carry the allele (Not present). Estimates of sHLA-G concentrations that were more than 1.5 interquartile ranges from the quartiles are shown individually as outliers (indicated by filled squares).

$P = 0.19$). Circulating sHLA-G concentrations during pregnancy did not differ significantly as a function of the estimated gestational age ($P = 0.50$) or trimester ($P = 0.53$) in which the sample was collected (Supplementary data, Fig. S1).

Discussion

Identification of immunogenetic factors that contribute to PE risk and severity will provide insight into the etiology of this multifactorial disorder, and may ultimately lead to improved methods for early diagnosis or treatment. In this study, we evaluated the association between genetic variation in the *HLA-G* gene and PE in two independent samples of African-American women. In both, we observed an increased risk of PE when the mother carried the 1597ΔC allele, which results from a single-base-pair deletion of a cytosine in exon 3 that abolishes the expression of the full-length HLA-G protein isoforms containing the α2 domain, i.e. HLA-G1 and -G5 (Ober *et al.*, 1998). In contrast, the presence of the 1597ΔC allele in the fetus was not associated with PE risk. The observation that increasing frequency of the maternal, but not the fetal, 1597ΔC allele was associated with increasing severity of PE suggests that maternal-derived HLA-G may serve functions different from those of the placental-derived isoforms.

The lack of association between the 1597ΔC allele and PE in African-American infants is not new. We previously studied this polymorphism in 39 African-American infants with intrauterine growth restriction, 57 African-American infants from pre-eclamptic pregnancies and 111 African-American normal-weight term infants, ascertained from the same clinics as the CLIPP sample (Aldrich *et al.*, 2000). This earlier study, which did not include the mothers, did not show an association with either disorder (Aldrich *et al.*, 2000). We previously showed that the shorter HLA-G isoforms, G2 and G6, are expressed in the placenta of fetuses who are homozygous for the 1597ΔC allele, and suggested that those isoforms may compensate for reduced levels of HLA-G1 and -G5 in the placentas of fetuses carrying the 1597ΔC allele (Ober *et al.*, 1998). These shorter HLA-G2 and -G6 isoforms also circulate in the maternal periphery during pregnancy (Hunt *et al.*, 2000) and could explain why the presence of the 1597ΔC allele in the fetus is not associated with preeclampsia, as we have observed. Thus, the combined results of these studies support our suggestion that the effects of the 1597ΔC allele on PE risk is due to the maternal, but not the fetal, genotype.

To directly assess the effects of the maternal or fetal 1597ΔC allele on circulating sHLA-G levels, we measured concentrations of the HLA-G1 and -G5 isoforms in pregnancy serum from 51 control women. The maternal 1597ΔC allele was associated with lower circulating sHLA-G levels in these uncomplicated, normotensive pregnancies, indicating that maternal sHLA-G contributes to risk of PE but is not causal. This is also consistent with previous studies that reported lower levels of circulating sHLA-G during pregnancy in women with PE (Yie *et al.*, 2005; Hackmon *et al.*, 2007; Steinborn *et al.*, 2007; Rizzo *et al.*, 2009). In our study, we could not study circulating sHLA-G concentrations in the pre-eclamptic pregnancies, but the associations between 1597ΔC and PE risk, and between 1597ΔC and reduced levels of sHLA-G, are consistent with these earlier studies reporting reduced levels of sHLA-G being a risk factor for PE.

The results of the present study of African-American women did not confirm associations reported in previous *HLA-G* genetic association studies, such as those involving the 14 bp insertion/deletion polymorphism (rs66554220) or the Thr258Met variant (rs12722482). The presence of the 14 bp insertion in the fetal genome had been associated with increased risk of PE in women of European ancestry (Hylenius *et al.*, 2004; Moreau *et al.*, 2008; Larsen *et al.*, 2010), but we did not observe differences in 14 bp insertion allele frequencies in the African-American PE cases compared with controls when considering either fetal or maternal genotype. The increased frequency of the fetal G*0106 haplotype (which is defined by the Thr258Met variant allele) in pre-eclamptic pregnancies reported previously (Moreau *et al.*, 2008; Tan *et al.*, 2009) was also not apparent in our African-American data, perhaps because of the fact that the Met allele occurred at very low frequencies (<2%) in the African-American women and infants in our study.

A potential limitation of our study is that we could not formally assess differences in local ancestry at the *HLA-G* locus in the case and control samples because we did not have genotype data for SNPs spanning this region. It is possible that the increased frequency of the 1597ΔC allele in the cases was due to increased African ancestry in those individuals, because that allele occurs at higher frequencies in populations of African descent (Aldrich *et al.*, 2002). However, we think this is unlikely for three reasons. First, the −725T allele also occurs at significantly higher frequencies in African Americans compared with European Americans (10.2 versus 2.2%, respectively; Tan *et al.*, 2005). Yet, the frequency of this allele was 4.9% in the pre-eclamptic cases and 10.0% in the normotensive term controls in the CLIPP sample. Second, we did not observe a significant difference in the 1597ΔC allele frequencies between infants born to pre-eclamptic and control pregnancies. Third, we demonstrated associations between the 1597ΔC allele and PE in two independent

African-American samples, each with cases and controls recruited from within the same medical center, and it is unlikely that the effects of population stratification would be consistent in direction and magnitude in both populations (Thomas and Witte, 2002; Wacholder *et al.*, 2002).

This study provides new insight into the genetic factors that contribute to PE risk. The differences between the current results and those reported for other populations further support the idea that PE is a multifactorial disorder that develops as a result of complex interactions between genetic and environmental factors, with potentially different contributions from maternal and fetal genes. Indeed, significant heterogeneity in the strength of associations has been observed in studies of candidate genes and PE risk. For studies of HLA-G, this heterogeneity may be due to the effects of small sample size, differences in population genetic structure or ancestry, differential impact of environmental influences or even maternal–fetal interactions (Tan *et al.*, 2009). Thus, it is important for future studies of HLA-G and PE risk to be adequately powered in terms of sample size and genetic coverage and to include subjects of diverse ancestry to allow assessment of alleles that are rare in Europeans but common in other groups, such as the 1597ΔC allele described in this report.

Supplementary data

Supplementary data are available at http://molehr.oxfordjournals.org/.

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Authors' roles

D.A.L., R.R. and C.O. were involved in study design, execution and presentation. K.M. and T.C. performed the patient consenting and acquisition of biological samples. D.A.L., C.B. and K.P. completed the genotyping and ELISA studies. D.A.L. and C.O. performed data processing and statistical analysis. D.A.L. and C.O. wrote and revised the manuscript. All the authors contributed critical discussion and manuscript review and gave final approval of the version to be published.

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Conflict of interest

None declared.

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