GENETIC DETERMINANTS OF PRE-ECLAMPSIA: *HLA-G* AND *KIR2DL4* ALLELES/HAPLOTYPES AND RISK OF PRE-ECLAMPSIA IN A MALAY POPULATION

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Table of contents

Abstract of thesis

Pre-eclampsia (PE) is a leading cause of maternal and fetal mortality and morbidity. HLA-G is expressed predominantly on fetal extravillous trophoblasts that invade the maternal decidua during pregnancy and has been postulated to be important in the maintenance of a healthy pregnancy. It has been thought that HLA-G exerts its protective functions through its inhibitory receptor, KIR2DL4, expressed on maternal natural killer cells. Therefore, alleles/haplotypes of *HLA-G* and *KIR2DL4* were tested in a case-control study of 83 PE and 240 normotensive Malay women to determine if particular alleles or combinations of different alleles may predispose women to PE. Case-control comparisons showed that risk for PE was significantly associated with fetal allele G*0106 ($p=0.002$, OR=5.0, 95%CI=1.8-13.8) but not maternal *HLA-G*. No significant association was observed between *KIR2DL4* alleles and PE in both maternal and fetal groups. Gene-gene interaction analyses showed that combinations of maternal *2DL4*006* and fetal *G*0106* significantly increases risk of PE ($p<0.001$). Therefore, fetal *G*0106* significantly increases risk for PE in pregnancies where the mother carries the *2DL4*006* allele.

Summary

Pre-eclampsia (PE) is a leading cause of maternal and fetal mortality and morbidity and occurs only during pregnancy. Although extensive studies have been carried out, the cause of PE is still unknown. Accumulative evidence implicates that the placenta plays a role in the development of PE. Human Leukocyte Antigen (HLA)-G expressed predominantly on fetal extravillous trophiblast cells from the placenta that invade the maternal decidua during pregnancy has been postulated to be important in the maintenance of a healthy pregnancy. Structural or functional alterations of HLA-G may predispose women to PE. It has been thought that HLA-G exerts its protective functions through its inhibitory receptor, killer-cell immunoglobulin-like receptor (KIR)2DL4, expressed on maternal natural killer cells. Pregnancy is the only physiological situation where KIRs may meet cognate non-self variants of HLA allotypes.

Therefore, alleles/haplotypes of *HLA-G* and *KIR2DL4* were tested in a case-control study of 83 PE and 240 normotensive Malay women to determine if particular alleles or combinations of different alleles may predispose women to PE. *HLA-G* and *KIR2DL4* genes were amplified separately in 2 single-tube multiplex-PCR reactions and genotyped for 18 and 23 single nucleotide polymorphisms (SNPs), respectively, using multiplex-minisequencing strategy. Case-control comparisons showed that risk for PE was significantly associated with fetal allele G*0106, interestingly only in multigravid pregnancies (p=0.002, OR=5.0, 95%CI=1.8-13.8) but no significance was observed in the maternal group. Among multigravid pregnancies, the frequency of PE babies heterozygous or homozygous for G*0106 was also significantly higher compared to normal control babies (p=0.002. OR=5.4, 95%CI=1.9-15.4).

Multivariate analyses with adjustment for factors associated with PE revealed similar results (p=0.003, OR=10.1, 95%CI=2.2-46.8). Additionally, a significantly higher frequency of fetal-maternal G*0106 genotype mismatch was observed in pre-eclamptic compared to normal multigravid pregnancies (p=0.001, OR=9.6, 95%CI=2.4-38.7). No significant association was observed between *KIR2DL4* alleles and PE in both maternal and fetal groups. Gene-gene interaction analyses showed that combinations of maternal *2DL4*006* and fetal *G*0106* significantly increases risk of PE (p<0.001). Therefore, the presence of paternal *G*0106* significantly increases risk for PE in pregnancies where the mother lacks the *G*0106* allele and carries the *2DL4*006* allele.

This study was carried out to test a larger sample size as well as to include HLA-G's receptor, KIR2DL4, following a preliminary study on 31 PE and 164 controls of the *HLA-G* gene where it was observed that there was significantly higher proportion of PE babies carrying the *G*0106* allele.

The work on *HLA-G* alleles/ haplotype in PE and controls as well as data on the frequencies of *HLA-G* alleles/ haplotypes in 3 local populations were published in Molecular Human Reproduction 2008: **14**(5); 317-324 (Tan, Ho et al. 2008) and the work on *KIR2DL4* alleles and PE was submitted to the Reproductive Sciences on the 4th of March, 2009.

List of Tables

List of Figures

1.0 Introduction

1.1 Pre-clampsia

Pre-eclampsia (PE) is a major cause of maternal and perinatal mortality and morbidity, causing 15 – 20 % of maternal death in developed countries each year (Sibai, Dekker et al. 2005). PE occurs only during pregnancy and affects about 5-8% of healthy nulliparous women and the rate increases substantially in women with previous pre-eclampsia (18%) as well as women with twin gestation (14%) (Hauth, Ewell et al. 2000; Sibai, Hauth et al. 2000; Hnat, Sibai et al. 2002). It is a multisystemic disorder that can manifest as either a maternal syndrome (hypertension and proteinuria, with or without other multisystem abnormalities) or fetal syndrome (fetal growth restriction, reduced amniotic fluid, and abnormal oxygenation) (2000; Sibai 2003; Sibai, Dekker et al. 2005).

PE is defined as blood pressure of at least 140 mm Hg systolic or at least 90 mm Hg diastolic measured on at least two occasions and at least 4 to 6 hours apart after the 20^{th} week of gestation in women known to be normotensive beforehand in the presence of proteinuria of at least 300 mg per 24-hour period or a concentration of at least 30 mg/dL (or at least 1+ on dipstick) in two or more random urine samples collected at least 6 hours apart (2000; Sibai 2003). PE is considered severe if there is severe gestational hypertension in association with severe proteinuria of at least 5 g per 24-hour period. In addition to that, multiorganic involvement such as pulmonary edema, seizures, oliguria (less than 500 mL per 24-hour period), thrombocytopenia (platelet count less than 100,000/mm^3), abnormal liver enzymes in association with persistent epigastric or right upper quadrant pains or persistent severe central nervous

system symptoms (altered mental status, headaches, blurred vision or blindness) may be observed in patients with severe PE (SPE) (Sibai 2003).

There are 2 forms of PE, namely early and late onset with symptoms occurring before or after week 34 respectively (Redman and Sargent 2005; Oudejans, van Dijk et al. 2007). The early onset form of PE is more severe and the fetus may suffer nutritional and respiratory insufficiency, resulting in a higher rate of neonates that are smaller size compared to neonates of the same gestational age. Early onset PE also has higher recurrence rate compared with the late onset form of the disease (Redman and Sargent 2005).

Risk factors for PE include primiparity, primipaternity, extremes of maternal age, PE in a previous pregnancy, family history of PE, multifetal gestations, long intervals between pregnancies, high pre-pregnancy body mass index (BMI), pre-existing medical conditions such as chronic hypertension, diabetes, renal disease and urinary tract infection (Conde-Agudelo and Belizan 2000; Lee, Hsieh et al. 2000; Anorlu, Iwuala et al. 2005; Duckitt and Harrington 2005; Funai, Paltiel et al. 2005; Sibai, Dekker et al. 2005). Interestingly, cigarette smoking during pregnancy was reported to be a protective factor against the development of PE (Conde-Agudelo and Belizan 2000).

Although the cause of PE is unknown, accumulative evidence strongly implicates the placenta (Redman 1991). It has been suggested that PE is caused by the presence of the placenta itself or due to the maternal response to placentation, as PE occurs only during pregnancy and also, the fact that PE is promptly resolved after

the delivery of the placenta. Moreover, development of PE does not require the presence of fetus as an increased risk of PE was observed in molar pregnancies (Ness and Roberts 1996). Also, the uterus is not necessarily involved as PE can occur in extra-uterine pregnancies (Emembolu 1989; Piering, Garancis et al. 1993; Seki, Kuromaki et al. 1997).

In normal pregnancy, placentation takes places before 20 weeks of gestation where extravillous trophoblast (EVT) cells from the placenta invade the maternal spiral arteries in the myometrium and remodels the arteries extensively causing them to lose their smooth muscle and becomes greatly dilated. Proper trophoblast invasion to the inner third of the myometrium ensures sufficient blood flow to the feto-placental unit, which in turns, ensures proper growth of the fetus (Trundley and Moffett 2004). However, in pre-eclampsia, poor placentation occurs where the spiral arteries are poorly remodelled due to shallow trophoblastic invasion of the spiral arteries at the maternal-fetal interface (Redman 1991; Naicker, Khedun et al. 2003). This results in a markedly reduced volume of the uteroplacental circulation and failure of the EVT in gaining full access to maternal supplies.

Although extensive research addressing this disorder has been carried out in the past decade, the etiology and pathogenesis of PE remains unknown. It has been suggested that the development of PE may be due to maternal immune maladaptation where a maternal alloimmune reaction takes place triggered by a rejection of the fetal allograft. The immune maladaptation hypothesis is supported by findings of the protective effect of sperm exposure (Klonoff-Cohen, Savitz et al. 1989; Smith, Walker et al. 1997; Dekker 2002; Wang, Knottnerus et al. 2002; Einarsson, Sangi-

Haghpeykar et al. 2003), higher incidence of PE in nulliparous women (Campbell, MacGillivray et al. 1985) and in women who are less exposed to their partners' antigens (Robillard, Hulsey et al. 1994; Trupin, Simon et al. 1996; Smith, Walker et al. 1997; Lie, Rasmussen et al. 1998; Wang, Knottnerus et al. 2002; Saftlas, Levine et al. 2003) as well as an increased risk of PE in changing paternity (Trupin, Simon et al. 1996; Tubbergen, Lachmeijer et al. 1999). Furthermore, a genetic basis for PE has been demonstrated as a family history of PE increases the risk for developing the condition (Chesley, Annitto et al. 1968; Cincotta and Brennecke 1998).

1.2 PE Genes

Genome-wide scans have been performed in several association studies including the Dutch Preeclampsia study, the British Genetics of Pre-Eclampsia (GOPEC) consortium, the Norwegian HUNT cohort, Australian/New Zealand cohort, Finland, Iceland and other countries resulting in the identification of several susceptibility locus including 2p13, 2q22, 2p25, 4q32, 9p13 and 10q22 (Arngrimsson, Sigurard ttir et al. 1999; Moses, Lade et al. 2000; Laasanen, Hiltunen et al. 2003; Laivuori, Lahermo et al. 2003; Oudejans, Mulders et al. 2004; 2005; Moses, Fitzpatrick et al. 2006). The GOPEC study genotyped 28 SNPs in 7 candidate genes conferring susceptibility to PE and concluded that none of the genetic variants tested in their study of strictly defined PE pregnancies confers a high risk of disease (2005).

To date, more than 50 candidate genes for pre-eclampsia have been reported (Chappell and Morgan 2006). Several of these genes account for the majority of all pre-eclampsia candidate gene studies, including genes involved in the renin-angiotensin system such as the angiotensinogen, angiotensin-converting enzyme and angiotensin receptors (AGTR1 and AGTR2) (Ward, Hata et al. 1993; Morgan,

Crawshaw et al. 1998; Plummer, Tower et al. 2004); in inherited thrombophilias such as coagulation factor V Leiden cariant, prothrombin and methylene tetrahydrofolate reductase (MTHFR) (Dizon-Townson, Nelson et al. 1996; Sohda, Arinami et al. 1997; Kupferminc, Eldor et al. 1999); in regulation of the synthesis of the vasorelaxant eNOS (endothelial nitric oxide synthase) such as the *NOS3* gene (Yoshimura, Yoshimura et al. 2000) and in immunogenetics such as the *Human Leukocyte Antigen (HLA)-DR, -DQ and -DP* (Kilpatrick, Gibson et al. 1990; de Luca Brunori, Battini et al. 2000; de Luca Brunori, Battini et al. 2003; Ooki, Takakuwa et al. 2008), *HLA-C* (Takakuwa, Arakawa et al. 1997; Hiby, Walker et al. 2004) and *HLA-G* genes.

1.3 Human Leukocyte Antigens

Human Leukocyte Antigen (HLA) genes are part of the human major histocompatibility complex (MHC) located on the short arm of chromosome 6 on the 6p21.3 region (Robinson, Waller et al. 2003). The *HLA* genes are divided into 2 main classes (I and II) and among these 2 classes, class I genes are further sub-grouped into classical class Ia (*HLA-A*, *-B* and *-C*) and non-classical class Ib (*HLA-E*, *-F* and *–G*) genes whereas Class II genes includes *HLA-DP*, *-DQ* and *–DR* (Baines and Ebringer 1992; 1999).

Classical class Ia genes share some characteristics with the non-classical class Ib gene but the 2 groups have different expression patterns and also, class Ib genes have a lower allelic polymorphism compared to the former group (Geraghty, Koller et al. 1987; Koller, Geraghty et al. 1988; Geraghty, Wei et al. 1990; Heinrichs and Orr 1990). It is thought that the highly polymorphic classical class I molecules HLA-A, -B, -C, which are expressed on almost all somatic cells, play a role in the induction of a specific immune response by presenting peptide antigens to T cells. In contrast, the

non-classical HLA class I molecules HLA-G and HLA-E are thought to be involved in the induction of immune tolerance by acting as ligands for inhibitory receptors present on natural killer (NK) cells and macrophages.

Among the HLA genes, the classical class Ia and II genes (*HLA-A*, *-B*, -C, *-DP*, *-DQ* and *-DR*) genes are most widely studied due to their role in organ transplantation (Doherty and Zinkernagel 1975; Hurley, Wade et al. 1999; Schreuder, Hurley et al. 2005) and antigen-peptide presentation (Morris, Shaman et al. 1994; Chen and Jensen 2008) as well as their association with a range of autoimmune diseases (Manabe, Donaldson et al. 1993; Czaja, Santrach et al. 1995; Strettell, Thomson et al. 1997; Hunt, Marshall et al. 2001). In addition to that, certain *HLA* genes, especially class Ib genes, has also been of much interest in studies of diseases in pregnancies such as recurrent spontaneous abortion (RSA) and PE as it is thought that the semiallogenic fetus carrying paternal genes foreign to the mother may trigger an alloimmune response in the mothers during pregnancy (Ober 1998; Ober, Hyslop et al. 1998; Ishitani, Sageshima et al. 2003; Ishitani, Sageshima et al. 2006).

At the maternal-fetal interface, EVT cells are the only fetal cell type that is exposed to the maternal uterine decidua and comes into direct contact with maternal tissues in the pregnant uterus. These cells exert a crucial role during implantation and placentation and are thought to play a role in the protection of the semiallogenic fetus from the maternal immune surveillance. On the EVT, a unique combination of HLA class I molecules is expressed: the non-classical class Ib molecules, HLA-G, HLA-E and HLA-F, as well as the classical class Ia molecule, HLA-C (Kovats, Main et al. 1990; Yelavarthi, Fishback et al. 1991; King, Boocock et al. 1996; Proll, Blaschitz et

al. 1999; King, Allan et al. 2000; King, Burrows et al. 2000; Blaschitz, Hutter et al. 2001; Ishitani, Sageshima et al. 2003). Classical class Ia genes expressed in nearly all other nucleated cells such as the *HLA-A* and *HLA-B*, as well as all HLA class II genes such as *HLA-DR*, *HLA-DQ* and *HLA-DP* are absent on the EVTs (Redman, McMichael et al. 1984).

Interestingly, only HLA-G protein expression is primarily restricted to EVT (McMaster, Librach et al. 1995) whereas HLA-C and HLA-E have ubiquitous distribution (Koller, Geraghty et al. 1988; Kariyone, Tanabe et al. 1990) and HLA-F have been detected on a number of diverse tissues (Lury, Epstein et al. 1990). Therefore, the immunomodulatory role of HLA-G in complications of human pregnancies has been of much interest given its restricted expression on trophoblast cells that form the physical interface between fetus and mothers.

1.4 HLA-G

HLA-G is a member of the non-classical MHC class Ib genes consisting of 6 exons (O'Callaghan and Bell 1998; Robinson and Marsh 2007). The *HLA-G* gene has almost the same structure as classical class Ia genes and shares more than 86% homology with *HLA-A*, *-B* and *-C* (Geraghty, Koller et al. 1987). However, there are several features that sets *HLA-G* apart from classical class Ia genes. Firstly, transcripts of HLA-G is able to undergo alternative splicing to generate at least 7 distinct splice variants (HLA-G1 through HLA-G7) (Ishitani and Geraghty 1992; Fujii, Ishitani et al. 1994; Kirszenbaum, Moreau et al. 1994; Moreau, Carosella et al. 1995; Hviid, Moller et al. 1998; Hiby, King et al. 1999; Paul, Cabestre et al. 2000), of which HLA-G1 to -G4 are membrane bound isoforms whereas HLA-G5 to -G7 are soluble isoforms due to the presence of a premature stop codon either in intron 2

(HLA-G7) or intron 4 (HLA-G5 and -G6). This leads to the production of proteins lacking the transmembrane region. Among the 7 isoforms, HLA-G1 isoform encoding the full length protein is the most abundant and may also be the only form expressed on cell surface (Bainbridge, Ellis et al. 2000; Mallet, Proll et al. 2000).

Also, in contrast to other HLA class Ia molecules, HLA-G has limited allelic polymorphism and due to the presence of a stop codon in exon 7, HLA-G has a shortened cytoplasmic tail. As a result, HLA-G proteins lack the endocytosis motifs found in the cytoplasmic tail of other HLA class Ia molecules. Absence of these motifs enables HLA-G proteins to have an extended surface half-life compared to other HLA molecules (Park, Lee et al. 2001).

HLA-G transcripts were found to be upregulated in tumor tissues in the breast, kidney, lung, lymphoid, gastrointestinal tract and skin. (Paul, Rouas-Freiss et al. 1998; Davies, Hiby et al. 2001; Ibrahim, Guerra et al. 2001; Urosevic, Kurrer et al. 2001; Lefebvre, Antoine et al. 2002; Amiot, Le Friec et al. 2003; Ibrahim, Aractingi et al. 2004; Hansel, Rahman et al. 2005). In addition to that, HLA-G has also been associated with a range of diseases including HIV-1 infection (Aikhionbare, Kumaresan et al. 2006; Tripathi and Agrawal 2007), systemic lupus erythematosus (Rizzo, Hviid et al. 2008), asthma (Ober 2005), juvenile idiopathic arthritis (Veit, Vianna et al. 2008), inflammatory diseases (Baricordi, Stignani et al. 2008), ulcerative colitis and Crohn's disease (Rizzo, Melchiorri et al. 2008) among others.

The most interesting feature of HLA-G is that its protein expression is restricted to the trophoblast cells of the fetal placenta and this has been shown repeatedly in different studies using different anti-HLA-G monoclonal antibodies as

well as different techniques. In addition to that, transcripts of *HLA-G* have also been identified in a wide variety of tissues, including human oocytes, pre-implantation embryos (Jurisicova, Casper et al. 1996), maternal plasma, amniotic fluid (McMaster, Zhou et al. 1998; Rebmann, Pfeiffer et al. 1999), keratinocytes (Ulbrecht, Rehberger et al. 1994), peripheral blood B and T cells (Kirszenbaum, Moreau et al. 1994), fetal and adult thymus (Crisa, McMaster et al. 1997), kidney and eyes as well as fetal liver (Houlihan, Biro et al. 1992), lung and spleen (Onno, Guillaudeux et al. 1994). However, the expression of these transcripts in different tissues has been controversial because some of the anti-HLA-G antibodies used in the earlier studies were later shown to cross-react with other classical HLA molecules due to the high homology among members of the HLA family (Real, Cabrera et al. 1999; Apps, Gardner et al. 2008). Therefore, the expression of HLA-G in various tissues apart from trophoblast cells and perhaps also, its role in tumor development remains inconclusive.

As *HLA-G* is the main HLA Class Ib gene being expressed by the fetal trophoblasts at the materno-fetal placental interface (King, Boocock et al. 1996), it is possible that maternal NK cells found at the placental interface do not lyse the semiallogenic invasive fetal cytotrophoblasts due to their expression of *HLA-G*. Interestingly, the expression of *HLA-G* in the EVT is reduced in PE (Colbern, Chiang et al. 1994; Hara, Fujii et al. 1996; Goldman-Wohl, Ariel et al. 2000; Yie, Li et al. 2004; Hackmon, Koifman et al. 2007). It is possible that these cells are more susceptible to the attack by the maternal immune system and thereby results in the reduced invasion and poor remodeling of maternal spiral arteries as observed in PE placentas. Other possible roles of *HLA-G* in the maintenance of pregnancy include participating in vascular remodeling through inhibition of angiogenesis (Fons, Chabot

et al. 2006; Le Bouteiller, Fons et al. 2007), influencing the maternal NK cell production of cytokines and angiogenic factors (Chumbley, King et al. 1994; Li, Charnock-Jones et al. 2001; Le Bouteiller, Pizzato et al. 2003) and inhibiting the transendothelial migration of NK cells across the placenta (Dorling, Monk et al. 2000), thereby enhancing maternal tolerance to the fetus.

In addition to that, *HLA-G* may also play a role in ensuring maternal tolerance to paternal alloantigens by reducing the population of activated CD4+ and CD8+ killer T cells that could be present in the blood in the intervillous space and decidua (Le Bouteiller, Legrand-Abravanel et al. 2003). The finding that only embryos that express *HLA-G* are implanted successfully after *in vitro* fertilization (Fuzzi, Rizzo et al. 2002; Yie, Balakier et al. 2005) and have an increased cleavage rate (Jurisicova, Casper et al. 1996) as well as the reduced expression of *HLA-G* in PE (Colbern, Chiang et al. 1994; Hara, Fujii et al. 1996; Goldman-Wohl, Ariel et al. 2000; Yie, Li et al. 2004; Hackmon, Koifman et al. 2007), further highlights the importance of *HLA-G* in establishing and maintaining pregnancy.

According to the World Health Organization (WHO) Nomenclature Committee for Factors of the HLA System, a total of 36 *HLA-G* alleles have been reported to date, of which 16 are major *HLA-G* alleles (*HLA-G*0101* to *HLA-G*0116*) (Robinson, Waller et al. 2003). These alleles are characterized by single nucleotide polymorphisms (SNPs) that change the amino acid sequence of the HLA-G protein and these SNPs are distributed primarily between α1, α2 and α3 domains encoded by exons 2 to 4 (Table 1), unlike polymorphisms in classical class Ia molecules that are concentrated around the peptide binding groove.

Several studies have been performed to determine the association of *HLA-G* alleles with PE and the results have been inconclusive thus far, as positive associations of certain *HLA-G* alleles with PE are observed only in some study populations and not found in others. For example, fetal inheritance of maternal G*0104 allele was reported to increase risk of PE in a study by Carreiras *et al.* (Carreiras, Montagnani et al. 2002) but a lack of association was observed in other studies (Hylenius, Andersen et al. 2004). Therefore, further studies are necessary to determine if alleles of *HLA-G* is linked to PE.

In addition to the SNPs coding for different *HLA-G* alleles, variations in the 5' upstream region (UR), promoter and 3' untranslated region (UTR) has been of much interest in association studies as well. In contrast to the low level of polymorphism in the coding region, the 5' flanking sequences of HLA-G is highly polymorphic, with 18 SNPs identified in the region approximately 1500 base-pairs (bp) upstream of exon 1 (Hviid, Sorensen et al. 1999). Moreover, it has been proposed that this upstream region contains an important regulatory element that regulates the transcription and expression pattern of HLA-G (Schmidt, Ehlenfeldt et al. 1993; Moreau, Paul et al. 1997).

Table 1. *HLA-G* alleles defined by SNPs (highlighted in bold) based on the WHO nomenclature**.**

Protein domain	Leader signal peptide		α1 domain							α2 domain								α3 domain				Transmembrane	
Exon	Exon 1		Exon 2							Exon 3								Exon 4				Exon 5	
Nucleotide	15	36	37	90	104	122	160	170	206	278	320	327	350	387	443	474	506	563	707	772	800	869	926
Codon			13	31	35	41	54	57	69	93	107	110	117	130	148	159	169	188	236	258	267	290	309
HLA G *01010101	GCG	CTG	TCC	ACG	CGG	GCG	CAG	CCG	GCC	CAC	GGA	CTC	GCC	CTG	GAG	TAC	CAC	CAC	GCA	ACG	CCG	GGC	AGA
HLA G *01010201	GC**A**	CT**A**						CC**A**		CA**T**												GG**T**	AG**G**
HLA G *010103								CC**A**			GG**T**												
HLA G *010104									GC**T**														
HLA G *010105											GG**T**												
HLA G *010106																		CA**T**					AG**G**
HLA G *010107								CC**A**		CA**T**	GG**T**												
HLA G *010108								CC**A**															
HLA G *010109															GA**A**								
HLA G *010111													GC**G**										
HLA G *010112						GC**T**		CC**A**		CA**T**													
HLA G *010113								CC**A**		CA**T**							CA**T**						
HLA G *010114					CG**A**			CC**A**		CA**T**													
HLA G *0102							C**G**G												GC**C**				
HLA G *0103				**T**CG														CA**T**					AG**G**
HLA G *010401	GC**A**	CT**A**						CC**A**				**ATC**											AG**G**
HLA G *010402								CC**C**				**ATC**						CA**T**					
HLA G *010403												**ATC**											
HLA G *010404	GC**A**	CT**A**						CC**A**				**A**TC									CC**A**		AG**G**
HLA G *0105N	GC**A**	CT**A**						CC**A**		CA**T**				***TG								GG**T**	AG**G**
HLA G *0106		A						CC**A**		CA**T**										A**T**G		GG**T**	AG**G**
HLA G *0107	GC**A**	CT**A**	T**T**C					CC**A**				**A**TC											AG**G**
HLA G *0108								CC**A**		CA**T**													
HLA G *0109								CC**A**		CA**T**						**C**AC							
HLA G *0110				A**T**G																			
HLA G *0111				A**T**G				CC**A**				**A**TC											

* Deletion

Reference:

1. IMGT/ HLA database

A 14 bp insertion/ deletion polymorphism (5'-ATTTGTTCATGCCT-3') in the 3'UTR at nucleotide position 2961 (NP2961) of the *HLA-G* gene (Harrison, Humphrey et al. 1993) have been reported to affect HLA-G isoform splicing patterns and HLA-G transcript stability. *HLA-G* mRNA transcripts with the 14-bp insertion undergoes further splicing of 92 bp in the 5' UTR region (Hviid, Hylenius et al. 2003), resulting in a more stable transcript compared to the complete form (Rousseau, Le Discorde et al. 2003). Therefore, this polymorphism had been speculated to be involved in complications of pregnancies such as RSA and PE as it is associated with reduced expression of HLA-G.

Although it has been reported that certain *HLA-G* alleles/ polymorphisms are associated with increased risk for PE, the results have not been conclusive. Furthermore, most previous studies have looked at individual *HLA-G* SNPs and not alleles. Therefore, in this study, a more comprehensive approach is used to study polymorphisms of *HLA-G* at the haplotypes/ alleles level in addition to the individual SNPs to search for possible association with PE using a case-control approach.

On the other hand, HLA-G may exert its protective functions by inhibiting maternal NK cells via interaction with inhibitory receptors expressed on NK cells and this may explain maternal tolerance of the semiallogenic fetus in the maintenance of a healthy pregnancy. Inhibitory receptors of HLA-G are the immunoglobulin-like transcript ILT2 (also known as leukocyte Ig-like receptor 1, LILRB1, or CD58j) (Colonna, Navarro et al. 1997), ILT4 (also known as LILRB2 or CD85d) (Colonna, Samaridis et al. 1998; Allan, Colonna et al. 1999), and the killer-cell immunoglobulin-like receptor KIR2DL4 (CD158d) (Rajagopalan and Long 1999).

Both ILT2 and ILT4 are able to interact with classical HLA class I molecules although they have a higher affinity for HLA-G (Shiroishi, Tsumoto et al. 2003). In contrast, KIR2DL4 has only one known ligand, HLA-G. Furthermore, expression of KIR2DL4 is mainly restricted to NK cells. Therefore, the role of this inhibitory receptor, KIR2DL4, in association with PE was also included in this study.

1.5 Natural Killer (NK) cells

NK cells are a highly specialized lymphoid population that is an important part of the innate immune defenses. NK cells constitute an average of 10-15% of peripheral blood lymphocytes (PBL) from healthy individuals (Trinchieri 1989) and are found in many lymphoid tissues, including the spleen and lymph nodes, as well as in blood, lung, peritoneal cavity in human (Trinchieri 1989). NK cells are involved in two major effector functions, cytotoxicity and cytokine production, regulated by a series of activating and inhibitory cell surface receptors that is recognized by specific MHC class I or non-MHC ligands (Moretta and Moretta 2004).

Apart from its presence in the peripheral circulation, a large population of NK cells are also found in the uterus (Trinchieri 1989) to prevent allograft rejection of the fetus and yet still maintain a competent immune defense against micro-organisms at the maternal-fetal interface. Uterine NK (uNK) cells are phenotypically and functionally distinct from NK cells in peripheral blood (Moffett-King 2002) and they have been shown to be a distinct NK cell lineage (Koopman, Kopcow et al. 2003). These cells emerge as the most prominent subpopulation in the uterine in the beginning of pregnancy, constituting 50 to 90% of total leukocytes in the decidua (Parham 2004). During early pregnancy, uNK cells accumulated as a dense infiltrate around trophoblast cells and from mid-gestation onwards, these cells progressively

disappear and are absent at term (Kam, Gardner et al. 1999). Hence, the presence of uNK cells in the uterus during pregnancy is coincident with the period of trophoblast invasion, as placentation is complete by about 20 weeks gestation (Moffett-King 2002). The exact functions of uNK cells are not know but it has been suggested that they influence maternal mucosal and arterial function and/or regulate placental trophoblast invasion (Moffett-King 2002).

There are 5 main families of NK cell receptors, namely the C-type lectin heterodimer family (CD94/NKGs), the natural killer cytotoxicity receptors (NCR), the glycosylphosphatidylinositol-anchored CD160 receptor, the immunoglobulin-like transcripts (ILT) and the killer-immunoglobulin-like receptors (KIR) (Tabiasco, Rabot et al. 2006).

1.6 Killer-Cell Immunoglobulin-like Receptors (KIR)

KIR genes are located on chromosome 19q13.4 within the Leukocyte Receptor Complex (LRC) and are classified into 3 groups by the HUGO Genome Nomenclature Committee (HGNC) based on their number of extracellular Ig-like domains, cytoplasmic tail length, and sequence similarity (Robinson, Waller et al. 2005). According to the nomenclature, the first digit following the KIR acronym corresponds to the number of Ig-like domains in the molecule where the 'D' denotes 'domain'. The D is followed by either a L (long cytoplasmic tail), S (short cytoplasmic tail) or P (pseudogenes). The final digit indicates the number of the gene encoding a protein with this structure (Vilches and Parham 2002).

KIRs with long cytoplasmic tails contains Immune Tyrosine-based Inhibitory Motifs (ITIM), which are tyrosine phosphorylated upon receptor engagement, and

subsequently bind the SH2 domains of protein-tyrosine phosphatases (SHP-1 and SHP-2) to transduce inhibitory signals to the NK cell (Vivier and Daeron 1997). On the other hand, those with short (S) cytoplasmic tails activate NK cell cytoxicity through interactions with the adaptor molecule known as DAP12, which contains the immunoreceptor tyrosine-based activation motif (ITAM) (Lanier, Corliss et al. 1998).

The majority of human KIR has 2 extracellular Ig-like domains and theis group of 2 domain genes are further divided into 2 types: Type 1 and Type II KIR2D. Type I KIR2D has a D1 and D2 conformation and members of this group include *KIR2DL1-3*, *KIR2DS1-5* and pseudogene *KIR2DP1* whereas Type II KIR2D has D0 and D2 conformation and its members are the *KIR2DL*4 and *KIR2DL5*. In addition to having a different domain conformation, Type II KIR2D genes differs from Type I KIR2D genes by having a deletion in the region coresponding to exon 4 in all other KIR as well as an untranslated pseudoexon 3 sequence in place of the translated exon 3. Apart from the 11 KIR2D genes, there is a group of 4 KIR genes with 3 domains, namely the KIR3D genes. These genes have 3 extracellular Ig-like domains with a D0, D1 and D2 conformation and members of this group includes the structurally related *KIR3DL2, KIR3DS1, KIR3DL3* and *KIR3DP1*.

All known KIR haplotypes are flanked at their centromeric end by *KIR3DL3* and their telomeric end by *KIR3DL2*, together with the centric *KIR3DP1* and *KIR2DL4*. These 4 *KIR* genes constitute the framework genes found to be present in all haplotypes and they define the two intervals of the *KIR* gene complex containing genes that vary between haplotypes (Martin, Freitas et al. 2000; Wilson, Torkar et al. 2000; Barten, Torkar et al. 2001). The conservation of gene structures and sequence homologies between the different KIR receptor haplotypes indicates that the LRC

evolved by extensive gene duplication and recombination with insertion and deletion mechanisms leading to KIR gene diversification (Shilling, Guethlein et al. 2002).

1.7 KIR2DL4

KIR2DL4 is unique among members of the KIR family as it consists of 8 exons compared to the 9 exons found on other two domain KIR molecules due to the complete absence of exon 4. Also, as previously mentioned, KIR2DL4 is a Type II KIR with two Ig domains in a D0-D2 configuration whereas other two domain members of the family have a D1-D2 configuration and also, it is a framework gene that is present on all KIR haplotypes along with KIR3DL3, KIR3DP1 and KIR3DL2 (Martin, Freitas et al. 2000; Wilson, Torkar et al. 2000; Barten, Torkar et al. 2001). In addition to that, it possesses both an arginine residue in the transmembrane region as well as a long cytoplasmic tail with a single ITIM. The combination of both the arginine residue and ITIM allows *KIR2DL4* to elicit both inhibitory (Faure and Long 2002) and activating (Rajagopalan, Fu et al. 2001; Kikuchi-Maki, Yusa et al. 2003) signals.

The expression of *KIR2DL4* had been reported in several studies, whilst one report had described expression on decidual NK cells during the first trimester of pregnancy and also on all NK cells obtained from the placenta at term, but not on NK cells obtained from the mother's peripheral blood (Ponte, Cantoni et al. 1999), other studies found *KIR2DL4* to be constitutively expressed in all NK cells at the transcriptional level (Valiante, Uhrberg et al. 1997; Rajagopalan and Long 1999; Goodridge, Witt et al. 2003). Expression of *KIR2DL4* in NK cells at the maternal-fetal interface suggests that *KIR2DL4* might play an important role in the maintenance of pregnancy.

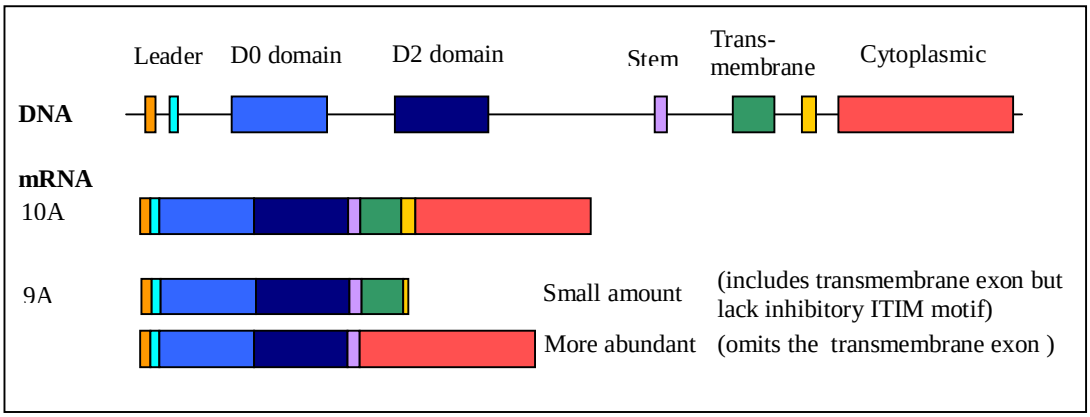
Functionally, *KIR2DL4* has been reported to be an inhibitory receptor for peripheral NK cells (Riteau, Menier et al. 2001) and uterine NK cells (Ponte, Cantoni et al. 1999). Additionally, *KIR2DL4* has been shown to activate cytokine production, but not cytotoxicity, in resting NK cells from peripheral blood (Rajagopalan, Fu et al. 2001). Production of cytokines such as TNFα, IL-1β and IFN-γ induces vascular endothelial growth factor production in trophoblast cells and thereby influences uterine angiogenesis (Choi, Park et al. 2002). These different functional activities are consistent with the fact that *KIR2DL4* has both an ITIM and a charged residue, allowing it to mediate different functions under different circumstances. A recent study on the possible roles of KIR2DL4 expression on human uNK cells demonstrated that KIR2DL4 may be important in the maintenance of human pregnancy as it was observed that KIR2DL4 protein levels in isolated uNK cells is much higher in normal controls as compared to patients with a pregnancy disorder (Yan, Lin et al. 2007).

According to the Immuno Polymorphism Database (IPD), 12 major alleles of *KIR2DL4* characterized by 23 SNPs/polymorphisms have been identified based on various studies (Selvakumar, Steffens et al. 1996; Selvakumar, Steffens et al. 1997; Valiante, Uhrberg et al. 1997; Cantoni, Verdiani et al. 1998; Rajalingam, Gardiner et al. 2001; Gedil, Steiner et al. 2005; Robinson, Waller et al. 2005). Of these 25 *KIR2DL4* SNPs/polymorphisms, 15 results in non-synonymous substitution of amino acids while the others are synonymous SNPs (Table 2).

Among the 23 SNPs/polymorphisms, codon 248 in exon 6 is of much interest as it consists of two polymorphisms: a dA→dG transition that results in a non-conservative amino acid substitution of Asn to Asp, and a single dA deletion within a

stretch of 10 adenines that results in a frameshift. This deletion polymorphism results in 2 variants of the transmembrane exon, namely the 9A and 10A alleles (Witt, Martin et al. 2000), each with a population frequency of 50 % (Witt, Whiteway et al. 2002). The 10A allele encodes the full-length classical membrane-bound receptor with an ITIM motif in its cytoplasmic tail, whereas the 9A allele encodes a completely different amino acid sequence in exons 7 and 8 as a result of the frameshift mutation (Figure 1), leading to the production of a protein without an ITIM or alternatively, a more abundantly expressed mRNA lacking the transmembrane exon.

Figure 1. Gene structure of *KIR2DL4* and the 9A/10A splice variants.



The association between alleles of *KIR2DL4* and PE had only been reported in one study (Witt, Whiteway et al. 2002) and no association was detected between the two. The authors hypothesized that the 9A alleles lacking the transmembrane exon would fail to prevent maternal NK cells from attacking the trophoblast and thereby lead to development of PE. However, comparisons of the frequencies of the 9A/10A variants, polymorphisms in exons 3 and 4 as well as the KIR gene repertoire between PE and normotensive groups showed no significant difference between the 2 groups.

Table 2. *KIR2DL4* alleles defined by SNPs (highlighted in bold) based on the IPD-KIR database.

Protein domain	Extracellular Ig domain												Stem	Transmembrane			Cytoplasmic								
	D0 domain					D2 domain																			
Exon	Exon3					Exon 4							Exon 5	Exon 6			Exon 7	Exon 8							
Nucleotide	82	183	191	207	226	34	51	119	145	189	254	264	32	56	65	105	29	22	153	154	159	168	235	246	247
Alleles/ Codon	30	64	66	72	78	109	115	137	146	161	182	186	206	231	234	248	257	273	316	317	318	321	344	347	348
KIR2DL4 *00101	TAT	GTG	CCA	TAC	CAC	CCG	ACA	GAA	CCT	CTG	GAA	GCG	ACT	TTT	CTT	AAT	GCG	CAG	AGA	GCG	TTG	GCC	CTT	TCT	AAT
KIR2DL4 *00102								GA**G**			GA**G**			TT**C**							TT**A**				
KIR2DL4 *0010301								GA**G**			GA**G**			TT**C**											
KIR2DL4 *00104											GA**G**			TT**C**											
KIR2DL4 *00105								GA**G**			GA**G**			TT**C**			GC**A**				TT**A**				
KIR2DL4 *00201							**G**CA				GA**G**	**C**CG													
KIR2DL4 *00202							**G**CA	GA**G**			GA**G**	**C**CG													
KIR2DL4 *00203							**G**CA	GA**G**			GA**G**	**C**CG		TT**C**											
KIR2DL4 *003		**C**TG	CC**T**					GA**G**			GA**G**		AC**A**	TT**C**	CT**C**	**G**AT					TT**A**	GC**G**		TC**C**	**C**AT
KIR2DL4 *004		**C**TG					**G**CA	GA**G**			GA**G**	**C**CG	AC**A**	TT**C**								GC**G**		TC**C**	**C**AT
KIR2DL4 *00501	T**G**T						**G**CA	GA**G**			GA**G**	**C**CG		TT**C**											
KIR2DL4 *00502	T**G**T						**G**CA	GA**G**			GA**G**	**C**CG		TT**C**							TT**A**				
KIR2DL4 *00601						C**T**G	**G**CA	GA**G**			GA**G**	**C**CG	AC**A**	TT**C**											
KIR2DL4 *00602						C**T**G	**G**CA	GA**G**			GA**G**	**C**CG		TT**C**											
KIR2DL4 *007				**A**AC	C**G**C		**G**CA	GA**G**			GA**G**	**C**CG				*			AG**T**					***	***
KIR2DL4 *0080101							**G**CA	GA**G**			GA**G**	**C**CG				*									
KIR2DL4 *0080102							**G**CA	GA**G**			GA**G**	**C**CG				*				**C**CG					
KIR2DL4 *0080103							**G**CA	GA**G**			GA**G**	**C**CG				*									
KIR2DL4 *0080104							**G**CA	GA**G**			GA**G**	**C**CG				*							**T**TT		
KIR2DL4 *0080201							**G**CA				GA**G**	**C**CG				*									
KIR2DL4 *009							**G**CA	GA**G**		**G**TG	GA**G**	**C**CG				*									
KIR2DL4 *010						C**T**G	**G**CA	GA**G**	C**A**T		GA**G**	**C**CG		TT**C**											
KIR2DL4 *011	T**G**T						**G**CA	GA**G**			GA**G**	**C**CG				*									
KIR2DL4 *012	T**G**T						**G**CA	GA**G**			GA**G**	**C**CG		TT**C**				**G**AG							

* deletion

References:

1. IPD-KIR database

1.8 KIR2DL4-HLA-G interaction

HLAG exerts its function through interaction with *KIR2DL4* and this signaling is supported by the fact that NK cells represent more than 80% of decidual lymphocytes and contact invading fetal trophoblast cells during pregnancy (King, Burrows et al. 1998; Loke, Hiby et al. 1999). Interactions between HLA-G and its inhibitory receptor, KIR2DL4, has been shown in various reports (Rajagopalan and Long 1999; LeMaoult, Zafaranloo et al. 2005; Yan and Fan 2005). Residues Met76 and Gln79 in the α1 domain unique to HLA-G was observed to play an important role in the recognition of KIR2DL4 (Yan and Fan 2005) and may be involved in the regulation of NK functions. Interestingly, all reported isoforms of HLA-G contains the α1 domain, which may explain why individuals that are homozygous for the HLA-G null allele (*HLA-G*0105N*) did not show any immunodeficiency and had healthy pregnancies (Ober, Aldrich et al. 1998; Casro, Morales et al. 2000).

1.9 SNP Genotyping

SNPs are an invaluable tool for gene-disease associations as they are the most common type of genetic variation in the human genome and hence, many different methods are available for SNP genotyping. Among them are hybridization-based methods whereby complementary DNA probes are utilized for hybridization to corresponding SNPs and these assays includes the use of molecular beacons (Abravaya, Huff et al. 2003) and SNP microarrays (Shen, Fan et al. 2005; Xiao, Segal et al. 2007). In addition, enzyme-based methods such as restriction fragment length polymorphism (RFLP), 5'-nuclease allelic discrimination assay (Taqman) (Hui, DelMonte et al. 2008), primer extension as well as other methods such as high

resolution melting analysis (Liew, Pryor et al. 2004), and direct DNA sequencing are also frequently used for SNP genotyping.

Genotyping of *HLA-G* and *KIR2DL4* SNPs were performed using a primer extension-based method known as minisequencing using a commercially available assay, the ABI PRISM® SnaPShot Multiplex kit by Applied Biosystems. This single-base extension assay was used as it is able to genotype multiple SNPs at known locations in a single tube using a small amount of amplified template and is relatively inexpensive for the detection of a fairly large number of SNPs in one reaction.

1.10 Aims of study

Pregnancy is the only physiological situation where HLA-specific inhibitory receptors expressed on maternal NK cells such as the Killer-cell Immunoglobulin-like Receptors (KIRs) may meet non-self variants of their otherwise cognate HLA allotypes, therefore, studies are currently underway to determine whether some combinations of maternal KIR genes and fetal HLA allotypes are less favorable for healthy pregnancy compared to others (Hiby, Walker et al. 2004; Trundley and Moffett 2004). Inhibition of NK cells by *HLA-G* at the maternal-fetal interface presents an attractive hypothesis to explain maternal tolerance of the trophoblast as the trophoblast cells lack the highly polymorphic classical class Ia genes (apart from *HLA-C*), expressing only non-classical class Ib genes *HLA-E*, *HLA-F* and *HLA-G* with limited allelic variations (Yelavarthi, Fishback et al. 1991; King, Boocock et al. 1996; King, Allan et al. 2000; Blaschitz, Hutter et al. 2001; Ishitani, Sageshima et al. 2003).

Certain combinations of HLA-KIR may be favorable in the maintenance of a healthy pregnancy, as shown in a study of the effect of HLA-C and KIR haplotypes in association with PE (Hiby, Walker et al. 2004). Therefore, it is possible that certain HLA-KIR combinations confer higher risk of PE in pregnancies. This study proposes to investigate the relationship between particular *HLAG* and *KIR2DL4* haplotypes in the development and risk for PE in the Malay population. Studies examining the association of *HLAG* with pregnancy-related hypertensive disorders have been inconcordant and contradictory thus far whereas studies of *KIR2DL4* alleles with PE are limited. Nevertheless, we hypothesize that certain *HLAG* and/or *KIR2DL4* haplotypes are associated with the development of PE.

2.0 Materials and Methods

2.1 Sample Collection

For the *HLA-G* and *KIR2DL4* case-control studies, 83 Malay women with PE and 240 healthy normotensive Malay women being cared for in the Department of Obstetrics and Gynaecology in the National University Hospital (NUH, Singapore) and Hospital Sultanah Aminah (HSA, Malaysia) were recruited antenatally or in labour. Among the PE group, 68 samples were collected from HSA and 15 samples were from NUH whereas for controls, a total of 125 subjects were recruited from HSA while 115 subjects were recruited from NUH. Only subjects of Malay ethnicity were selected as we had more access to Malay patients. The ethnicity of all subjects in this study was determined based on hospital records. This study had been approved by the Domain Specific Review Board of the National Healthcare Group of Singapore and Ethics Review Board of Hospital Sultanah Aminah. Informed written consent was obtained from all participants of the study.

Clinical phenotyping was carried out by doctors of respective hospitals. Details of participant's past obstetrics and medical history were noted to reduce other potential causes of hypertension such as autoimmune diseases, cardiac or renal complications. Diagnosis of PE was made if there was a gestational hypertension, with a systolic blood pressure of at least 140 mmHg and/ or a diastolic blood pressure of at least 90 mmHg, measured on two separate occasions at least 6 hours apart after the 20^{th} week of gestation, as well as proteinuria of at least 300 mg per day. Diagnosis of severe PE (SPE) was made if, in addition to symptoms above, signs of eclampsia such as headache, vomiting, visual disturbance and/ or epigastric pain were present.

Population *HLA-G* allele, genotype, and haplotype frequencies were also determined to confirm observed frequencies in the control group, and to compare against observed frequencies in other populations. Population allele, genotype and haplotype frequencies of *KIR2DL4* were not determined as *KIR2DL4* was not significantly associated with PE. Anonymized (except for gender and ethnicity) and unselected cord blood samples from a total of 90 Malay, 94 Chinese, and 90 Indian newborns were used to represent the 3 major ethnic groups in Singapore and Malaysia.

2.2 DNA Extraction

About 5 mL of venous blood was collected from each woman either in the delivery suite or in the antenatal ward, whilst 7 mL of blood from the umbilical cord was collected after the delivery of the baby. Genomic DNA extraction was carried from the blood samples using the phenol-chloroform method. First, blood samples with clots were passed through a 18g needle to shear the clots. Following that, for each blood sample, TKM buffer 1 (comprising of Tris-HCl, KCl, $MGCl_2$ and EDTA) and 2 % Triton X-100 was added to lyse the red blood cells and destabilize protein debris in the sample. The mixture were mixed well and centrifuged to remove the supernant. For the lysis of white blood cells, TKM buffer 2 (made up of Tris-HCl, KCl, $MGCl_2$, EDTA and NaCl) and sodium dodecyl sulfate (SDS) were added and incubated for 10 minutes at 55°C. After the incubation, RNase A was added to remove RNA in sample and the mixture was incubated at 37°C for up to 1 hour.

The supernatant containing DNA was added with a mixture of phenol: chloroform: iso-amylalcohol (25:24:1) and mixed for 10 minutes to remove protein

residues in the samples. Following that, the samples were centrifuged and the top aqueous phase containing DNA was transferred to a clean tube. Residual phenol in the sample was removed by the addition of choloform and iso-amylalcohol. The mixture was centrifuged separate the aqueous and organic phase. Finally, DNA was precipitated from the aqueous solution with the addition of ice-cold absolute ethanol. DNA sample was spooled out into a clean tube and 70% ethanol was added to wash the sample. After removing the 70% ethanol from the DNA samples, rehydrating solution (TE 10:1) was added to reconstitute the DNA samples. The extracted DNA samples were quantitated using the Spectramax M5 microplate reader after it is well dissolved in the rehydrating solution to obtain concentrations as well as the purity of the neat DNA.

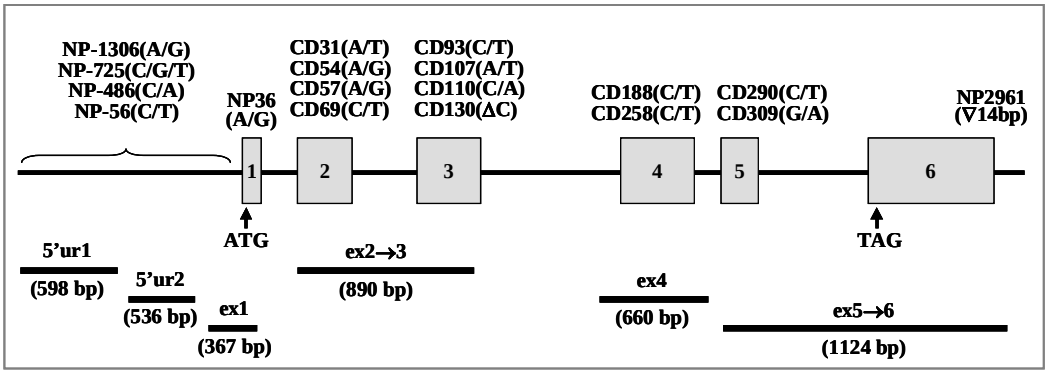
After that, the samples were divided into different groups based on the site of collection (NUH or HSA), diagnosis (SPE, PE or normal) as well as sample type (maternal or baby). Following that, the DNA samples were diluted into 96-well plates of 500 ng/µl and 50 ng/µl concentrations to be kept as stock and working plates respectively. The neat genomic DNA, stock and working DNA plates were then kept frozen in the -86ºC freezer until needed.

2.3 *HLA-G* PCR Amplification

Amplification of the *HLA-G* coding, 5' and 3' flanking regions containing the target single nucleotide polymorphism (SNP)/ polymorphisms was carried out using multiplex PCR. All 6 exons of the *HLA-G* gene were amplified in 6 fragments using specific primers in a single tube multiplex polymerase chain reaction (PCR) (Figure 2). Each 25 µl PCR reaction contained 50 ng of DNA, 0.2 mM of each deoxynucleotide triphosphate (dNTP), 1 Unit of HotStarTaq DNA polymerase in 1 X

supplied PCR buffer (Qiagen), and 6 pairs of specific forward and reverse primers with concentrations as shown in Table 3. Samples were amplified in a T3 thermal cycler (Biometra) with the following conditions: initial denaturation at 95 ºC for 15 min, and a subsequent denaturation at 94 ºC for 2 min, followed by 30 cycles of denaturation at 94 ºC for 1 min, annealing at 63ºC for 1 min, and extension at 72 ºC for 1.5 min, with a final extension at 72 ºC for 10 min. Following that, an 8 µl aliquot of each multiplex PCR product was analyzed by electrophoresis through a 1 % agarose gel in 1 X Tris-Borate-EDTA buffer at 15 volts/cm for an hour.

Figure 2. Gene structure of *HLA-G*: the 6 PCR amplicons and locations of polymorphisms are shown below and above the gene map, respectively.



2.4 *HLA-G* Minisequencing

Genotyping of the target *HLA-G* SNPs/ polymorphisms was carried out using a multiplex mini-sequencing strategy. The 18 SNPs/ polymorphisms of interest were genotyped in 2 panels, with each reaction assaying containing 9 polymorphic sites. Each minisequencing primer was designed to differ in length from the others, by the addition of non-specific GACT tetra-nucleotides at the 5' end, so that they could be clearly separated and differentiated based on size. Primers larger than 40 base-pairs (bp) were purified by polyacrylamide gel electrophoresis or HPLC to ensure that resulting primers are of specific desired lengths.

Table 3. Primers used in the multiplex PCR amplification of the *HLA-G* exons.

Primer Name	Primer Sequence (5'→3')	Length (bp)	Nucleotide Position[a]	Conc. (μM)[b]	Amplicon size (bp)	Region Amplified	Polymorphisms within Amplicon
HLAGu1F	GTCCCTACAATGAACCAGGTATG	23	-1470 to -1492	0.3	598	5'ur1	NP-1306 (A/G)
HLAGu1R	CAGCACAAACTTTATTCACCTCAC	24	-894 to -917	0.3			
HLAGu2F	AACTTAGGGCTACGGAATGAAGG	23	-810 to -822	0.6	536	5'ur2	NP-725 (C/G/T), NP-486 (C/A)
HLAGu2R	AGCGTTCTGTCTCAGTGTCTCC	22	-287 to -308	0.6			
HLAGe1F	CAGGGCCTCAAGCGTGGCTC	20	-234 to -253	0.2	367	5'ur3 and ex1	NP-56 (C/T), NP36 (A/G)
HLAGe1R	CCTCCGCGCAGGGGCTGTTT	20	95 to 114	0.2			
HLAGe2/3F	GCAGGACTCGGCAGCCGCG	19	140 to 158	0.8	890	ex2→3	CD31 (A/T), CD54 (A/G), CD57 (A/G), CD69 (C/T), CD93 (C/T), CD107 (A/T), CD110 (C/A), CD130 (ΔC)
HLAGe2/3R	GGCCAGGCTGAGAGGTCTACA	21	1009 to 1029	0.8			
HLAGe4F	GGTTCCCTTTGACCCCACAGC	21	1229 to 1249	0.2	660	ex4	CD188 (C/T), CD258 (C/T)
HLAGe4R	TTTCCCTAACAGACATGATGCCT	23	1866 to 1888	0.2			
HLAGe5-6F	TTTAACAGGGTCGGTGGTGAGG	22	1910 to 1931	0.3	1124	ex5→6	CD290 (C/T), CD309 (G/A), NP2961 (∇14bp)
HLAGe5-6R	GAAGGAATGCAGTTCAGCATGAG	23	3038 to 3060	0.3			

[a]With reference to the translation start site (+1).
[b]Concentration of primer in the multiplex-PCR reaction mixture.
ur: upstream region; ex: exon; NP: nucleotide position; CD: codon

The World Health Organization (WHO) allele assignments are based mainly on polymorphisms present in exons 2, 3 and 4 of the *HLA-G* gene. In recent years, more focus has been placed on the significance of a 14 base-pair (bp) insertion/ deletion in the 3'-untranslated region (3'UTR) and polymorphisms in the 5' upstream region. As such, these polymorphisms were included in the *HLA-G* haplotype profiles as well.

After PCR amplification, 2.5 µL of PCR product was incubated with 0.5 µL of *Exo*I nuclease (10 U/µl) and 1 µL of shrimp alkaline phosphatase (SAP) (1 U/µl) (United States Biochemical, USA) at 37 °C for 15 min, to remove and inactivate excess PCR primers and unincorporated dNTPs. The reaction was terminated by incubating the mixture at 80 °C for 15 min. Two multiplex genotyping reactions were prepared by mixing 1 µL of *Exo*I-SAP treated PCR product, 0.5 µL of either Panel 1 or 2 locus-specific detection primer mixture (Table 4), and 0.5 µL of SNaPshot™ Multiplex Ready Reaction Mix (Applied Biosystems, USA) containing AmpliTaq® DNA polymerase and fluorescently labeled dideoxynucleotide triphosphates (ddNTPs). Each 2 µL genotyping reaction mixture was subjected to 25 single-base extension (minisequencing) cycles consisting of a 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 30 s. One microliter of SAP (1 Unit) was added to the completed cycle minisequencing reaction, and the mixture was incubated at 37 °C for 1 h to inactivate unincorporated fluorescent ddNTPs, followed by a 75 °C incubation for 15 min to terminate the SAP treatment.

Table 4. Minisequencing primers used in multiplex genotyping of *HLA-G* polymorphisms.

	SNP ID	Primer Name[a]	Primer Sequence (5' to 3')[b]	Length (bp)	Annealing Site[c]	Conc. (μM)[d]	SNP allele / GS length[e]	SNP allele / GS length[e]
Panel 1	NP-1306 (A/G)	NP-1306F	$(gact)_4$GAACAGTGCTAGAGCCACA	35	-1307 to -1325	0.12	dA / 39.2	dG / 38.7
	NP-486 (C/A)	NP-486F	$(gact)_2$CGAGCTCACTCTCTGGCA	26	-504 to -487	0.12	dC / 31.2	dA / 31.9
	NP-56 (C/T)	NP-56R	$(gact)_7$GCGAGGACTTTAGAACCAG	47	-55 to -37	0.05	dG / 51.0	dA / 52.1
	CD31 (A/T)	CD31F	t$(gact)_{11}$ATGGGCTACGTGGACGAC	63	274 to 291	0.08	dA / 67.0	dT / 67.5
	CD57 (A/G)	CD57R	GTCTCCTCTTCCCAATACTC	20	373 to 392	0.30	dT / 28.0	dC / 25.6
	CD110 (C/A)	CD110R	ct$(gact)_9$ATACTGTTCATACCCGCGGA	58	756 to 775	0.08	dG / 60.0	dT / 61.7
	CD130 (ΔC)	CD130F	$(gact)_3$CTCGCCCTGAACGAGGAC	30	797 to 814	0.20	dC[f] / 34.9	dT[f] / 36.1
	CD188 (C/T)	CD188F	act$(gact)_8$AGACCCCCCCAAGACACA	53	1570 to 1587	0.08	dC / 54.5	dT / 55.6
	CD258 (C/T)	CD258F	$(gact)_5$TGGAGAGGAGCAGAGATACA	40	1779 to 1798	0.80	dC / 44.2	dT / 45.4
Panel 2	NP-725 (C/G/T)	NP-725R	actgactTGCATCTAAAAGCATTACAACA	29	-724 to -705	0.10	dG / 29.9	dC / 31.1 (dA / 30.9)
	NP36 (A/G)	NP36R	ct$(gact)_8$GTCAGGGCCCCCGAGAG	51	37 to 53	0.12	dT / 55.5	dC / 55.1
	CD54 (A/G)	CD54F[g]	$(gact)_{10}$GCGCCGTGGGTGGAGC	56	346 to 361	0.04	dA / 60.6	-
	CD69 (C/T)	CD69R[g]	$(gact)_5$ATTCTGTCAGTCTGTGCGTG	40	409 to 428	0.25	dG / 45.3	-
	CD93 (C/T)	CD93R	$(gact)_3$CAATCATCCACTGGAGGGT	31	707 to 725	0.10	dG / 35.5	dA / 37.2
	CD107 (A/T)	CD107F	t$(gact)_7$GACCTGGGGTCCGACGG	46	731 to 747	0.20	dA / 50.7	dT / 50.8
	CD290 (C/T)	CD290F	$(gact)_3$ATCATGGGTATCGTTGCTGG	32	1998 to 2017	0.60	dC / 38.8	dT / 40.3
	CD309 (G/A)	CD309F	act$(gact)_{10}$CGCTGCTGTGCTGTGGAG	61	346 to 361	0.08	dG / 65.4	dA / 66.2
	NP2961 (∇14bp)	NP2961R	GTTCTTGAAGTCACAAAGGGA	21	2960 to 2980	0.80	dA[h] / 28.3	dC[h] / 25.9

[a]Primer names ending in F are forward primers, names ending in R are reverse complement primers
[b]Sequences in lowercases represent non-specific tails; subscripted numbers represent multiples of the GACT tetranucleotide.
[c]With reference to the translation start site (+1).
[d]Concentration of primer in the multiplex minisequencing reaction mixture.
[e]Mean apparent nucleotide length of extended primer as analyzed by GeneScan (GS).
[f]dC represents allele containing the C nucleotide at the insertion/deletion polymorphism site in exon 3. dT represents allele without the C nucleotide at the insertion/deletion polymorphism site.
[g]These primers detected only one allele in our population.
[h]dA represents allele with the 14bp sequence at the insertion/deletion polymorphism site in the 3'UTR. dC represents allele without the 14bp sequence at the insertion/deletion polymorphism site.
ex: exon; NP: nucleotide position; CD: codon

2.5 *KIR2DL4* PCR Amplification

A multiplex PCR strategy was used to amplify exons 3 to 8 of the *KIR2DL4* gene containing the SNPs/ polymorphisms of interest in 5 fragments (Figure 3). Primers were designed in the intronic region as the *KIR2DL4* gene is highly homologous to other genes of the *KIR* family and it is essential that the primers used are KIR2DL4 specific to avoid incorrect amplification and genotyping subsequently. A single tube multiplex PCR was carried out in a T3 thermal cycler (Biometra) in a total volume of 25 µl containing 50 ng of DNA, 0.2 mM of each dNTP, 1 Unit of HotStarTaq DNA polymerase in 1 X supplied PCR buffer (Qiagen), and 5 pairs of forward and reverse primers with concentrations as shown in Table 5. PCR cycling conditions were as follow: an initial denaturation at 95 ºC for 15 min, followed by 30 cycles of denaturation at 95 ºC for 45 sec, annealing at 60 ºC for 1 min and extension at 72 ºC for 1 min, with a final extension at 72 ºC for 7 min. A 8 µl aliquot of each amplified product was resolved on a 2 % agarose gel in 1 X Tris-borate-EDTA at 15 volts/cm for an hour.

Figure 3. Gene structure of *KIR2DL4*: the 5 PCR amplicons and locations of polymorphisms are shown below and above the gene map, respectively

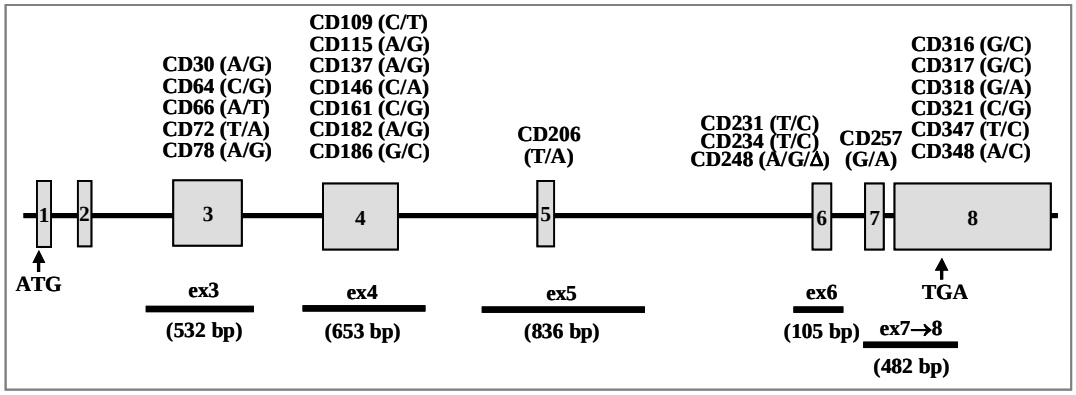


Table 5. Primers used in the multiplex PCR amplification of the *KIR2DL4* exons.

Primer name	Primer Sequence (5' → 3')	Length (bp)	Nucleotide Position[a]	Conc (μM)[b]	Amplicon size (bp)	Region Amplified	Polymorphisms within Amplicon
2DL4-Ex3F	GGA GAC GCC ACG TCT ATG CG	20	981-1000	0.2	532	ex 3	Cd30, Cd64, Cd66. Cd72, Cd78
2DL4-Ex3R	TGA TCG GAC TCT GGT GGA CAC	21	1512-1492	0.2			
2DL4-Ex4F	GGG ATC GAC AGG AAG AGT TGG	21	2151-2171	0.2	653	ex 4	Cd109, Cd115, Cd137, Cd146, Cd161, Cd182, Cd186
2DL4-Ex4R	GGA GCC CTT ACT GCA AGC TTC	21	2803-2783	0.2			
2DL4-Ex5F	CCA GAT TGT AGA TTC TTC GAA CC	23	4819-4841	0.5	836	ex 5	Cd206
2DL4-Ex5R	AAC CAG GGT TGG ATC ATG ACA G	22	5654-5633	0.5			
2DL4-Ex6F	ACA CCC CTC CCA ATA GGC AC	20	9198-9217	0.2	446	ex 6	Cd231, Cd234, Cd248
2DL4-Ex6R	CCA TCC TGC TTC CGC ACA GG	20	9643-9624	0.2			
2DL4-Ex7F	GTT TYG ATT GCT TCC GTC TCC	21	10022-10042	0.5	482	ex 7 → 8	Cd257, Cd316, Cd317, Cd318, Cd321, Cd348
2DL4-Ex8R	GAA GAG TGA TGC TCT AAG ATG G	22	10503-10482	0.5			

[a]With reference to the translation start site (+1).
[b]Concentration of primer in the multiplex-PCR reaction mixture.
ex: exon; CD: codon

2.6 *KIR2DL4* Minisequencing

The PCR-amplified products were genotyped for 23 *KIR2DL4* polymorphisms in 2 multiplex minisequencing reactions, with reactions assaying 12 and 11 polymorphic sites for Panels 1 and 2, respectively. For the genotyping of each PCR product, a total of 24 mini-sequencing primers were used to detect the 22 SNPs and 1 insertion/ deletion polymorphism that characterizes the 12 *KIR2DL4* major alleles. For the detection of the SNP at codon 317, 2 minisequencing primers were used because this SNP is located next to a SNP at codon 316. Therefore, the last nucleotide of the codon 317 minisequencing primer was designed with either of the 2 possible nucleotides of codon 316 SNP (A or T) to enable phase detection of the codons 316 and 317 in addition to the detection of the codon 317 alleles. In addition to that, for the detection of polymorphisms at codon 248, 2 different primers (1 in each panel) were also used because this codon consists of a SNP and a deletion polymorphism.

Minisequencing primers for the detection of *KIR2DL4* polymorphisms were designed to be similar to the primers used for genotyping the *HLA-G* SNPs/ polymorphisms whereby each minisequencing primer was added with different lengths of non-specific GACT nucleotides at the 5' end to enable separation and differentiation. The minisequencing protocol and analysis of *KIR2DL4* genotype data were performed as described for the detection of *HLA-G* SNPs/ polymorphisms with the exception that different locus-specific detection primer mixtures were used for the detection of *KIR2DL4* SNPs (Table 6).

Table 6. Minisequencing primers used in multiplex genotyping of *KIR2DL4* polymorphisms.

	SNP ID	Primer Name[a]	Primer Sequence (5' to 3')[b]	Length (bp)	Annealing Site[c]	Conc. (μM)[d]	SNP allele / GS length[e]	SNP allele / GS length[e]
Panel 1	CD30 (A/G)	CD30F	GTGACTCTTCGGTGTCACT	19	1204-1222	0.2	dG / 22.4	dA / 25.5
	CD66 (A/T)	CD66F[f]	$(gact)_{11}$gTCATTAGCCCTSTGACCCC	64	1313-1331	0.3	dA / 67.6	-
	CD72 (T/A)	CD72F[f]	$(gact)_{2}$gaCWGCACACGCAGGGACC	27	1331-1347	0.2	dT / 31.4	-
	CD115 (A/G)	CD115F	$(gact)_{4}$gaGGGCCCCACGGTTCGC	34	2340-2355	0.2	dG / 37.8	dA / 39.5
	CD146 (C/A)	CD146F[f]	act$(gact)_{4}$GCCCATGAACTTAGGCTCC	38	2431-2449	0.2	dC / 41.2	-
	CD182 (A/G)	CD182F[f]	$(gact)_{5}$gacTTCCATGGATCTCCCTACGA	43	2539-2558	0.6	dG / 46.3	-
	CD206 (T/A)	CD206F[f]	t$(gact)_{6}$gacGTAGTTGGCCTTCACCCAC	47	5200-5218	0.4	dT / 51.4	-
	CD231 (T/C)	CD231F	$(gact)_{8}$TCAGTGGCCATCATCCTCTT	52	9517-9536	0.2	dC / 56.0	dT / 56.8
	CD248 (A/G)	CD248R-2[f]	$(gact)_{10}$gTCTGCTTCGTGAGGCTTAC	60	9605-9587	0.8	dT/ 64.4	-
	CD317 (G/C)	CD317F-A[f]	$(gact)_{2}$gacTCCAAATGCTGAGCCCAGA	30	10334-10352	0.2	dG / 33.6	-
	CD321 (C/G)	CD321R[F]	act$(gact)_{11}$gaCCTGACTGTGGTGCTCATG	68	10386-10368	0.3	dG / 71.4	-
	CD348 (A/C)	CD348R[f]	act$(gact)_{8}$gGATTCCAGCTGCTGGTACAT	56	10466-10447	0.2	dT / 60.0	-
Panel 2	CD64 (G/C)	CD64F[f]	AACAGTTTCCTCATTAGCCCT	21	1303-1323	0.08	dG / 24.6	-
	CD78 (A/G)	CD78R[f]	t$(gact)_{2}$gCAGTGGGGGAGTGCGGG	27	1384-1368	0.1	dT / 36.8	-
	CD109 (C/T)	CD109F	$(gact)_{4}$gaCTTCGCTTACAGCCCGGC	36	2321-2338	0.5	dC / 39.7	dA / 40.8 (dT / 40.9)
	CD137 (A/G)	CD137F	$(gact)_{5}$ATCTACCATCTATCCAGGGA	40	2404-2423	0.4	dG / 43.9	dA / 45.0
	CD161 (C/G)	CD161F[f]	$(gact)_{6}$gaATTCCAGGCCGACTTCCCT	45	2475-2493	0.2	dC / 48.8	-
	CD186 (G/C)	CD186R	$(gact)_{8}$gCAGGCAGTGGGTCACTCG	51	2587-2570	0.3	dG / 54.6	dC / 55.0
	CD234 (T/C)	CD234R[f]	act$(gact)_{8}$CGATGAAGGAGAAAGAAGGG	55	9566-9547	0.4	dA / 59.5	-
	CD248 (A/C)	CD248R-1	TTCGTGAGGCTTACYTTTTTTTT	23	9600-9578	0.2	dG[g] / 28.1	dT[g] / 30.9
	CD257 (G/A)	CD257R[f]	$(gact)_{10}$gCTGTTCACTGTTCTGTGTCC	61	10096-10077	0.5	dC / 64.7	-
	CD316 (A/T)	CD316F[f]	$(gact)_{11}$gTTCCAAATGCTGAGCCCAG	64	10333-10351	0.8	dA / 68.0	-
	CD317 (G/C)	CD317F-T[h]	$(gact)_{2}$gacTCCAAATGCTGAGCCCAGT	30	10334-10352	0.1	-	-
	CD318 (G/A)	CD318R	ct$(gact)_{12}$GTGCTCATGSGCAGGAGA	68	10376-10359	2.0	dC / 71.5	dT / 72.5

[a]Primer names ending in F are forward primers, names ending in R are reverse complement primers
[b]Sequences in lowercases represent non-specific tails; subscripted numbers represent multiples of the GACT tetranucleotide.
[c]With reference to the translation start site (+1).
[d]Concentration of primer in the multiplex minisequencing reaction mixture.
[e]Mean apparent nucleotide length of extended primer as analyzed by GeneScan (GS).
[f]These primers detected only one allele in our population.
[g]dG represents allele with a series of 9 adenines at the 9A/10A polymorphism site in exon 6. dT represents allele with a series of 10 adenines at the 9A/10A polymorphism site.
[h]This primer did not detect any allele in our population.
ex: exon; NP: nucleotide position; CD: codon

2.7 Capillary Electrophoresis and Genotype Analysis

For the genotype analysis of *HLA-G* and *KIR2DL4*, a 1.5 µL aliquot of treated multiplex minisequencing product was mixed with 9 µL of HiDi™ formamide and 0.1 µL of GeneScan-120 LIZ internal size calibrator (Applied Biosystems, USA). The mixture was heated at 95 °C for 5 min, snap-cooled on ice, then resolved by automated capillary electrophoresis on an ABI PRISM® 3100 Genetic Analyzer and analyzed using GeneScan™ application software (Applied Biosystems, USA). Called alleles of samples were randomly selected for further confirmation by direct sequencing.

2.8 Statistical Analysis

Allele/ haplotype frequencies for *HLA-G* as well as *KIR2DL4* were estimated based on an expectation-maximization (EM) algorithm using the SNPHap software (Clayton). Only samples in which all loci were successfully genotyped were included in the allele/ haplotype frequency estimation. Statistical analyses of *HLA-G* and *KIR2DL4* allele/ haplotype as well as individual SNP frequencies were performed using SPSS 15.0 for Windows (SPSS Inc., USA). Due to small number of subjects for certain alleles/ haplotypes, Fisher's exact test was used to compare *HLA-G* as well as *KIR2DL4* allele/ haplotype and genotype frequencies between case and control mothers, as well as between case and control babies to test for paternal contribution to disease development. Logistic regression analyses were performed to determine the association between the presence of particular haplotypes in an individual (and thus genotype frequencies) and the development of PE with adjustments for maternal age, body mass index (BMI), maternal history of PE or pregnancy induced hypertension

(PIH), history of abortion, primigravidity and change of partner. Deviation of *HLA-G* SNPs from Hardy-Weinberg equilibrium (HWE) was tested using the χ^2 Goodness-of-Fit test.

In addition, for the statistical analysis of *HLA-G* alleles/ haplotypes and the risk of PE, the defining SNP for the particular haplotype showing significant association with PE was further analyzed at the mother-child genotype pair levels to test for association with disease.

On the other hand, alleles of *KIR2DL4* receptor were further analyzed with alleles of its ligand, *HLA-G*, to test the effect of gene-gene interaction on risk for PE. Logistic regression analyses were performed to compare maternal *KIR2DL4* alleles with fetal *HLA-G* alleles to test for risk of PE. Individual *HLA-G* or *KIR2DL4* alleles that showed significant risk of PE following logistic regression analyses were further analyzed by comparing different fetal ligand-maternal receptor allele combinations to test for possible gene-gene interactions using Fisher's exact test.

The effect of an allele or genotype in relation to disease risk/ susceptibility was expressed as an odds ratio (OR) http://www.hutchon.net/ConfidOR.htm. Statistical significance was set at a more conservative $p<0.01$ to adjust for type I error in multiple comparisons.

3.0 Results

3.1 Group-specific demographic and clinical characteristics

Demographic characteristics and pregnancy outcome of the PE cases and controls in this study are summarized in Table 7. The demographics characteristics of cases and controls were comparable with regards to their mean age. In contrast, there are a significantly higher proportion of PE women with previous history of PE or PIH and a higher BMI. Also, a nominally higher proportion of PE women was observed to be primigravids and had changed partners.

Table 7. Analysis of risk factors and pregnancy outcomes in pre-eclampsia cases and normal controls.

Risk Factors and Pregnancy Outcomes	Incidence		*P*-value
	Normal Controls N=240	Pre-eclampsia N=83	
Previous abortion	22.55%	20.51%	0.706
Previous history of PE or PIH	2.13%	20.51%	**<0.001**
Gender of baby – Male	49.79%	60.34%	0.149
– Female	50.21%	39.66%	0.149
Primigravidity	24.69%	38.27%	0.019
Change of partner	2.53%	8.75%	0.023
Maternal age (years, mean±SD)	28.70 ± 5.66	29.83 ± 6.13	0.140
BMI (kg/m^2, mean±SD)	25.03 ± 4.86	28.42 ± 5.89	**<0.001**
Gestational age (weeks, mean±SD)	39.08 ± 1.74	36.59 ± 3.43	**<0.001**
Baby birthweight (g, mean±SD)	3145.75 ± 469.42	2684.81 ± 742.03	**<0.001**

PE, pre-eclampsia; PIH, pregnancy induced hypertension; BMI, body mass index
P-values <0.01 are highlighted in bold.

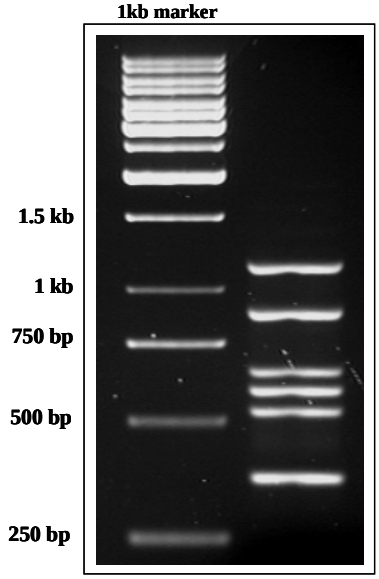
In addition to that, significantly different pregnancy outcomes were also observed between babies in the PE and normal control groups, including a shorter period of gestation and lower birthweight in PE babies. The majority of the PE cases analyzed in this study involved late onset PE (68.2%) with symptoms observed after 34 weeks' gestation.

3.2. *HLA-G*

3.2.1 Multiplex PCR Amplification and Genotyping

Amplification of the 6 *HLA-G* fragments containing the target SNPs in the coding, 5' and 3' flanking regions were carried out in a single tube multiplex PCR reaction and resolved on agarose gel electrophoresis (Figure 4). All samples analyzed in this study were succesfully amplified for the 6 fragments designed to genotype the SNPs of interest.

Figure 4. Multiplex PCR amplified fragments of the *HLA-G* gene.



Amplicon	SNPs present
Exon 5-6 (1124 bp)	CD209 CD309 NP2961
Exon 2,3 (890 bp)	CD31, CD54, CD57, CD69, CD93, CD107, CD110, CD130
Exon 4 (660 bp)	CD188, CD258
5'UR (598 bp)	NP-1306
5'UR (536 bp)	NP-725, NP-486
Exon 1 (367 bp)	NP-56, NP36

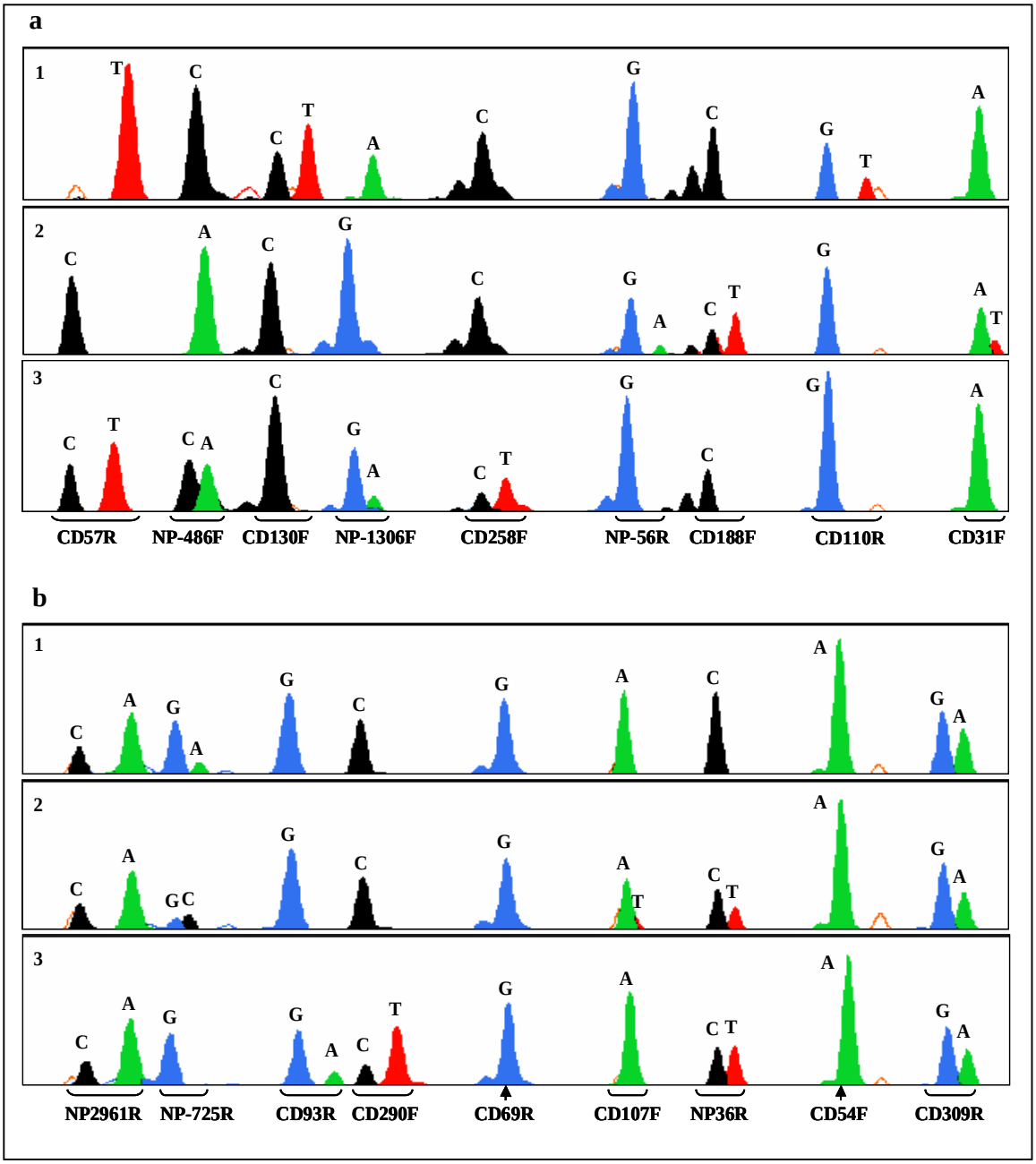
Genotyping of the 18 *HLA-G* SNPs were carried using a 2 panel multiplex mini-sequencing assay with 9 SNPs in each panel. Figure 5 displays minisequencing traces for Panels 1 and 2 following capillary electrophoresis. This minisequencing assay was designed to detect only 6 *HLA-G* alleles as other alleles were discovered more recently after the development of this assay. In our study population, 5 major *HLA-G* alleles were present, namely *G*0101*, *G*0103*, *G*0104*, *G*0105N* and *G*0106*. These alleles were further sub-grouped into haplotypes based on different SNPs in the 5' UR and 3' UTR.

3.2.2 Comparisons of HLA-G Allele/ Haplotype Frequencies in PE and Controls

Pair-wise comparisons revealed no significant differences in *HLA-G* allele frequencies between case and control mothers (Table 8). However, there was a nominally higher frequency of *G*0106* in PE babies compared with normal control babies (p=0.013) (Table 8). The frequency of PE babies heterozygous or homozygous for *G*0106* was also observed to be nominally higher than in the normal control group (p=0.012) (Table 9). When multivariate logistic regression analysis was performed, with adjustments for maternal age, BMI, history of PE or PIH, history of abortion, primigravidity and change of partner, the difference between PE and normal control babies reached statistical significance (p=0.004, OR=6.4, 95% CI 1.8-23.0) (Table 9). However, this association is not present in the maternal group (Table 10).

As primigravidity is known to be a risk factor for pre-eclampsia, we further analyzed the primigravida sub-group separately from the multigravida sub-group, to exclude confounding due to unequal proportions of primigravidas between case and control groups. No significant allele or genotype differences were observed between

Figure 5. GeneScan™ electropheromgram traces of panel 1 (a1-a3) and 2 (b1-b3) after multiplex minisequencing of *HLA-G* polymorphisms from 3 different individuals.



cases and controls in either primigravid or multigravid mother sub-groups (Table 10). However, a significantly higher frequency of *G*0106* allele was observed in PE babies compared to normal control babies, but only in the multigravid sub-group (p=0.002, OR=5.0, 95% CI 1.8-13.8) (Table 9). A significantly higher frequency of babies homozygous or heterozygous for G*0106 was also observed in the PE multigravid sub-group compared to babies in the normal control multigravid sub-group (p=0.002, OR=5.4, 95% CI 1.9-15.4) (Table 9). Logistic regression analysis with adjustments for maternal age, BMI, history of PE or PIH, history of abortion and change of partner also yielded similarly significant association between presence of fetal *G*0106* in multigravid pregnancies and PE (p=0.003, OR=10.1, 95% CI 2.2-46.8) (Table 9).

Table 8. Analysis of maternal and fetal *HLA-G* allele/haplotype frequencies in cases and controls.

Allele/Haplotype[a]	Polymorphisms																			Maternal Frequencies Number (%)			Fetal Frequencies Number (%)		
	5' Upstream Region				Exon 1	Exon 2				Exon 3				Exon 4		Exon 5		Exon 6							
	NP-1306	NP-725	NP-486	NP-56	NP36	CD31	CD54	CD57	CD69	CD93	CD107	CD110	CD130	CD188	CD258	CD290	CD309	NP2961[b]		Normal Controls n=480	Pre-eclampsia n=166	Fisher's Exact Test *P*-value	Normal Controls n=480	Pre-eclampsia n=166	Fisher's Exact Test *P*-value
G*0101																				250 (52.08)	81 (48.80)	0.473	244 (50.83)	85 (51.20)	1.000
G*010101																				139 (28.96)	45 (27.11)	0.691	117 (24.38)	47 (28.31)	0.352
*G*010101a*	G	C	A	C	CTG	ACG	CAG	CCG	GCC	CAC	GGA	CTC	CTG	CAC	ACG	GGC	AGA	G		136 (28.33)	45 (27.11)	0.841	113 (23.54)	47 (28.31)	0.251
*G*010101b*	G	G	A	C	CTG	ACG	CAG	CCG	GCC	CAC	GGA	CTC	CTG	CAC	ACG	GGC	AGA	G		3 (0.63)	0 (0.00)	0.573	4 (0.83)	0 (0.00)	0.577
G*010102																				22 (4.58)	5 (3.01)	0.502	24 (5.00)	5 (3.01)	0.385
*G*010102a*	A	C	C	C	CT**A**	ACG	CAG	CC**A**	GCC	CA**T**	GGA	CTC	CTG	CAC	ACG	GGC	AG**G**	**T**		7 (1.46)	0 (0.00)	0.200	10 (2.08)	1 (0.60)	0.305
*G*010102b*	A	C	C	C	CT**A**	ACG	CAG	CC**A**	GCC	CA**T**	GGA	CTC	CTG	CAC	ACG	GG**T**	AG**G**	**T**		15 (3.13)	4 (2.41)	0.793	9 (1.88)	3 (1.81)	1.000
*G*010102d*	G	C	C	C	CT**A**	ACG	CAG	CC**A**	GCC	CA**T**	GGA	CTC	CTG	CAC	ACG	GGC	AG**G**	**T**		0 (0.00)	1 (0.60)	0.257	4 (0.83)	1 (0.60)	1.000
*G*010102e*	A	C	C	C	CT**A**	ACG	CAG	CC**A**	GCC	CA**T**	GGA	CTC	CTG	CAC	ACG	GG**T**	AG**G**	G		0 (0.00)	0 (0.00)	1.000	1 (0.21)	0 (0.00)	1.000
G*010103																				87 (18.13)	30 (18.07)	1.000	102 (21.25)	33 (19.88)	0.741
*G*010103a*	A	C	C	C	CT**A**	ACG	CAG	CC**A**	GCC	CAC	GG**T**	CTC	CTG	CAC	ACG	GGC	AG**G**	**T**		87 (18.13)	29 (17.47)	0.907	102 (21.25)	30 (18.07)	0.435
*G*010103b*	G	C	A	C	CTG	ACG	CAG	CCG	GCC	CAC	GG**T**	CTC	CTG	CAC	ACG	GGC	AGA	**T**		0 (0.00)	1 (0.60)	0.257	0 (0.00)	3 (1.81)	0.017
G*010108a	A	C	C	C	CT**A**	ACG	CAG	CC**A**	GCC	CAC	GGA	CTC	CTG	CAC	ACG	GGC	AG**G**	G		2 (0.42)	1 (0.60)	1.000	1 (0.21)	0 (0.00)	1.000
G*0103																				1 (0.21)	1 (0.60)	0.448	3 (0.63)	1 (0.60)	1.000
*G*0103b*	G	T	A	T	CTG	**T**CG	CAG	CCG	GCC	CAC	GGA	CTC	CTG	CA**T**	ACG	GGC	AG**G**	**T**		1 (0.21)	1 (0.60)	0.448	2 (0.42)	1 (0.60)	1.000
*G*0103c*	A	T	A	T	CTG	**T**CG	CAG	CCG	GCC	CAC	GGA	CTC	CTG	CA**T**	ACG	GGC	AG**G**	**T**		0 (0.00)	0 (0.00)	1.000	1 (0.21)	0 (0.00)	1.000
G*010401a	A	C	C	C	CT**A**	ACG	CAG	CC**A**	GCC	CAC	GGA	**A**TC	CTG	CAC	ACG	GGC	AG**G**	G		213 (44.38)	78 (46.99)	0.588	219 (45.63)	70 (42.17)	0.469
G*0105N																				4 (0.83)	1 (0.60)	1.000	5 (1.04)	0 (0.00)	0.335
*G*0105Na*	A	C	C	C	CT**A**	ACG	CAG	CC**A**	GCC	CA**T**	GGA	CTC	-TG	CAC	ACG	GG**T**	AG**G**	**T**		3 (0.63)	1 (0.60)	1.000	5 (1.04)	0 (0.00)	0.335
*G*0105Nb*	A	C	C	C	CT**A**	ACG	CAG	CC**A**	GCC	CAC	GGA	CTC	-TG	CAC	ACG	GG**T**	AG**G**	**T**		1 (0.21)	0 (0.00)	1.000	0 (0.00)	0 (0.00)	1.000
G*0106																				12 (2.50)	5 (3.01)	0.779	9 (1.88)	10 (6.02)	0.013
*G*0106a*	A	C	C	C	CT**A**	ACG	CAG	CC**A**	GCC	CA**T**	GGA	CTC	CTG	CAC	A**T**G	GG**T**	AG**G**	**T**		10 (2.08)	5 (3.01)	0.550	8 (1.67)	9 (5.42)	0.020
*G*0106b*	A	C	C	C	CT**A**	ACG	CAG	CC**A**	GCC	CA**T**	GGA	CTC	CTG	CAC	A**T**G	GGC	AG**G**	G		0 (0.00)	0 (0.00)	1.000	0 (0.00)	1 (0.60)	0.257
*G*0106c*	A	C	C	C	CT**A**	ACG	CAG	CC**A**	GCC	CA**T**	GGA	CTC	CTG	CAC	A**T**G	GGC	AGA	**T**		2 (0.42)	0 (0.00)	1.000	1 (0.21)	0 (0.00)	1.000

[a]Sub-haplotypes of the WHO defined alleles are indicated by the letters a, b, c, d, and e at the end.
Nucleotide numberings are with reference to the translation start site; n represents the number of chromosomes examined.
[b]T represents the allele containing the 14 bp insertion.
NP: nucleotide position; CD: codon

Table 9. Analysis of fetal *HLA-G* allele and genotype frequencies.

	Fetal Allele Frequencies					Fetal Genotype Frequencies[a]					
	Normal Controls		Pre-eclampsia		Fisher's Exact Test	Normal Controls		Pre-eclampsia		Fisher's Exact Test	Logistic Regression
All Pregnancies											
	n=480	%	n=166	%	*P*-value	N=240	%	N=83	%	*P*-value	*P*-value[b]
*G*0101*	244	50.83	85	51.20	1.000	182	75.83	62	74.70	0.882	0.752
*G*0103*	3	0.63	1	0.60	1.000	3	1.25	1	1.20	1.000	0.690
*G*0104*	219	45.63	70	42.17	0.469	169	70.42	52	62.65	0.218	0.757
*G*0105N*	5	1.04	0	0.00	0.335	5	2.08	0	0.00	0.333	0.999
*G*0106*	9	1.88	10	6.02	0.013	9	3.75	10	12.05	0.012	**0.004**
Primigravid Pregnancies											
	n=118	%	n=62	%	*P*-value	N=59	%	N=31	%	*P*-value	*P*-value[c]
*G*0101*	63	53.39	38	61.29	0.345	47	79.66	27	87.10	0.563	0.953
*G*0104*	53	44.92	23	37.10	0.344	42	71.19	19	61.29	0.353	0.360
*G*0106*	2	1.69	1	1.61	1.000	2	3.39	1	3.23	1.000	0.678
Multigravid Pregnancies											
	n=360	%	n=100	%	*P*-value	N=180	%	N=50	%	*P*-value	*P*-value[d]
*G*0101*	180	50.00	45	45.00	0.429	134	74.44	34	68.00	0.372	0.781
*G*0103*	3	0.83	1	1.00	1.000	3	1.67	1	2.00	1.000	0.840
*G*0104*	165	45.83	45	45.00	0.910	126	70.00	32	64.00	0.491	0.747
*G*0105N*	5	1.39	0	0.00	0.590	5	2.78	0	0.00	0.588	0.999
*G*0106*	7	1.94	9	9.00	**0.002**	7	3.89	9	18.00	**0.002**	**0.003**

n, number of chromosomes examined.
N, number of individuals examined.
[a]Heterozygosity or homozygosity for a particular allele.
[b]*P*-value of multivariate analysis after adjustment of maternal age, body mass index, history of PE or PIH, history of abortion, primigravidity and change of partner.
[c]*P*-value of multivariate analysis after adjustment of maternal age and body mass index.
[d]*P*-value of multivariate analysis after adjustment of maternal age, body mass index, history of PE or PIH, history of abortion and change of partner.
P-values <0.01 are highlighted in bold.

Table 10. Analysis of maternal *HLA-G* allele and genotype frequencies.

	Maternal Allele Frequency					Maternal Genotype Frequency[a]					
	Normal Controls		Pre-eclampsia		Fisher's Exact Test	Normal Controls		Pre-eclampsia		Fisher's Exact Test	Logistic Regression
All Pregnancies											
	n=480	%	n=166	%	*P*-value	N=240	%	N=83	%	*P*-value	*P*-value[b]
*G*0101*	250	52.08	81	48.80	0.473	182	75.83	64	77.11	0.882	0.692
*G*0103*	1	0.21	1	0.60	0.448	1	0.42	1	1.20	0.448	0.938
*G*0104*	213	44.38	78	46.99	0.588	162	67.50	65	78.31	0.071	0.014
*G*0105N*	4	0.83	1	0.60	1.000	4	1.67	1	1.20	1.000	0.410
*G*0106*	12	2.50	5	3.01	0.779	11	4.58	5	6.02	0.568	0.053
Primigravid Pregnancies											
	n=118	%	n=62	%	*P*-value	N=59	%	N=31	%	*P*-value	*P*-value[c]
*G*0101*	66	55.93	31	50.00	0.529	46	77.97	24	77.42	1.000	0.440
*G*0104*	49	41.53	29	46.77	0.529	37	62.71	24	77.42	0.235	0.052
*G*0106*	3	2.54	2	3.23	1.000	2	3.39	2	6.45	0.606	0.066
Multigravid Pregnancies											
	n=360	%	n=100	%	*P*-value	N=180	%	N=50	%	*P*-value	*P*-value[d]
*G*0101*	183	50.83	48	48.00	0.652	135	75.00	38	76.00	1.000	0.900
*G*0103*	1	0.28	1	1.00	0.388	1	0.56	1	2.00	0.388	0.927
*G*0104*	163	45.28	47	47.00	0.821	124	68.89	39	78.00	0.225	0.164
*G*0105N*	4	1.11	1	1.00	1.000	4	2.22	1	2.00	1.000	0.498
*G*0106*	9	2.50	3	3.00	0.729	9	5.00	3	6.00	0.727	0.300

n, number of chromosomes examined.
N, number of individuals examined.
[a]Heterozygosity or homozygosity for a particular allele.
[b]*P*-value of multivariate analysis after adjustment of maternal age, body mass index, history of PE or PIH, history of abortion, primigravidity and change of partner.
[c]*P*-value of multivariate analysis after adjustment of maternal age and body mass index.
[d]*P*-value of multivariate analysis after adjustment of maternal age, body mass index, history of PE or PIH, history of abortion and change of partner.

3.2.3 Comparisons of HLA-G SNP Frequencies in PE and Controls

Comparisons of individual *HLA-G* SNPs did not reveal any significant difference between PE and control groups among the maternal samples (Table 11). In the fetal group, a nominal increase was observed at codon 258 in the PE group compared to controls (p=0.013) (Table 12). This is consistent with the observation of an association of fetal *G*0106* allele with PE because based on WHO definitions, *G*0106* is defined by a variant T-nucleotide at the second position of codon 258 instead of a C-nucleotide, which results in a non-conservative threonine to methionine amino acid change.

Table 11. Analysis of allele/genotype frequencies of individual *HLA-G* polymorphisms in case and control mothers.

SNP Position	Groups	Number of samples	Genotype frequency (%)				Allele frequency (%)			Fisher's exact test P-value
			AA	GG	AG		A	G		
NP-1306	Normal	240	47.50	5.83	46.67		70.83	29.17		1.000
	PE	83	51.81	9.64	38.55		71.08	28.92		
			CC	CG	CT	GG	C	G	T	
NP-725	Normal	240	98.33	0.83	0.42	0.42	98.96	0.83	0.21	1.000
	PE	83	97.59	1.20	1.20	0.00	98.80	0.60	0.60	
			AA	CC	AC		A	C		
NP-486	Normal	240	5.83	47.50	46.67		29.17	70.83		0.921
	PE	83	9.64	53.01	37.35		28.31	71.69		
			CC	CT			C	T		
NP-56	Normal	240	99.58	0.42			99.79	0.21		0.448
	PE	83	98.80	1.20			99.40	0.60		
			AA	GG	AG		A	G		
NP36	Normal	240	47.50	5.83	46.67		70.83	29.17		0.921
	PE	83	53.01	9.64	37.35		71.69	28.31		
			AA	AT			A	T		
CD31	Normal	240	99.58	0.42			99.79	0.21		0.448
	PE	83	98.80	1.20			99.40	0.60		
			AA				A			
CD54	Normal	240	100.00				100.00			1.000
	PE	83	100.00				100.00			
			AA	GG	AG		A	G		
CD57	Normal	240	47.50	5.83	46.67		70.83	29.17		0.921
	PE	83	53.01	9.64	37.35		71.69	28.31		
			CC				C			
CD69	Normal	240	100.00				100.00			1.000
	PE	83	100.00				100.00			
			CC	TT	CT		C	T		
CD93	Normal	240	86.25	0.83	12.92		92.71	7.29		0.862
	PE	83	86.75	0.00	13.25		93.37	6.63		
			AA	TT	AT		A	T		
CD107	Normal	240	67.50	3.75	28.75		81.88	18.13		1.000
	PE	83	67.47	3.61	28.92		81.93	18.07		
			AA	CC	AC		A	C		
CD110	Normal	240	21.25	32.50	46.25		44.38	55.63		0.588
	PE	83	15.66	21.69	62.65		46.99	53.01		
			CC	CT			C	T		
CD130	Normal	240	98.33	1.67			99.17	0.83		1.000
	PE	83	98.80	1.20			99.40	0.60		
			CC	CT			C	T		
CD188	Normal	240	99.58	0.42			99.79	0.21		0.448
	PE	83	98.80	1.20			99.40	0.60		
			CC	TT	CT		C	T		
CD258	Normal	240	95.42	0.42	4.17		97.50	2.50		0.779
	PE	83	93.98	0.00	6.02		96.99	3.01		
			CC	TT	CT		C	T		
CD290	Normal	240	87.92	0.83	11.25		93.54	6.46		1.000
	PE	83	87.95	0.00	12.05		93.98	6.02		
			AA	GG	AG		A	G		
CD309	Normal	240	5.83	47.92	46.25		28.96	71.04		0.842
	PE	83	9.64	54.22	36.14		27.71	72.29		
			GG	TT	GT		G	T		
NP2961	Normal	240	53.33	5.83	40.83		73.75	26.25		0.759
	PE	83	54.22	4.82	40.96		74.70	25.30		

Table 12. Analysis of allele/genotype frequencies of individual *HLA-G* polymorphisms in case and control babies.

SNP Position	Groups	Number of samples	Genotype frequency (%)			Allele frequency (%)			Fisher's exact test P-value
			AA	GG	AG	A	G		
NP-1306	Normal	240	52.50	3.75	43.75	74.38	25.63		0.157
	PE	83	46.99	9.64	43.37	68.67	31.33		
			CC	CG	CT	C	G	T	
NP-725	Normal	240	96.25	2.50	1.25	98.13	1.25	0.63	0.466
	PE	83	98.80	0.00	1.20	99.40	0.00	0.60	
			AA	CC	AC	A	C		
NP-486	Normal	240	3.75	53.75	42.50	25.00	75.00		0.154
	PE	83	8.43	46.99	44.58	30.72	69.28		
			CC	CT		C	T		
NP-56	Normal	240	98.75	1.25		99.38	0.63		1.000
	PE	83	98.80	1.20		99.40	0.60		
			AA	GG	AG	A	G		
NP36	Normal	240	53.75	3.75	42.50	75.00	25.00		0.154
	PE	83	46.99	8.43	44.58	69.28	30.72		
			AA	AT		A	T		
CD31	Normal	240	98.75	1.25		99.38	0.63		
	PE	83	98.80	1.20		99.40	0.60		1.000
			AA			A			
CD54	Normal	240	100.00			100.00			1.000
	PE	83	100.00			100.00			
			AA	GG	AG	A	G		
CD57	Normal	240	53.75	3.75	42.50	75.00	25.00		0.154
	PE	83	46.99	8.43	44.58	69.28	30.72		
			CC			C			
CD69	Normal	240	100.00			100.00			1.000
	PE	83	100.00			100.00			
			CC	TT	CT	C	T		
CD93	Normal	240	85.00	0.42	14.58	92.29	7.71		0.620
	PE	83	84.34	2.41	13.25	90.96	9.04		
			AA	TT	AT	A	T		
CD107	Normal	240	63.33	5.83	30.83	78.75	21.25		0.741
	PE	83	63.86	3.61	32.53	80.12	19.88		
			AA	CC	AC	A	C		
CD110	Normal	240	20.83	29.58	49.58	45.63	54.38		0.469
	PE	83	21.69	37.35	40.96	42.17	57.83		
			CC	CT		C	T		
CD130	Normal	240	97.92	2.08		98.96	1.04		0.335
	PE	83	100.00	0.00		100.00	0.00		
			CC	CT		C	T		
CD188	Normal	240	98.75	1.25		99.38	0.63		1.000
	PE	83	98.80	1.20		99.40	0.60		
			CC	CT		C	T		
CD258	Normal	240	96.25	3.75		98.13	1.88		0.013
	PE	83	87.95	12.05		93.98	6.02		
			CC	TT	CT	C	T		
CD290	Normal	240	88.33	0.42	11.25	93.96	6.04		0.582
	PE	83	87.95	2.41	9.64	92.77	7.23		
			AA	GG	AG	A	G		
CD309	Normal	240	3.33	54.58	42.08	24.38	75.63		0.124
	PE	83	8.43	46.99	44.58	30.72	69.28		
			GG	TT	GT	G	T		
NP2961	Normal	240	50.83	10.00	39.17	70.42	29.58		0.921
	PE	83	49.40	7.23	43.37	71.08	28.92		

3.2.4 Comparisons of HLA-G 14 bp Insertion/Deletion Polymorphism Frequencies

No significant differences were observed for the frequencies of the 14bp insertion/deletion polymorphism (NP2961) between PE and controls in both maternal and fetal groups. Further analysis of primigravid and multigravid sub-groups did not reveal any significant association between cases and control groups (Table 13).

Table 13. Analysis of maternal and fetal 14 bp insertion/deletion polymorphism in cases and controls.

	Maternal					Fetal				
	Normal Controls		Pre-eclampsia		Fisher's Exact Test	Normal Controls		Pre-eclampsia		Fisher's Exact Test
All Pregnancies										
Allele	n=480	%	n=166	%	*P*-value	n=480	%	n=166	%	*P*-value
G	354	73.75	124	74.70	0.838	338	70.42	118	71.08	0.921
T	126	26.25	42	25.30		142	29.58	48	28.92	
Genotype	n=240	%	n=83	%	*P*-value	n=240	%	n=83	%	*P*-value
GG	128	53.33	45	54.22	0.899	122	50.83	41	49.40	0.899
TT	14	5.83	4	4.82	1.000	24	10.00	6	7.23	0.519
GT	98	40.83	34	40.96	1.000	94	39.17	36	43.47	0.518
Primigravid Pregnancies										
Allele	n=118	%	n=62	%	*P*-value	n=118	%	n=62	%	*P*-value
G	69	58.47	33	53.23	0.529	65	55.08	39	62.90	0.344
T	49	41.53	29	46.77		53	44.92	23	37.10	
Genotype	n=59	%	n=31	%	*P*-value	n=59	%	n=31	%	*P*-value
GG	22	37.29	7	22.58	0.235	17	28.81	12	38.71	0.353
TT	12	20.33	5	16.13	0.779	11	18.64	4	12.90	0.565
GT	25	42.37	19	61.29	0.121	31	52.54	15	48.39	0.825
Multigravid Pregnancies										
Allele	n=360	%	n=100	%	*P*-value	n=360	%	n=100	%	*P*-value
G	197	54.72	53	53.00	0.821	195	54.17	55	55.00	0.910
T	163	45.28	47	47.00		165	45.83	45	45.00	
Genotype	n=180	%	n=50	%	*P*-value	n=180	%	n=50	%	*P*-value
GG	56	31.11	11	22.00	0.225	54	30.00	18	36.00	0.491
TT	39	21.67	8	16.00	0.434	39	21.67	13	26.00	0.567
GT	85	47.22	31	62.00	0.079	87	48.33	19	38.00	0.204

3.2.5 Maternal-fetal Histo-incompatibility Effect of HLA-G in PE and Controls

Analysis of this SNP also revealed that the frequency of mother-child *HLA-G* genotype mismatches among multigravid pregnancies, where mothers who were homozygous C/C (i.e. G*0106-negative) carried fetuses who were heterozygous C/T (i.e. G*0106-positive), was significantly higher in PE pregnancies compared to normal pregnancies (p=0.001, OR=9.6, 95% CI 2.4-38.7) (Table 14).

Table 14. Analysis of mother-child genotype pairs at the codon 258 SNP locus in case and control pregnancies.

Genotype Pair (Mother/Child)	Normal Controls		Pre-eclampsia		Fisher's Exact Test
All Pregnancies					
	N=240	%	N=83	%	*P*-value
CC/CC	226	94.17	71	85.54	0.019
CC/CT	3	1.25	7	8.43	**0.004**
CT/CC	5	2.08	2	2.41	1.000
CT/CT	5	2.08	3	3.61	0.428
TT/CT	1	0.42	0	0.00	1.000
Primigravid Pregnancies					
	N=59	%	N=31	%	*P*-value
CC/CC	57	96.61	29	93.55	0.606
CT/CC	0	0.00	1	3.23	0.344
CT/CT	1	1.69	1	3.23	1.000
TT/CT	1	1.69	0	0.00	1.000
Multigravid Pregnancies					
	N=180	%	N=50	%	*P*-value
CC/CC	168	93.33	40	80.00	0.011
CC/CT	3	1.67	7	14.00	**0.001**
CT/CC	5	2.78	1	2.00	1.000
CT/CT	4	2.22	2	4.00	0.613

N, number of individuals examined.
P-values <0.01 are highlighted in bold.

3.2.6 Comparisons of HLA-G Allele/ Haplotype Frequencies in 3 Local Populations

As an independent verification of the observed *HLA-G* allele/ haplotype frequencies in control mothers and babies, the corresponding population allele/ haplotype frequencies in the Southeast Asian Malays, Chinese and Indians, the 3 main population groups in Singapore and Malaysia were also determined. Consistent with the minimally polymorphic state of *HLA-G*, five or fewer WHO-designated *HLA-G* alleles accounted for at least 85% of the chromosomes in all 3 populations. All polymorphisms were in HWE except for three SNPs in the Malay population, namely NP-486 ($p=0.03$), NP36 ($p=0.03$) and CD309 ($p=0.04$). This observation is unlikely to be a result of sample bias as the majority of the SNPs genotyped were in HWE. Moreover, as the same genotyping strategy was applied to all samples, it is also unlikely that this deviation from HWE is due to genotyping artefact. Hence, it is more likely that this deviation is due to chance as the statistical analysis performed was based on a 95% confidence interval. Pair-wise comparisons between populations showed significant differences for certain haplotypes (Table 15). Importantly, with the exception of the presumptive PE risk allele G*0106 in the PE babies, frequencies of the other *HLA-G* alleles in all case and control groups were very similar to each other and to the observed frequencies in the Malay population.

Table 15. Analysis of *HLA-G* allele/haplotype frequencies in the Southeast Asian Chinese (CH), Indian (IN), and Malay (ML) populations.

Allele/Haplotype[a]	Polymorphisms																		Frequency			Fisher's Exact Test *P*-value		
	5' Upstream Region				Exon 1	Exon 2				Exon 3				Exon 4		Exon 5		Exon 6						
	NP-1306	NP-725	NP-486	NP-56	NP36	CD31	CD54	CD57	CD69	CD93	CD107	CD110	CD130	CD188	CD258	CD290	CD309	NP2961[b]	Chinese n=188 (%)	Indian n=180 (%)	Malay n=180 (%)	CH vs IN	CH vs ML	IN vs ML
G*0101																			127 (67.55)	110 (61.11)	92 (51.11)	0.231	**0.002**	0.071
G*010101																			78 (41.49)	57 (31.67)	45 (25.00)	0.052	**<0.001**	0.198
*G*010101a*	G	C	A	C	CTG	ACG	CAG	CCG	GCC	CAC	GGA	CTC	CTG	CAC	ACG	GGC	AGA	G	77 (40.96)	47 (26.11)	45 (25.00)	**0.003**	**0.001**	0.904
*G*010101b*	G	G	A	C	CTG	ACG	CAG	CCG	GCC	CAC	GGA	CTC	CTG	CAC	ACG	GGC	AGA	G	1 (0.53)	10 (5.56)	0 (0.00)	**0.005**	1.000	**0.002**
G*010102																			11 (5.85)	20 (11.11)	11 (6.11)	0.090	1.000	0.132
*G*010102a*	A	C	C	C	CT**A**	ACG	CAG	CC**A**	GCC	CA**T**	GGA	CTC	CTG	CAC	ACG	GGC	AG**G**	**T**	0 (0.00)	2 (1.11)	0 (0.00)	0.239	1.000	0.499
*G*010102b*	A	C	C	C	CT**A**	ACG	CAG	CC**A**	GCC	CA**T**	GGA	CTC	CTG	CAC	ACG	GG**T**	AG**G**	**T**	10 (5.4)	17 (9.44)	10 (5.56)	0.162	1.000	0.229
*G*010102c*	A	G	C	C	CT**A**	ACG	CAG	CC**A**	GCC	CA**T**	GGA	CTC	CTG	CAC	ACG	GG**T**	AG**G**	**T**	1 (0.53)	0 (0.00)	0 (0.00)	1.000	1.000	1.000
*G*010102d*	G	C	C	C	CT**A**	ACG	CAG	CC**A**	GCC	CA**T**	GGA	CTC	CTG	CAC	ACG	GGC	AG**G**	**T**	0 (0.00)	0 (0.00)	1 (0.56)	1.000	0.489	1.000
*G*010102e*	A	C	C	C	CT**A**	ACG	CAG	CC**A**	GCC	CA**T**	GGA	CTC	CTG	CAC	ACG	GG**T**	AG**G**	G	0 (0.00)	1 (0.56)	0 (0.00)	0.489	1.000	1.000
G*010103																			38 (20.21)	31 (17.22)	35 (19.44)	0.505	0.896	0.683
*G*010103a*	A	C	C	C	CT**A**	ACG	CAG	CC**A**	GCC	CAC	GG**T**	CTC	CTG	CAC	ACG	GGC	AG**G**	**T**	37 (19.68)	31 (17.22)	35 (19.44)	0.592	1.000	0.683
*G*010103c*	G	C	C	C	CT**A**	ACG	CAG	CC**A**	GCC	CAC	GG**T**	CTC	CTG	CAC	ACG	GGC	AGA	**T**	1 (0.53)	0 (0.00)	0 (0.00)	1.000	1.000	1.000
G*010108b	A	C	C	C	CT**A**	ACG	CAG	CC**A**	GCC	CAC	GGA	CTC	CTG	CAC	ACG	GGC	AG**G**	**T**	0 (0.00)	1 (0.56)	0 (0.00)	0.489	1.000	1.000
G*010109a	A	C	C	C	CT**A**	ACG	CAG	CCG	GCC	CAC	GGA	CTC	CTG	CAC	ACG	GG**T**	AG**G**	**T**	0 (0.00)	0 (0.00)	1 (0.56)	1.000	0.489	1.000
G*010110a	A	G	A	C	CTG	ACG	CAG	CCG	GCC	CA**T**	GGA	CTC	CTG	CAC	ACG	GG**T**	AGA	**T**	0 (0.00)	1 (0.56)	0 (0.00)	0.489	1.000	1.000
G*0103																			2 (1.06)	3 (1.67)	1 (0.56)	0.679	1.000	0.623
*G*0103a*	G	C	A	T	CTG	**T**CG	CAG	CCG	GCC	CAC	GGA	CTC	CTG	CA**T**	ACG	GGC	AG**G**	**T**	2 (1.06)	3 (1.67)	0 (0.00)	0.679	0.499	0.248
*G*0103c*	A	T	A	T	CTG	**T**CG	CAG	CCG	GCC	CAC	GGA	CTC	CTG	CA**T**	ACG	GGC	AG**G**	**T**	0 (0.00)	0 (0.00)	1 (0.56)	1.000	0.489	1.000
G*010401																			52 (27.66)	47 (26.11)	81 (45.00)	0.814	**<0.001**	**<0.001**
*G*010401a*	A	C	C	C	CT**A**	ACG	CAG	CC**A**	GCC	CAC	GGA	**A**TC	CTG	CAC	ACG	GGC	AG**G**	G	52 (27.66)	47 (26.11)	80 (44.44)	0.814	**0.001**	**<0.001**
*G*010401b*	A	C	C	C	CT**A**	ACG	CAG	CC**A**	GCC	CAC	GGA	**A**TC	CTG	CAC	ACG	GGC	AG**G**	**T**	0 (0.00)	0 (0.00)	1 (0.56)	1.000	0.489	1.000
G*0105Na	A	C	C	C	CT**A**	ACG	CAG	CC**A**	GCC	CA**T**	GGA	CTC	-TG	CAC	ACG	GG**T**	AG**G**	**T**	5 (2.66)	5 (2.78)	2 (1.11)	1.000	0.449	0.449
G*0106																			2 (1.06)	15 (8.33)	4 (2.22)	**<0.001**	0.440	0.016
*G*0106a*	A	C	C	C	CT**A**	ACG	CAG	CC**A**	GCC	CA**T**	GGA	CTC	CTG	CAC	A**T**G	GG**T**	AG**G**	**T**	2 (1.06)	14 (7.78)	4 (2.22)	**0.002**	0.440	0.027
*G*0106d*	A	G	C	C	CT**A**	ACG	CAG	CC**A**	GCC	CA**T**	GGA	CTC	CTG	CAC	A**T**G	GGC	AG**G**	G	0 (0.00)	1 (0.56)	0 (0.00)	0.489	1.000	1.000

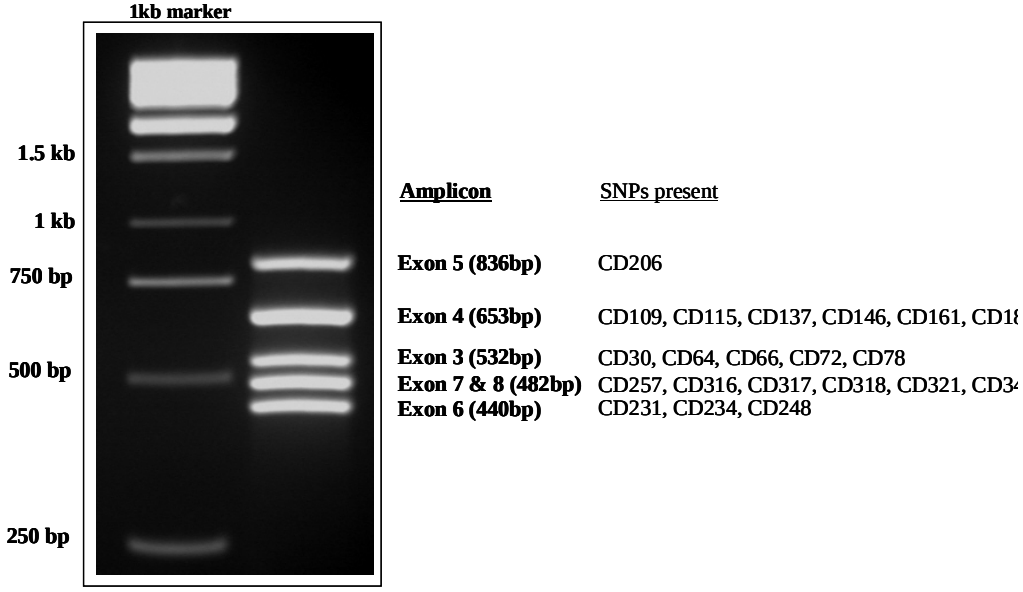
[a]Sub-haplotypes of the WHO defined alleles are indicated by the letters a, b, c, d, and e at the end.
Nucleotide numberings are with reference to the translation start site; n represents the number of chromosomes examined.
n, number of chromosomes examined; CH, Chinese; IN, Indian; ML, Malay.
[b]T represents the allele containing the 14 bp insertion.
P-values <0.01 are highlighted in bold.
NP: nucleotide position; CD: codon

3.3 *KIR2DL4*

3.3.1 Multiplex PCR Amplification and Genotyping

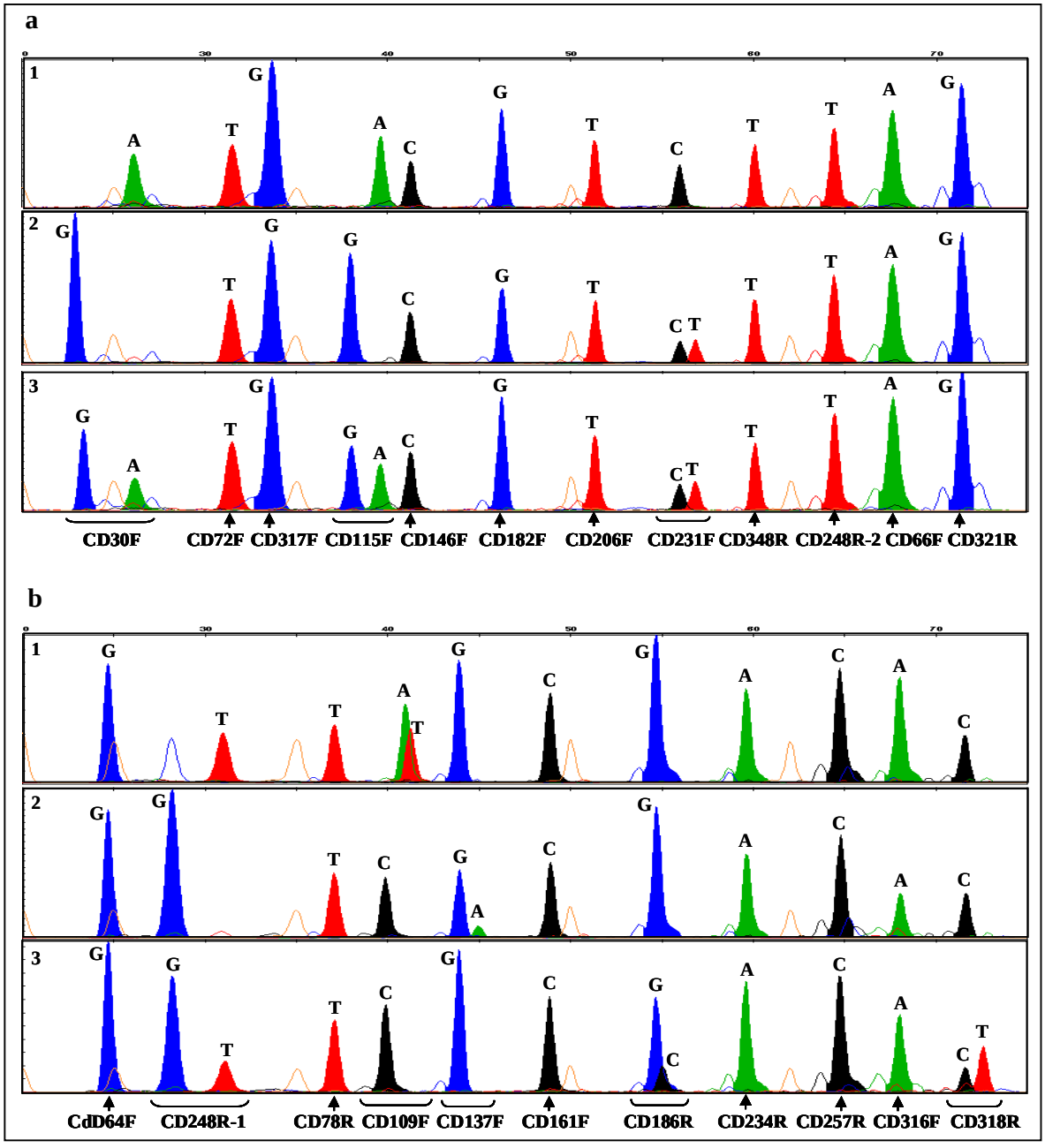
Fragments of *KIR2DL4* gene were amplified in a single tube multiplex PCR reaction to amplify exons 3 through 8 in 5 fragments containing SNPs of interest. PCR products were then resolved on agarose gel electrophoresis as shown in Figure 6. Amplification of all the 5 fragments containing the SNPs of interest was successful in all samples analyzed in this study.

Figure 6. Multiplex PCR amplified fragments of the *KIR2DL4* gene.



For the genotyping of the 23 SNPs in the *KIR2DL4* gene, a 2 panel multiplex-minisequencing strategy assaying 11 and 12 SNPs in Panels 1 and 2, respectively, was used. Minisequencing products were resolved on capillary electrophoresis as shown in Figure 7. This minisequencing assay was design to genotype all the *KIR2DL4* alleles reported to date, with the exception of *2DL4*0080104* (characterized by codon 344) and *2DL4*012* (characterized by codon 273), which were discovered very recently.

Figure 7. GeneScan™ electropheromgram traces of panel 1(a1-a3) and 2 (b1-b3) after multiplex minisequencing of *KIR2DL4* polymorphisms from 3 different individuals.



3.3.2 Comparisons of KIR2DL4 Allele Frequencies in PE and controls

Analysis of *KIR2DL4* allele frequencies revealed no significant differences between PE and control groups in both maternal and fetal samples (Table 16). As primigravidity is known to be a PE risk factor, we performed separate analyses of primigravida and multigravida sub-groups. No significant difference in maternal allele or genotype frequencies between cases and controls was detected in either sub-group, although there was a nominally lower frequency of PE mothers carrying the *2DL4*001* allele in the primigravid sub-group (Table 17). However, logistic regression analysis of genotype frequencies with adjustments for factors associated with PE did not reveal any significant association of the allele with PE (Tables 17).

Among the fetal group, a nominal increase was observed in the frequencies of PE babies in the primigravid sub-group carrying the *2DL4*011* allele (Table 18). However, further analysis of genotype frequency showed no association between this allele and the development of PE (Table 18).

Table 16. Analysis of maternal and fetal *KIR2DL4* allele frequencies in cases and controls.

Allele/Haplotype[a]	Polymorphisms																							Maternal Frequencies Number (%)			Fetal Frequencies Number (%)		
	Exon 3					Exon 5							Exon 6	Exon 7			Exon 8	Exon 9											
	CD30	CD64	CD66	CD72	CD78	CD109	CD115	CD137	CD146	CD161	CD182	CD186	CD206	CD231	CD234	CD248	CD257	CD316	CD317	CD318	CD321	CD348	Normal Controls n=480	Pre-eclampsia n=166	Fisher's Exact Test *P*-value	Normal Controls n=480	Pre-eclampsia n=166	Fisher's Exact Test *P*-value
2DL4*001																								171 (35.63)	56 (33.73)	0.706	170 (35.42)	55 (33.13)	0.637
2DL4*00102																								158 (32.92)	49 (29.52)	0.441	152 (31.67)	48 (28.92)	0.559
*2DL4*00102a*	TAT	GTG	CCA	TAC	CAC	CCG	ACA	GA**G**	CCT	CTG	GA**G**	GCG	ACT	TT**C**	CTT	AAT	GCG	AGA	GCG	TT**A**	GCC	AAT		158 (32.92)	49 (29.52)	0.441	151 (31.46)	48 (28.92)	0.560
*2DL4*00102b*	TAT	GTG	CCA	TAC	CAC	C**T**G	ACA	GA**G**	CCT	CTG	GA**G**	GCG	ACT	TT**C**	CTT	AAT	GCG	AGA	GCG	TT**A**	GCC	AAT		0 (0.00)	0 (0.00)	1.000	1 (0.21)	0 (0.00)	1.000
2DL4*00103																								13 (2.71)	7 (4.22)	0.311	18 (3.75)	7 (4.22)	0.816
*2DL4*00103a*	TAT	GTG	CCA	TAC	CAC	CCG	ACA	GA**G**	CCT	CTG	GA**G**	GCG	ACT	TT**C**	CTT	AAT	GCG	AGA	GCG	TTG	GCC	AAT		9 (1.88)	6 (3.61)	0.231	17 (3.54)	6 (3.61)	1.000
*2DL4*00103b*	TAT	GTG	CCA	TAC	CAC	CCG	ACA	GA**G**	CCT	CTG	GA**G**	**C**CG	ACT	TT**C**	CTT	AAT	GCG	AGA	GCG	TTG	GCC	AAT		1 (0.21)	1 (0.60)	0.448	0 (0.00)	0 (0.00)	1.000
*2DL4*00103c*	TAT	GTG	CCA	TAC	CAC	C**A**G	ACA	GA**G**	CCT	CTG	GA**G**	GCG	ACT	TT**C**	CTT	AAT	GCG	AGA	GCG	TTG	GCC	AAT		3 (0.63)	0 (0.00)	0.573	0 (0.00)	1 (0.60)	0.257
*2DL4*00103d*	TAT	GTG	CCA	TAC	CAC	C**T**G	ACA	GA**G**	CCT	CTG	GA**G**	**C**CG	ACT	TT**C**	CTT	AAT	GCG	AGA	GCG	TTG	GCC	AAT		0 (0.00)	0 (0.00)	1.000	1 (0.21)	0 (0.00)	1.000
2DL4*00202	TAT	GTG	CCA	TAC	CAC	CCG	**G**CA	GA**G**	CCT	CTG	GA**G**	**C**CG	ACT	TT**C**	CTT	AAT	GCG	AGA	GCG	TTG	GCC	AAT		0 (0.00)	1 (0.60)	0.257	0 (0.00)	0 (0.00)	1.000
2DL4*005																								126 (26.25)	47 (28.31)	0.612	120 (25.00)	48 (28.92)	0.356
*2DL4*005a*	T**G**T	GTG	CCA	TAC	CAC	CCG	**G**CA	GA**G**	CCT	CTG	GA**G**	**C**CG	ACT	TT**C**	CTT	AAT	GCG	AGA	GCG	TTG	GCC	AAT		123 (25.63)	47 (28.31)	0.540	119 (24.79)	48 (28.92)	0.305
*2DL4*005b*	T**G**T	GTG	CCA	TAC	CAC	C**T**G	**G**CA	GA**G**	CCT	CTG	GA**G**	**C**CG	ACT	TT**C**	CTT	AAT	GCG	AGA	GCG	TTG	GCC	AAT		1 (0.21)	0 (0.00)	1.000	1 (0.21)	0 (0.00)	1.000
*2DL4*005c*	T**G**T	GTG	CCA	TAC	CAC	C**T**G	ACA	GA**G**	CCT	CTG	GA**G**	GCG	ACT	TT**C**	CTT	AAT	GCG	AGA	GCG	TTG	GCC	AAT		2 (0.42)	0 (0.00)	1.000	0 (0.00)	0 (0.00)	1.000
2DL4*00602																								53 (11.04)	15 (9.04)	0.558	48 (10.00)	15 (9.04)	0.879
*2DL4*00602a*	TAT	GTG	CCA	TAC	CAC	C**T**G	**G**CA	GA**G**	CCT	CTG	GA**G**	**C**CG	ACT	TT**C**	CTT	AAT	GCG	AGA	GCG	TTG	GCC	AAT		48 (10.00)	13 (7.83)	0.446	39 (8.13)	13 (7.83)	0.865
*2DL4*00602b*	TAT	GTG	CCA	TAC	CAC	C**A**G	**G**CA	GA**G**	CCT	CTG	GA**G**	**C**CG	ACT	TT**C**	CTT	AAT	GCG	AGA	GCG	TTG	GCC	AAT		4 (0.83)	2 (1.20)	0.650	9 (1.88)	2 (1.20)	0.738
*2DL4*00602c*	TAT	GTG	CCA	TAC	CAC	C**A**G	**G**CA	GA**G**	CCT	CTG	GA**G**	**C**CG	ACT	TT**C**	CTT	AAT	GCG	AGA	GCG	TT**A**	GCC	AAT		1 (0.21)	0 (0.00)	1.000	0 (0.00)	0 (0.00)	1.000
2DL4*008																								37 (7.71)	9 (5.42)	0.384	46 (9.58)	12 (7.23)	0.432
*2DL4*00801*																								37 (7.71)	9 (5.42)	0.384	44 (9.17)	12 (7.23)	0.524
*2DL4*0080101a*	TAT	GTG	CCA	TAC	CAC	CCG	**G**CA	GA**G**	CCT	CTG	GA**G**	**C**CG	ACT	TTT	CTT	***C**	GCG	AGA	GCG	TTG	GCC	AAT		36 (7/5)	9 (5.42)	0.479	44 (9.17)	12 (7.23)	0.524
*2DL4*0080101b*	TAT	GTG	CCA	TAC	CAC	C**A**G	**G**CA	GA**G**	CCT	CTG	GA**G**	**C**CG	ACT	TTT	CTT	***C**	GCG	AGA	GCG	TTG	GCC	AAT		1 (0.21)	0 (0.00)	1.000	0 (0.00)	0 (0.00)	1.000
*2DL4*00802*	TAT	GTG	CCA	TAC	CAC	CCG	**G**CA	GAA	CCT	CTG	GA**G**	**C**CG	ACT	TTT	CTT	***C**	GCG	AGA	GCG	TTG	GCC	AAT		0 (0.00)	0 (0.00)	1.000	2 (0.42)	0 (0.00)	1.000
2DL4*011																								93 (19.38)	38 (22.89)	0.370	96 (20.00)	36 (21.69)	0.656
*2DL4*011a*	T**G**T	GTG	CCA	TAC	CAC	CCG	**G**CA	GA**G**	CCT	CTG	GA**G**	**C**CG	ACT	TTT	CTT	***C**	GCG	AGA	GCG	TTG	GCC	AAT		93 (19.38)	37 (22.29)	0.433	96 (20.00)	36 (21.69)	0.656
*2DL4*011b*	T**G**T	GTG	CCA	TAC	CAC	CCG	**G**CA	GA**G**	CCT	CTG	GA**G**	GCG	ACT	TTT	CTT	***C**	GCG	AGA	GCG	TTG	GCC	AAT		0 (0.00)	1 (0.60)	0.257	0 (0.00)	0 (0.00)	1.000

[a]Sub-haplotypes of the WHO defined alleles are indicated by the letters p, q, r, and s at the end.
Nucleotide numberings are with reference to the translation start site; n represents the number of chromosomes examined.
CD: codon.

Table 17. Analysis of maternal *KIR2DL4* allele and genotype frequencies.

	Maternal Allele Frequency					Maternal +- or ++ Genotype Frequency					
	Normal Controls		Pre-eclampsia		Fisher's Exact Test	Normal Controls		Pre-eclampsia		Fisher's Exact Test	Logistic Regression
All Pregnancies											
	n=480	%	n=166	%	*P*-value	N=240	%	N=83	%	*P*-value	*P*-value[*]
*2DL4*001*	282	58.75	86	51.81	0.123	141	58.75	43	51.81	0.304	0.104
*2DL4*002*	0	0.00	2	1.20	0.066	0	0.00	1	1.20	0.257	1.000
*2DL4*005*	226	47.08	74	44.58	0.589	113	47.08	37	44.58	0.704	0.286
*2DL4*006*	100	20.83	30	18.07	0.501	50	20.83	15	18.07	0.637	0.523
*2DL4*008*	66	13.75	18	10.84	0.422	33	13.75	9	10.84	0.574	0.685
*2DL4*011*	166	34.58	66	39.76	0.260	83	34.58	33	39.76	0.427	0.445
Primigravid Pregnancies											
	n=118	%	n=62	%0.017	*P*-value	N=59	%	N=31	%	*P*-value	*P*-value[†]
*2DL4*001*	76	64.41	28	45.16	0.017	38	64.41	14	45.16	0.115	0.147
*2DL4*005*	54	45.76	28	45.16	1.000	27	45.76	14	45.16	1.000	0.894
*2DL4*006*	20	16.95	10	16.13	1.000	10	16.95	5	16.13	1.000	0.840
*2DL4*008*	8	6.78	6	9.68	0.562	4	6.78	3	9.68	0.688	0.960
*2DL4*011*	46	38.98	30	48.39	0.267	23	38.98	15	48.39	0.501	0.915
Multigravid Pregnancies											
	n=360	%	n=100	%	*P*-value	N=180	%	N=50	%	*P*-value	*P*-value[‡]
*2DL4*001*	204	56.67	56	56.00	0.910	102	56.67	28	56.00	1.000	0.243
*2DL4*002*	0	0.00	2	2.00	0.047	0	0.00	1	2.00	0.217	1.000
*2DL4*005*	172	47.78	44	44.00	0.571	86	47.78	22	44.00	0.749	0.173
*2DL4*006*	80	22.22	20	20.00	0.683	40	22.22	10	20.00	0.847	0.335
*2DL4*008*	58	16.11	12	12.00	0.349	29	16.11	6	12.00	0.656	0.382
*2DL4*011*	118	32.78	32	32.00	0.905	59	32.78	16	32.00	1.000	0.278

+- or ++, heterozygosity or homozygosity for a particular allele.
n, number of chromosomes examined.
N, number of individuals examined.
[*], *P*-value of multivariate analysis after adjustment of maternal age, body mass index, history of PE or PIH, history of abortion, primigravidity and change of partner.
[†], *P*-value of multivariate analysis after adjustment of maternal age and body mass index.
[‡], *P*-value of multivariate analysis after adjustment of maternal age, body mass index, history of PE or PIH, history of abortion and change of partner.

Table 18. Analysis of fetal *KIR2DL4* allele and genotype frequencies.

	Fetal Allele Frequency					Fetal +- or ++ Genotype Frequency					
	Normal Controls		Pre-eclampsia		Fisher's Exact Test	Normal Controls		Pre-eclampsia		Fisher's Exact Test	Logistic Regression
All Pregnancies											
	n=480	%	n=166	%	*P*-value	N=240	%	N=83	%	*P*-value	*P*-value[*]
*2DL4*001*	270	56.25	86	51.81	0.365	135	56.25	43	51.81	0.523	0.848
*2DL4*005*	216	45.00	88	53.01	0.086	108	45.00	44	53.01	0.251	0.104
*2DL4*006*	86	17.92	28	16.87	0.814	43	17.92	14	16.87	1.000	0.461
*2DL4*008*	86	19.92	22	13.25	0.185	43	17.92	11	13.25	0.395	0.710
*2DL4*011*	166	34.58	68	40.96	0.160	83	34.58	34	40.96	0.354	0.197
Primigravid Pregnancies											
	n=118	%	n=62	%	*P*-value	N=59	%	N=31	%	*P*-value	*P*-value[†]
*2DL4*001*	66	55.93	34	54.84	1.000	33	55.93	17	54.84	1.000	0.829
*2DL4*005*	52	44.07	26	41.94	0.874	26	44.07	13	41.94	1.000	0.836
*2DL4*006*	26	22.03	6	9.68	0.042	13	22.03	3	9.68	0.245	0.391
*2DL4*008*	18	15.25	8	12.90	0.824	9	15.25	4	12.90	1.000	0.363
*2DL4*011*	34	28.81	30	48.39	0.014	17	28.81	15	48.39	0.104	0.222
Multigravid Pregnancies											
	n=360	%	n=100	%	*P*-value	N=180	%	N=50	%	*P*-value	*P*-value[‡]
*2DL4*001*	204	56.67	52	52.00	0.427	102	56.67	26	52.00	0.630	0.705
*2DL4*005*	164	45.56	58	58.00	0.032	82	45.56	29	58.00	0.150	0.076
*2DL4*006*	60	16.67	22	22.00	0.238	30	16.67	11	22.00	0.406	0.186
*2DL4*008*	68	18.89	12	12.00	0.135	34	18.89	6	12.00	0.298	0.978
*2DL4*011*	130	36.11	36	36.00	1.000	65	36.11	18	36.00	1.000	0.438

+- or ++, heterozygosity or homozygosity for a particular allele.
n, number of chromosomes examined.
N, number of individuals examined.
[*], *P*-value of multivariate analysis after adjustment of maternal age, body mass index, history of PE or PIH, history of abortion, primigravidity and change of partner.
[†], *P*-value of multivariate analysis after adjustment of maternal age and body mass index.
[‡], *P*-value of multivariate analysis after adjustment of maternal age, body mass index, history of PE or PIH, history of abortion and change of partner.
P-values <0.01 are highlighted in bold.

3.3.3 Comparisons of KIR2DL4 SNP Frequencies in PE and controls

The *KIR2DL4* gene is minimally polymorphic in our study population, as 14 of the 23 genotyped SNPs were monomorphic in all samples tested. The monomorphic SNPs in our study population are codons 64, 66, 72, 78, 146, 161, 182, 206, 234, 257, 316, 317, 321 and 348. Pair-wise comparisons of *KIR2DL4* SNPs between PE and controls revealed no significant differences in both maternal and fetal groups for all SNPs genotyped (Table 19 and 20).

Table 19. Analysis of individual *KIR2DL4* SNPs/polymorphisms in case and control mothers.

SNP position	Groups	Number of samples	Genotype frequency (%)				Allele frequency (%)			Fisher's exact test p-value
			AA	**GG**	**AG**		**A**	**G**		
CD30	Normal	240	27.50	18.75	53.75		54.38	45.63		0.241
	PE	83	26.51	28.92	44.58		48.80	51.20		
			GG				**G**			
CD64	Normal	240	100.00				100.00			1.000
	PE	83	100.00				100.00			
			AA				**A**			
CD66	Normal	240	100.00				100.00			1.000
	PE	83	100.00				100.00			
			TT				**T**			
CD72	Normal	240	100.00				100.00			1.000
	PE	83	100.00				100.00			
			TT				**T**			
CD78	Normal	240	100.00				100.00			1.000
	PE	83	100.00				100.00			
			TT	**CC**	**CA**	**CT**	**C**	**A**	**T**	
Cd109	Normal	240	1.25	77.08	4.17	17.50	87.92	2.08	10.00	0.322
	PE	83	0.00	81.93	2.41	15.66	90.96	1.20	7.83	
			AA	**GG**	**AG**		**A**	**G**		
CD115	Normal	240	12.50	40.00	47.50		36.25	63.75		0.574
	PE	83	15.66	48.19	36.14		33.73	66.27		
			GG				**G**			
CD137	Normal	240	100.00				100.00			1.000
	PE	83	100.00				100.00			
			CC				**C**			
CD146	Normal	240	100.00				100.00			1.000
	PE	83	100.00				100.00			
			CC				**C**			
Cd161	Normal	240	100.00				100.00			1.000
	PE	83	100.00				100.00			
			GG				**G**			
CD182	Normal	240	100.00				100.00			1.000
	PE	83	100.00				100.00			
			GG	**CC**	**CG**		**G**	**C**		
CD186	Normal	240	40.00	12.08	47.92		63.96	36.04		0.638
	PE	83	48.19	15.66	36.14		66.27	33.73		
			TT				**T**			
CD206	Normal	240	100.00				100.00			1.000
	PE	83	100.00				100.00			
			CC	**TT**	**CT**		**C**	**T**		
CD231	Normal	240	54.58	8.75	36.67		72.92	27.08		0.763
	PE	83	51.81	8.43	39.76		71.69	28.31		
			AA				**A**			
CD234	Normal	240	100.00				100.00			1.000
	PE	83	100.00				100.00			
			TT	**GG**	**GT**		**T**	**G**		
CD248-1	Normal	240	54.58	8.75	36.67		72.92	27.08		0.763
	PE	83	51.81	8.43	39.76		71.69	28.31		
			TT				**T**			
CD248-2	Normal	240	100.00				100.00			1.000
	PE	83	100.00				100.00			
			C				**C**			
CD257	Normal	240	100.00				100.00			1.000
	PE	83	100.00				100.00			
			AA				**A**			
CD316	Normal	240	100.00				100.00			1.000
	PE	83	100.00				100.00			
			GG				**G**			
CD317	Normal	240	100.00				100.00			1.000
	PE	83	100.00				100.00			
			TT	**CC**	**TC**		**T**	**C**		
CD318	Normal	240	10.83	43.75	45.42		33.54	66.46		0.387
	PE	83	10.84	51.81	37.35		29.52	70.48		
			GG				**G**			
CD321	Normal	240	100.00				100.00			1.000
	PE	83	100.00				100.00			
			TT				**T**			
CD348	Normal	240	100.00				100.00			1.000
	PE	83	100.00				100.00			

Table 20. Analysis of individual *KIR2DL4* SNPs/polymorphisms in case and control babies.

SNP position	Groups	Number of samples	Genotype frequency (%)					Allele frequency (%)			Fisher's exact test p-value
			AA	**GG**	**AG**			**A**	**G**		
CD30	Normal	240	27.92	17.92	54.17			55.00	45.00		0.241
	PE	83	24.10	25.30	50.60			49.40	50.60		
			GG					**G**			
CD64	Normal	240	100.00					100.00			1.000
	PE	83	100.00					100.00			
			AA					**A**			
CD66	Normal	240	100.00					100.00			1.000
	PE	83	100.00					100.00			
			TT					**T**			
CD72	Normal	240	100.00					100.00			1.000
	PE	83	100.00					100.00			
			TT					**T**			
CD78	Normal	240	100.00					100.00			1.000
	PE	83	100.00					100.00			
			CA	**CC**	**TT**	**CT**	**AT**	**C**	**A**	**T**	
Cd109	Normal	240	3.75	81.25	1.67	12.92	0.42	89.58	2.08	8.33	0.882
	PE	83	3.61	81.93	1.20	13.25	0.00	90.36	1.81	7.83	
			AA	**GG**	**AG**			**A**	**G**		
CD115	Normal	240	14.58	43.33	42.08			35.63	64.38		0.573
	PE	83	14.46	48.19	37.35			33.13	66.87		
			GG	**GA**				**G**	**A**		
CD137	Normal	240	98.33	1.67				99.17	0.83		1.000
	PE	83	100.00	0.00				100.00	0.00		
			CC					**C**			
CD146	Normal	240	100.00					100.00			1.000
	PE	83	100.00					100.00			
			CC					**C**			
Cd161	Normal	240	100.00					100.00			1.000
	PE	83	100.00					100.00			
			GG					**G**			
CD182	Normal	240	100.00					100.00			1.000
	PE	83	100.00					100.00			
			GG	**CC**	**CG**			**G**	**C**		
CD186	Normal	240	40.00	12.08	47.92			63.96	36.04		0.638
	PE	83	48.19	15.66	36.14			66.27	33.73		
			TT					**T**			
CD206	Normal	240	100.00					100.00			1.000
	PE	83	100.00					100.00			
			CC	**TT**	**CT**			**C**	**T**		
CD231	Normal	240	50.83	10.00	39.17			70.42	29.58		0.921
	PE	83	49.40	7.23	43.37			71.08	28.92		
			AA					**A**			
CD234	Normal	240	100.00					100.00			1.000
	PE	83	100.00					100.00			
			TT	**GG**	**GT**			**T**	**G**		
CD248-1	Normal	240	50.83	10.00	39.17			70.42	29.58		1.000
	PE	83	49.40	8.43	42.17			70.48	29.52		
			TT					**T**			
CD248-2	Normal	240	100.00					100.00			1.000
	PE	83	100.00					100.00			
			C					**C**			
CD257	Normal	240	100.00					100.00			1.000
	PE	83	100.00					100.00			
			AA					**A**			
CD316	Normal	240	100.00					100.00			1.000
	PE	83	100.00					100.00			
			GG					**G**			
CD317	Normal	240	100.00					100.00			1.000
	PE	83	100.00					100.00			
			TT	**CC**	**TC**			**T**	**C**		
CD318	Normal	240	10.83	43.75	45.42			33.54	66.46		0.387
	PE	83	10.84	51.81	37.35			29.52	70.48		
			GG					**G**			
CD321	Normal	240	100.00					100.00			1.000
	PE	83	100.00					100.00			
			TT					**T**			
CD348	Normal	240	100.00					100.00			1.000
	PE	83	100.00					100.00			

3.3.3 Comparisons of KIR2DL4 9A/10A Allele Frequencies in PE and controls

For the comparison of the 9A/10A polymorphism in cases and controls, no significant association was observed in both maternal and fetal groups (Table 21).

Table 21. Analysis of maternal and fetal *KIR2DL4* 9A/10A allele frequencies

	Maternal					Fetal				
	Normal Controls		Pre-eclampsia		Fisher's Exact Test	Normal Controls		Pre-eclampsia		Fisher's Exact Test
All Pregnancies										
	n=480	%	n=166	%	*P*-value	n=480	%	n=166	%	*P*-value
9A	116	24.17	42	25.30	0.755	126	35.59	45	27.11	0.839
10A	364	76.83	124	74.70		354	73.75	121	72.89	
Primigravid Pregnancies										
	n=118	%	n=62	%	*P*-value	n=118	%	n=62	%	*P*-value
9A	27	22.88	18	29.03	0.371	26	22.03	19	30.65	0.211
10A	91	77.12	44	70.97		92	77.97	43	69.35	
Multigravid Pregnancies										
	n=360	%	n=100	%	*P*-value	n=360	%	n=100	%	*P*-value
9A	88	24.44	22	22.00	0.692	99	27.50	24	24.00	0.525
10A	272	75.56	78	78.00		261	72.50	76	76.00	

n, number of chromosomes examined.

3.4 Test of gene-gene interaction between *HLA-G* and *KIR2DL4*

As HLA-G is the only known ligand for KIR2DL4, we tested for gene-gene interaction effects of particular combinations of ligand and receptor variants on risk for PE. Fetal HLA-G and maternal KIR2DL4 come into contact only during pregnancy due to their predominant expression in EVT and uterine NK cells, respectively. Logistic regression analysis of fetal *HLA-G* and maternal *KIR2DL4* alleles showed that *HLA-G* allele **0106* is associated with PE in multigravid pregnancies (p=0.004), as previously observed, whereas no disease association was observed for any maternal *KIR2DL4* allele (Table 22).

Table 22. Analysis of fetal *HLA-G* and maternal *KIR2DL4* alleles as PE risk predictors.

	Alleles	*P*-value		
		All pregnancies	*Primigravids*	*Multigravids*
Fetal	*G*0101*	0.766	0.586	0.560
	*G*0103*	0.944	NA	0.909
	*G*0104*	0.189	0.670	0.376
	*G*0105N*	0.999	NA	0.999
	*G*0106*	0.014	0.828	**0.004**
Maternal	*2DL4*001*	0.093	0.137	0.265
	*2DL4*002*	1.000	NA	1.000
	*2DL4*005*	0.225	0.739	0.268
	*2DL4*006*	0.218	0.561	0.324
	*2DL4*008*	0.203	0.838	0.216
	*2DL4*011*	0.826	0.893	0.460

To exclude the possibility that potential ligand-receptor interactions could have been masked by sample size constraints, we further compared the effects of fetal *HLA-G*0106* allele in the presence or absence of different maternal *KIR2DL4* alleles on risk

for PE. For this gene-gene interaction analysis, *HLA-G* alleles **0103*, **0105N* and *KIR2DL4* allele **002* were omitted as there were 5 or less positives in all case and control groups studied. Presence of fetal *HLA-G*0106* was significantly associated with PE in the multigravid sub-group, either when maternal *KIR2DL4*001* is absent (p=0.008) or when the maternal *KIR2DL4*006* is present (p<0.001) (Table 23). Also, the analysis revealed that the absence of maternal *2DL4*001*, *2DL4*005*, *2DL4*008* and *2DL4*011* alleles contributes to an increased risk of PE in the multigravid sub-group (p=0.008, p=0.016, p=0.011 and p=0.015, respectively) (Table 23). Further analysis confirmed an interactive effect of fetal *HLA-G*0106* and maternal *KIR2DL4*006* in risk for PE among multigravidas (p=0.002) (Table 24). These results indicate that *KIR2DL4* itself does not contribute to risk of PE, but suggest that it may modulate the effect of *HLA-G* on PE risk.

Table 23. Effect of fetal *HLA-G*0106* in the presence or absence of particular maternal *KIR2DL4* alleles.

	Maternal *KIR2DL4*001 absent*					Maternal *KIR2DL4*005 absent*					Maternal *KIR2DL4*006 absent*					Maternal *KIR2DL4*008 absent*					Maternal *KIR2DL4*011 absent*				
	NC		PE		Fisher's exact test	NC		PE		Fisher's exact test	NC		PE		Fisher's exact test	NC		PE		Fisher's exact test	NC		PE		Fisher's exact test
All pregnancies																									
	n=99	%	n=40	%	*P*-value	n=127	%	n=46	%	*P*-value	n=190	%	n=68	%	*P*-value	n=207	%	n=74	%	*P*-value	n=157	%	n=50	%	*P*-value
Fetal *HLA-G*0106* absent	97	97.98	35	87.50	0.021	124	97.64	41	89.13	0.032	181	95.26	62	91.18	0.233	199	96.14	66	89.19	0.039	148	94.27	42	84.00	0.035
Fetal *HLA-G*0106* present	2	2.02	5	12.50		3	2.36	5	10.87		9	4.74	6	8.82		8	3.86	8	10.81		9	5.73	8	16.00	
Primigravid pregnancies																									
	n=21	%	n=17	%	*P*-value	n=32	%	n=17	%	*P*-value	n=49	%	n=26	%	*P*-value	n=55	%	n=28	%	*P*-value	n=36	%	n=16	%	*P*-value
Fetal *HLA-G*0106* absent	20	95.24	16	94.12	1.000	32	100.00	17	100.00	NA	47	95.92	25	96.15	1.000	53	96.36	27	96.43	1.000	34	94.44	15	93.75	1.000
Fetal *HLA-G*0106* present	1	4.76	1	5.88		0	0.00	0	0.00		2	4.08	1	3.85		2	3.64	1	3.57		2	5.56	1	6.25	
Multigravid pregnancies																									
	n=78	%	n=22	%	*P*-value	n=94	%	n=28	%	*P*-value	n=140	%	n=40	%	*P*-value	n=151	%	n=44	%	*P*-value	n=121	%	n=34	%	*P*-value
Fetal *HLA-G*0106* absent	77	98.72	18	81.82	**0.008**	91	96.81	23	82.14	0.016	133	95.00	35	87.50	0.142	145	96.03	37	84.09	0.011	114	94.21	27	79.41	0.015
Fetal *HLA-G*0106* present	1	1.28	4	18.18		3	3.19	5	17.86		7	5.00	5	12.5		6	3.97	7	15.91		7	5.79	7	20.59	

	Maternal *KIR2DL4*001* present					Maternal *KIR2DL4*005* present					Maternal *KIR2DL4*006* present					Maternal *KIR2DL4*008* present					Maternal *KIR2DL4*011* present				
	NC		PE		Fisher's exact test	NC		PE		Fisher's exact test	NC		PE		Fisher's exact test	NC		PE		Fisher's exact test	NC		PE		Fisher's exact test
All pregnancies																									
	n=141	%	n=43	%	*P*-value	n=113	%	n=37	%	*P*-value	n=50	%	n=15	%	*P*-value	n=33	%	n=9	%	*P*-value	n=83	%	n=33	%	*P*-value
Fetal *HLA-G*0106* absent	134	95.04	38	88.37	0.155	107	94.69	32	86.49	0.140	50	100.00	11	73.33	**0.002**	32	96.97	7	77.78	0.111	83	100.00	31	93.94	0.079
Fetal *HLA-G*0106* present	7	4.96	5	11.63		6	5.31	5	13.51		0	0.00	4	26.67		1	3.03	2	22.22		0	0.00	2	6.06	
Primigravid pregnancies																									
	n=38	%	n=14	%	*P*-value	n=27	%	n=14	%	*P*-value	n=10	%	n=5	%	*P*-value	n=4	%	n=3	%	*P*-value	n=23	%	n=15	%	*P*-value
Fetal *HLA-G*0106* absent	37	97.37	14	100.00	1.000	25	92.59	13	92.86	1000	10	100.00	5	100.00	1.000	4	100.00	3	100.00	1.000	23	100.00	15	100.00	1.000
Fetal *HLA-G*0106* present	1	2.63	0	0.00		2	7.41	1	7.14		0	0.00	0	0.00		0	0.00	0	0.00		0	0.00	0	0.00	
Multigravid pregnancies																									
	n=102	%	n=28	%	*P*-value	n=86	%	n=22	%	*P*-value	n=40	%	n=10	%	*P*-value	n=29	%	n=6	%	*P*-value	n=59	%	n=16	%	*P*-value
Fetal *HLA-G*0106* absent	96	94.12	23	82.14	0.058	82	95.35	18	81.82	0.053	40	100.00	6	60.00	**<0.001**	28	96.55	4	66.67	0.070	59	100.00	14	87.50	0.043
Fetal *HLA-G*0106* present	6	5.88	5	17.86		4	4.65	4	18.18		0	0.00	4	40.00		1	3.45	2	33.33		0	0.00	2	12.50	

NC: Normal controls; PE: Pre-eclampsia
n, number of individuals examined.

Table 24. Effect of fetal *HLA-G*0106* and maternal *KIR2DL4*006* alleles.

	NC		PE		Fisher's exact test
All pregnancies					
	n=240	%	n=83	%	*P*-value
b*G*0106*+/m*2DL4*006*+	0	0.00	4	4.82	**0.004**
b*G*0106*+/m*2DL4*006*-	9	3.75	6	7.23	0.226
b*G*0106*-/m*2DL4*006*+	50	20.83	11	13.25	0.145
b*G*0106*-/m*2DL4*006*-	181	75.42	62	74.70	0.884
Primigravid pregnancies					
	n=59	%	n=31	%	P-value
b*G*0106*+/m*2DL4*006*+	0	0.00	0	0.00	1.000
b*G*0106*+/m*2DL4*006*-	2	3.39	1	3.23	1.000
b*G*0106*-/m*2DL4*006*+	10	16.95	5	16.13	1.000
b*G*0106*-/m*2DL4*006*-	47	79.66	25	80.65	1.000
Multigravid pregnancies					
	n=180	%	n=50	%	*P*-value
b*G*0106*+/m*2DL4*006*+	0	0.00	4	8.00	**0.002**
b*G*0106*+/m*2DL4*006*-	7	3.89	5	10.00	0.141
b*G*0106*-/m*2DL4*006*+	40	22.22	6	12.00	0.160
b*G*0106*-/m*2DL4*006*-	133	73.89	35	70.00	0.592

b*G*0106+*: presence of fetal *HLA-G*0106* allele.
b*G*0106-*: absence of fetal *HLA-G*0106* allele.
m*2DL4*006+*: presence of maternal *KIR2DL4*006* allele.
m*2DL4*006-*: absence of maternal *KIR2DL4*006* allele.

4.0 Discussion

4.1 *HLA-G* haplotypes/ polymorphisms in PE case-control study

*4.1.1 Positive association of HLA-G*0106 allele with PE*

In this study, we observed a significant association between presence of *HLA-G* allele G*0106 in the fetus and an increased risk for PE, but only in multigravid pregnancies. This observation is supported by a recent report of a significant increase of G*0106 allele frequency in PE placentas (Moreau, Contu et al. 2008). However, two earlier studies of *HLA-G* polymorphisms in PE did not report any data to support an association with G*0106 (Hviid, Christiansen et al. 2001; Hylenius, Andersen et al. 2004). Hviid et al's study was based on the difference in the overall proportion of *HLA-G* alleles shared between couples, and no information on fetal genotype and its potential interaction with maternal genotype was provided. Similarly, information on paternal or maternal inheritance of G*0106 alleles was not provided in Hylenius et al's study of PE triads. As such, significant differences that were present could have been overlooked. Although the antigen-presenting functions of *HLA-G* are yet to be established, it is possible that mismatched fetal-maternal *HLA-G* genotypes may adversely affect the maternal tolerance of the semiallogenic fetus.

4.1.2 Positive association of codon 258 with PE

The *G*0106* allele is characterized by a non-synonymous substitution at codon 258. This codon 258 C→T change in the G*0106 peptide translates into a non-conservative threonine to methionine amino acid substitution, which could alter its structure and/or function. Interestingly, codon 258 is located in the α3 domain of

HLA-G, the candidate binding site for leukocyte Ig-like receptor 1 (LIR-1 or ILT2) and LIR-2 (or ILT4) (Clements, Kjer-Nielsen et al. 2005; Shiroishi, Kuroki et al. 2006). Both LIR1 and LIR2 receptors bind preferentially to HLA-G dimers (Shiroishi, Kuroki et al. 2006), the most common HLA-G conformation present on the surface of normal first trimester trophoblast cells (Apps, Gardner et al. 2007), suggesting that this antigen-receptor interaction is important in the maintenance of a healthy pregnancy. Therefore, a non-conservative amino acid substitution at this site may also alter HLA-G's binding affinity for its inhibitory receptors, thus adversely affecting its functions in protecting the semiallogenic fetus from maternal immune surveillance.

4.1.3 Histo-incompatibility in PE mother-child pairs

We also observed a significantly higher frequency of fetal-maternal *HLA-G* genotype mismatch in pre-eclamptic pregnancies compared to normal pregnancies, specifically involving G*0106-negative mothers with fetuses carrying paternally inherited G*0106. Again, this positive association involved only multigravid pregnancies. These observations strongly suggest that HLA-G variants foreign to the mother may lead to histoincompatibility between mother and child, suggesting an immunological basis for PE. Therefore, maternal rejection of the semiallogenic fetus could represent one of the major contributors to the development of PE. Also, it is important to consider the effects of paternal alleles in the fetus in studies of pregnancy complications and their interactions with the maternal genotype, which controls the *in utero* environment of the developing fetus.

*4.1.4 Association of G*0106 is only in the multigravids*

Although PE has been postulated to be a disease of first pregnancies due to the higher risk of PE in primigravids, we observed significant association only in the multigravid sub-group. The observation of an association between paternal G*0106 and PE only in multigravid but not in primigravid pregnancies suggests a gradual rather than immediate alloimmune response. This supposition is consistent with the observation that the G*0106 allele is unlikely to be highly immunogenic as this allele was not more likely to elicit HLA-G antibody production in mothers lacking G*0106 (Hunt, Pace et al. 2003). However, the lack of positive results in the Hunt et al. study could possibly be due to the failure of the wild-type sHLA-G*0101 protein used in the assay to recognize the anti-G*0106 antibodies generated in G*0106-naive mothers.

On the other hand, Hunt et al. demonstrated that tolerance to HLA-G can be overcome by exposure during pregnancy, thereby suggesting that it is possible that the risk of a severe alloimmune reaction to G*0106 leading to PE in G*0106-naive mothers increases with each repeated exposure to the foreign HLA-G antigen from previous pregnancies. This suggests that maternal-fetal incompatibility for G*0106 does not predispose to PE *per se* as no significant association was observed in primigravids, but the presence of this antigen in the fetus can lead to a recurrence in a woman with a previous PE pregnancy.

4.1.5 Population-specific differences in HLA-G haplotype/allele frequencies

The availability of population allele/haplotype frequencies serves a useful purpose in case-control association studies as an independent check to minimize false positive results arising from sampling error or differences in racial admixture between

case and control groups. Therefore, frequencies of *HLA-G* haplotype in the three main population groups in Singapore and Malaysia, namely the Southeast Asian Chinese, Indian and Malay populations, were determined. As the G*0106 allele had only been discovered relatively recently, few studies have examined its population frequencies and also its association with PE. A comparison of G*0106 allele frequencies among the Southeast Asian Chinese, Indian, Malay and Danish populations revealed differences, with frequencies of 1.1%, 8.3%, 2.2% and 4.0% respectively (Table 25).

Since the Indian G*0106 allele frequency is nominally higher compared to the Malay population (8.33% vs. 2.22%, p=0.016) (Table 15), a high Indian admixture could theoretically lead to a spuriously high G*0106 allele frequency within the PE babies group. However, the observed G*0104 allele frequencies in the various case, control, and population groups do not support this possibility. The G*0104 allele frequencies in normal control mothers (45.28%) and babies (45.83%), as well as PE mothers (47.00%) and babies (45.00%) were very similar to each other and to the Malay population (45.00%), but significantly different compared to the Indian population (26.11%) ($p<0.001$). A significant Indian admixture in the PE babies group would have been reflected by a significant, if not nominal, decrease in G*0104 allele frequency in this group, which we did not observe. Through the hospital's records, we have also re-confirmed the Malay ethnicity of the fathers of each of the G*0106-positive babies in both case and control groups, thus ruling out the possibility of Indian paternity among the G*0106-positive PE babies.

Table 25. *HLA-G* allele frequencies in different populations.

Allele[a]	Japanese[b] *n*=82	Hutterite[c] *n*=160	German / Croatian[d] *n*=344	Portugese[e] *n*=234	Spanish[f] *n*=228	Danish[g] *n*=154	African American[b] *n*=84	African Ghanaian[b] *n*=84	African Shona[h] *n*=216	Chinese[i] *n*=188	Indian[i] *n*=180	Malay[i] *n*=180
G*010101	42.7	45.5	32.1	37.2	30.2	61.7	70.2	83.3	39.3	41.5	31.7	25.0
G*010102	14.6	20.3	36.3	31.4	21.9	27.2	6.0	2.4	14.4	5.9	11.1	6.11
G*010103	4.9	2.0	6.8	17.4	7.0	5.1	2.4	0	0	20.2	17.2	19.4
G*010104	-	4.4	-	-	-	-	-	-	0	0	0	0
G*010105	-	-	-	-	-	-	-	-	0	0	0	0
G*010106	-	-	-	-	-	-	-	-	0	0	0	0
G*010107	-	-	1.9	-	-	-	-	-	0	0	0	0
G*010108	-	7.6	9.1	-	-	-	-	-	14.4	0	0.6	0
G*0102	-	-	-	-	-	-	-	-	0	0	0	0
G*0103		2.7	2.3	1.2	0	-			0	1.1	1.7	0.6
G*0104	37.8	12.6	6.1	12.8	10.5	4.5	13.1	9.5	20.8	27.7	26.1	45.0
G*0105	0	-	2.3	0	3.1	0.6	8.3	4.8	11.1	2.7	2.8	1.1
G*0106	-	-	-	-	-	4.0[i]	-	-	-	1.1	8.3	2.2

[a]Alleles are numbered according to WHO nomenclature.
n represents the number of chromosomes examined.
Dashes (-) indicate that genotyping for that haplotype was not performed.
[b](Ishitani, Kishida et al. 1999), [c](Ober, Rosinsky et al. 1996), [d](van der Ven, Skrablin et al. 1998), [e](Alvarez, Santos et al. 1999), [f](Suarez, Morales et al. 1997), [g](Hviid, Meldgaard et al. 1997), [h](Matte, Lacaille et al. 2000), [i]This study.

Given the variable but low frequency of the G*0106 haplotype in the different populations, its contribution to the total number of PE cases in any population group would be expected to be small. However, the clinical significance of these findings lies in the fact that in those pre-eclampsia cases caused in part by maternal alloimmune response to foreign HLA-G*0106 antigen, it may now be possible to provide improved prognosis and preventive intervention in subsequent at-risk pregnancies.

*4.1.6 Lack of association of G*0105N allele with PE*

Although the G*0105N haplotype contains a frameshift mutation that leads to a premature stop, resulting in reduced expression of the most abundant HLA-G1 isoform in heterozygotes (Ober, Aldrich et al. 1998), we did not find any association of this haplotype with PE in our study. Association studies involving different sample populations also yielded similar results (Aldrich, Verp et al. 2000; Hylenius, Andersen et al. 2004), suggesting that reduced or absence of HLA-G1 expression is not a contributory factor to PE development as these individuals could still produce HLA-G2, -G3, -G6 and –G7 isoforms. Furthermore, healthy multiparous individuals homozygous for the HLA-G*0105N null allele has also been reported (Ober, Aldrich et al. 1998; Casro, Morales et al. 2000). The fact that this allele encodes functional HLA-G proteins capable of inhibiting NK cell-mediated cytotoxicity (Le Discorde, Le Danff et al. 2005) as well as inducing cell surface expression of HLA-E molecules (Sala, Del Moral et al. 2004) indicates that this null mutation does not affect the maintenance of a healthy pregnancy.

4.1.7 Lack of association of the 5' UR polymorphism with PE

Variations in the 5' UR and promoter region of the *HLA-G* gene may influence the regulation of its transcription and expression. Polymorphisms located at this region include the NP-1306 SNP located within the locus control region that binds to nuclear factors to regulate the expression of HLA-G molecules (Hviid, Sorensen et al. 1999) as well as the NP-725 polymophism which forms a CpG dinucleotide flanking the interferon specific regulatory element (ISRE) and is associated with the status of methylation and expression of soluble HLA-G (Ober, Aldrich et al. 2003). In addition to that, the NP-486 SNP located very close to a putative heat shock element (HSE) was reported to bind heat shock factor 1 (HSF1) under stress (Hviid, Rizzo et al. 2006). Stressful condition such as heat shock was shown to induce the expression of HLA-G in tumor cells lines suggesting that a polymorphism at this region may affect binding of HSF1 to HSE leading to differences in HLA-G expression (Ibrahim, Morange et al. 2000). Also, a polymorphism at NP-56 located within the putative binding site for the polyomavirus enhancer-binding protein 2 (PEBP2) (Matte, Lacaille et al. 2002) may affect the binding of this protein to the enhancer core motif and thereby leading to reduced expression of HLA-G molecules. However, we did not observe any significant association between the 5' UR variations studied. Consistent with our observation, a study of the association of NP-56 SNP with PE also revealed no significant association between the two (Doherty, Rush et al. 2006).

4.1.8 Lack of association of the +/-14bp polymorphism with PE

The 14 bp insertion/deletion in the 3' UTR of the *HLA-G* gene has been reported to affect the stability of the *HLA-G* transcript as well as the splicing patterns of HLA-G isoform (Rousseau, Le Discorde et al. 2003) and has also been found to be associated with PE (O'Brien, McCarthy et al. 2001; Hylenius, Andersen et al. 2004). However, our data does not support this conclusion (Table 13), as did several previous reports in other population groups (Humphrey, Harrison et al. 1995; Bermingham, Jenkins et al. 2000; Lin, Yan et al. 2006; Vianna, Dalmaz et al. 2007; Iversen, Nguyen et al. 2008). The exact effect of this polymorphism remains unclear.

4.2 *KIR2DL4* haplotypes in PE case-control study

4.2.1 Lack of association of KIR2DL4 alleles with PE

In this study, we tested alleles of the *KIR2DL4* receptor for contribution to the development of PE using a case-control design, and observed a lack of association with disease in both mothers and babies. An earlier study that examined polymorphisms within exons 3 and 4 also found no significant association between *KIR2DL4* and PE (Witt, Whiteway et al. 2002). These 2 exons encode the extracellular immunoglobulin domains which are involved in HLA Class I ligand binding, and it was hypothesized that amino acid substitutions in these domains may affect KIR2DL4 binding to HLA-G molecules.

These observations implicate that KIR2DL4 receptor may not be involved in the development of PE. Of interest is a report of a multiparous woman who lacks this gene (Gomez-Lozano, de Pablo et al. 2003). Although very few individuals are

known to lack *KIR2DL4* (Norman, Carrington et al. 2002), these data suggests that KIR2DL4 may not be essential for the maintenance of human pregnancy, or that alternative genes such as *ILT2* or *ILT4* could function in place of *KIR2DL4* in its absence.

The *KIR2DL4* gene is minimally polymorphic in our study population, as 14 of the 23 genotyped SNPs were monomorphic in all samples tested. According to the Immuno Polymorphism Database (IPD), these 23 SNPs/polymorphisms characterize the 12 major *KIR2DL4* alleles (Selvakumar, Steffens et al. 1996; Selvakumar, Steffens et al. 1997; Valiante, Uhrberg et al. 1997; Cantoni, Verdiani et al. 1998; Rajalingam, Gardiner et al. 2001; Marsh, Parham et al. 2003; Gedil, Steiner et al. 2005; Robinson, Waller et al. 2005). However, in our study population, only 6 of the designated major alleles were observed (Table 16).

4.2.2 Lack of association of KIR2DL4 frameshift mutation and PE

The deletion polymorphism at codon 248 in exon 6 is of much interest as it results in 2 variants of the transmembrane exon, of which the 10A variant encodes the full-length classical membrane-bound receptor and the 9A variant either have a missing ITIM or lacking the transmembrane exon. Truncated proteins produced by the 9A allele of *KIR2DL4* may improperly insert into the cell membrane and/or bind its cognate ligand, and thus fail to prevent maternal NK cells from attacking the fetal trophoblasts. A previous study did not detect any association between the 9A alleles and PE (Witt, Whiteway et al. 2002). We also failed to observe an association between this allele and PE in both mothers and babies (Table 21).

4.3 HLA-G and KIR2DL4 gene-gene interaction

Pregnancy is a unique situation where KIRs may come into contact with non-self HLA ligands. As KIRs interact with HLA molecules and also, because the two gene families segregate independently on different chromosomes, therefore, it is important to consider combinations of HLA molecules and KIRs as risk factors, in addition to studying each of them separately. Several studies have examined whether some combinations of maternal KIR genes and fetal HLA allotypes may provide sub-optimal conditions for a healthy pregnancy (Hiby, Walker et al. 2004; Trundley and Moffett 2004). In this study, fetal *HLA-G* and maternal *KIR2DL4* alleles were analyzed together to determine if certain HLA-G ligand variants, in combination with specific KIR2DL4 receptor variants, might be less favorable for the maintenance of a healthy pregnancy compared to others.

Significant association with PE was observed for fetal *HLA-G*0106* in combination with maternal *KIR2DL4*006* allele among multigravid pregnancies ($p<0.001$). It should be noted, however, that only 4 PE pregnancies were positive for maternal *KIR2DL4*006* and fetal *HLA-G*0106*, and none in the controls. These results should, therefore, be interpreted conservatively in the context of the sample size constraints, and additional studies are required to verify these observations. Therefore, it is likely that KIR2DL4 is not associated with risk of PE.

Although HLA-G is the only known ligand of KIR2DL4, KIR2DL4 is not the sole receptor for HLA-G. It is possible that other inhibitory receptors such as ILT2 or ILT4, which bind preferentially to HLA-G dimers (Shiroishi, Kuroki et al. 2006), may also play a role in the development of PE. Furthermore, the interaction between

KIR2DL4 and its ligand HLA-G has been inconclusive. Some studies have reported KIR2DL4 to be a specific receptor for HLA-G (Rajagopalan and Long 1999; LeMaoult, Zafaranloo et al. 2005; Yan and Fan 2005) while others have disputed this receptor-ligand interaction (Allan, Colonna et al. 1999; Boyson, Erskine et al. 2002). It is possible that the differences observed in different studies are due to the different techniques used to detect the interactions between KIR2DL4 and HLA-G.

4.4 Limitation of this study

This study is limited by a small sample size and therefore, the strong association observed between G*0106 and PE should be interpreted conservatively. Similarly, as previously mentioned, the observation of an increase risk of PE in multigravid pregnancies with fetal *HLA-G*0106* in combination with maternal *KIR2DL4*006* allele should also be interpreted carefully due to the low number of pregnancies positive for this allele combinations. Therefore, a study with a larger sample size is necessary to confirm the observed results.

4.5 Conclusion

We conclude that the presence of paternal *HLA-G* G*0106 in the fetus significantly increases risk for PE in multigravidas who do not carry this allele. This increased risk for PE may be mediated by an increasing alloimmune response to the variant paternal HLA-G antigen expressed in the fetal trophoblast following repeated exposure to the foreign antigen. Presence of variant paternal HLA-G in the semiallogenic fetus may also cause a malfunctioning of the immune-protective effect of HLA-G due to possible changes in its interaction with maternal inhibitory

receptors, especially in pregnancies where the paternal allele in the fetus is foreign to the mother. These findings are consistent with clinical observations that women are more at risk of PE with certain men (Astin, Scott et al. 1981). On the other hand, KIR2DL4 is not associated with risk for PE. However, certain maternal *KIR2DL4* alleles may lead to increase disease risk in the presence of fetal *HLA-G*0106* allele.

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