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Association of maternal killer-cell immunoglobulin-like receptors and parental HLA-C genotypes with recurrent miscarriage

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BACKGROUND: The natural killer (NK) cells at the site of placentation express killer-cell immunoglobulin-like receptors (KIR) that can bind to human leukocyte antigen (HLA)-C molecules on trophoblast cells. Both these gene systems are polymorphic and an association of particular maternal KIR/fetal HLA-C genotypes has been shown in pre-eclampsia. Pre-eclampsia and recurrent miscarriage (RM) share the pathogenesis of defective placentation and therefore we have now genotyped couples with RM. METHODS AND RESULTS: DNA was obtained from the male ($n = 67$) and female ($n = 95$) partners of couples with three or more spontaneous miscarriages and genotyped for HLA-C groups and 11 KIR genes using the PCR-sequence-specific primer method (SSP). The frequency of the HLA-C2 group was increased in both parents (reaching significance only in the male partners, $P = 0.018$) compared with a parous control population. The KIR gene frequencies of the male partners were similar to controls, but the women had a high frequency of KIR AA haplotypes that lack activating KIR. In particular, the activating KIR for HLA-C2 groups (KIR2DS1) was significantly lower in these women ($P = 0.00035$, odds ratio 2.63, confidence interval 1.54–4.49). CONCLUSIONS: This is the first report to identify a genetic male factor that confers risk in RM. These findings support the idea that successful placentation depends on the correct balance of NK cell inhibition and activation in response to trophoblast.

Keywords: recurrent miscarriage; natural killer cells; HLA-C; trophoblast; killer-immunoglobulin-like receptor

Introduction

The idea that recurrent miscarriage (RM) has an immune aetiology that depends on incompatibility between the mother and her fetus is widespread, despite the paucity of supporting evidence (Porter *et al*., 2006; Rai and Regan, 2006). Likely effector cells could be the uterine natural killer (uNK) cells, a unique type of lymphoid cell present in great abundance in the decidua in the first trimester (Moffett-King, 2002; Koopman *et al*., 2003). NK cells amass at the site of placental implantation and are in close contact with invading trophoblast cells. Evidence that these uNK cells regulate placentation has come from functional and immunogenetic studies (Hiby *et al*., 2004; Hanna *et al*., 2006). NK cells have receptors for the three human leukocyte antigen (HLA) class I molecules (HLA-G, HLA-E and HLA-C) that are displayed by infiltrating extravillous trophoblast cells (Moffett and Loke, 2006). Of these three, only HLA-C is highly polymorphic and thus the HLA-C allotypes presented by trophoblast will vary depending on both the maternal and paternal contribution to the fetus.

The receptors on uNK cells that bind to HLA-C molecules belong to the killer-cell immunoglobulin-like receptor (KIR) family (Parham, 2005; Bashirova *et al*., 2006). Although the KIR locus contains variable numbers of genes (7–15), the haplotypes can be divided into two groups (A and B) (Fig. 1). The A haplotype contains mainly inhibitory KIR, whereas the B haplotype has additional KIR genes that are predominantly activating. Thus, KIR-mediated NK responses in individuals with two copies of the A haplotype (AA) are mainly inhibitory

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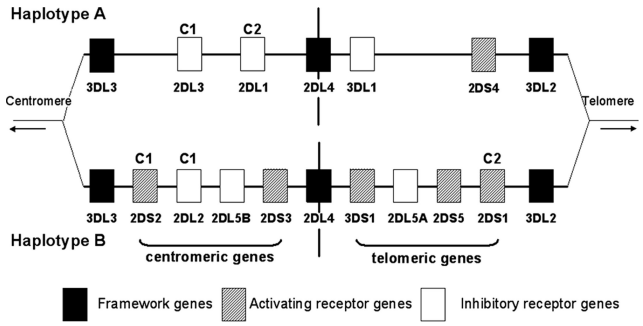

Figure 1: Schematic representation of a typical KIR AB genotype. The haplotypes are divided into centromeric and telomeric halves by the central framework gene, KIR2DL4. C1 and C2 are the two HLA-C ligands bound by both inhibitory and activating KIR as shown

in nature. NK KIR binding motifs to HLA-C are defined by a dimorphism in the α1 domain of the HLA-C heavy chain. HLA-C molecules with Ser^{77} Asn^{80} are in Group C1 (-Cw1, -Cw3, -Cw7, -Cw8, -Cw12, -Cw14 and -Cw1601). HLA-C molecules with Asn^{77} Lys^{80} are in Group C2 (-Cw2, -Cw4, -Cw5, -Cw6, -Cw15, -Cw1602, -Cw17 and -Cw18) (Parham, 2005). C1 allotypes are ligands for KIR2DL2/3 (inhibitory) and possibly KIR2DS2 (activating), whereas C2 allotypes are bound by KIR2DL1 (inhibitory) and KIR2DS1 (activating) (Fig. 1).

In pre-eclampsia, we have found that there was an association of maternal KIR AA with fetal HLA-C2 genotypes, genotype pairs that theoretically result in the inhibition of maternal NK cells and the lack of appropriate cytokines to induce proper invasion of fetal trophoblast cells into the uterus (Hiby *et al*., 2004). Pre-eclampsia and RM share a primary pathogenesis characterized by deficient trophoblast invasion into the uterus (Khong *et al*., 1986, 1987; Merviel *et al*., 2004; Norwitz, 2006). To determine if similar immunogenetic findings are associated with RM, we have now analysed the KIR and HLA-C genotypes in couples with RM.

Materials and Methods

Subjects

Women with three or more spontaneous miscarriages and no live births were recruited from St Mary's Hospital, London ($n = 95$). In 67 couples, the male partner also agreed to participate. Approximately 1000 new patients were screened during the period of the study and 9.5% were recruited after excluding those who declined or did not meet the criteria. Ninety-two percent of the selected patients had only first trimester miscarriages and 8% had both first and second trimester miscarriages. All the affected women had remained with the same partner. The following investigations were performed and were all normal (normal ranges shown): parental chromosomes, Day 2 hormone levels of FSH (3–11 U/l), LH (3–12 U/l) and testosterone (0.5–3 nmol/l), antiphospholipid antibodies including lupus anticoagulant (PLR, 0.8–1.05) and anticardiolipin antibodies [immunoglobulin (Ig)G 0–12 GPL units, IgM 0–5 MPL units] and prothrombotic risk factors screened for included activated protein C resistance (APCR, 2.6–4.36 ratio), factor V Leiden and prothrombin mutation. The uterine cavity was investigated by 2D ultrasound and there was no evidence of cervical incompetence.

Table I. PCR primers for human leukocyte antigen (HLA)-C1 and C2 amplification.

Primer code	Primer sequence	Amplicon size (bp)
P1: HLA-C forward (common)	5′-GCC GCG AGT CCR AGA GG-3′	
P2: HLA-C1 reverse	5′-GCG CAG GTT CCG CAG GC-3′	129
P3: HLA-C1 reverse	5′-GTT GTA GTA GCC GCG CAG G-3′	141
P4: HLA-C2 reverse	5′-CGC GCA GTT TCC GCA GGT-3′	130
P5: HLA-C2 reverse	5′-GTT GTA GTA GCC GCG CAG T-3′	141
P6: HLA-B forward	5′-CCA TGA GGT ATT TCT ACA CCT-3′	
P7: HLA-B reverse	5′-CCT CCT GCT CCA CCC AC-3′	156
P8: HLA-B forward	5′-GCC GCG AGT CCG AGA GG-3′	
P9: HLA-B reverse	5′-GCC ATA CAT CGT CTG CCA A-3′	425
DRB forward	5′-TGC CAA GTG GAG CAC CCA A-3′	
DRB reverse	5′-GCA TCT TGC TCT GTG CAG AT-3′	796

As controls we used a subset of women patients recruited from Leeds and Oxford as part of a wider study. They were all primiparae (no miscarriages or ectopic pregnancies) with an unremarkable pregnancy and delivery of a normal birthweight baby. All study participants were Caucasian. Ethical approval was obtained for the study from the London Multi-centre Research Ethics Committee and informed written consent was obtained in all cases (MREC No. 05/MRE02/20).

Genotyping

DNA was genotyped for KIR as previously described (Hiby *et al*., 2004). The HLA-C genotype was defined in terms of Group C1 or Group C2 as recognized by NK cells through the KIR receptors. The primers used to define these groups by PCR-SSP are given in Table I. Two reactions were used to amplify both C1 and C2. Rare HLA-B group alleles, which would also be amplified by the HLA-C primers, were identified with primers P6 with P7 and P8 with P9. An additional pair of PCR primers that amplified across the third intron of DRB1 genes was used in each reaction as a positive internal control for the PCR reaction. The HLA-C PCR method comprised 50 ng genomic DNA per 20 μl PCR, 200 μM dNTPs, 1 μM HLA-C primers with 350 nM DRB internal control primers and the Qiagen *Taq* kit (Qiagen Ltd, Crawley, UK). The PCR programme using a PE Applied Biosystems 9700 thermal cycler (Foster City, USA) was 96° 1 min; 4 cycles 96° 25 s, 70° 45 s, 72° 30 s; 26 cycles 96° 25 s, 65° 45 s, 72° 30 s; 5 cycles 96° 25 s, 58° 1 min, 72° 2 min and finally 72° 10 min.

Statistical analysis

The data were analysed using the chi-square and Fisher's exact test. A P-value of ≤ 0.05 was considered to be statistically significant. The magnitude of the effect was estimated by odds ratios (OR) and their 95% confidence intervals (CI) (Windows 11.0.0.2001; SPSS Inc.).

Results

The KIR AA genotype was found in 28% of the Caucasian controls (Fig. 2). Compared with the control population, we found a significant increase in the KIR AA genotype frequency in women with RM similar to our findings in pre-eclampsia (41% in RM compared with 35% for a pre-eclamptic cohort) (Hiby *et al*., 2004). Notably, the KIR AA genotype frequency was not increased in their male partners. The HLA-C2

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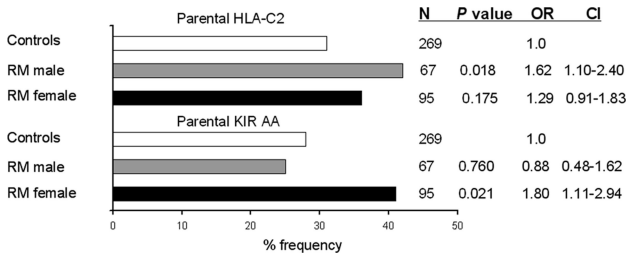

Figure 2: HLA-C2 group and KIR AA genotype frequencies in recurrent miscarriage (RM) couples and parous controls. Both affected women and men are more likely to carry an HLA-C2 group than the fertile controls but only the women have increased KIR AA genotype frequency. The statistical results are shown with numbers analysed (*N*), the significant *P*-values (Fisher's exact test) and the OR for all, with the 95% CI.

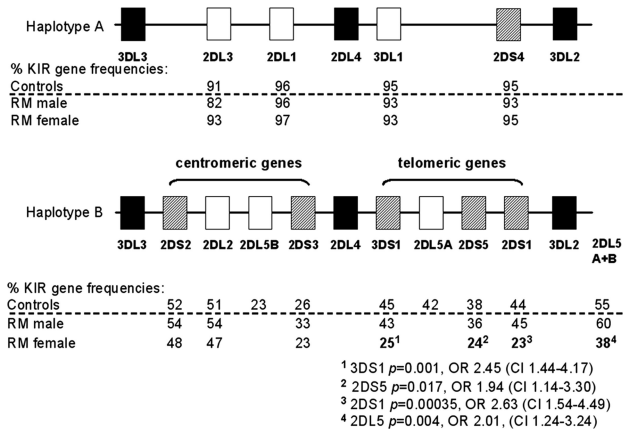

Figure 3: Individual KIR gene frequencies. The frequency of individual KIR genes in the telomeric half of the B haplotype is reduced in women with reproductive failure compared with controls. KIR gene frequencies in the male partners of women with RM are similar to controls. Statistics are given for the KIR frequencies that are significantly different between the affected women and the fertile controls.

frequencies were also analysed in both the female and the male partners of the RM couples and compared with controls (Fig. 2). As expected, the C2 frequencies were raised in both the females and the males (although only the difference in the male frequency reached significance), meaning that the chance of having a fetal C2 genotype is greater than in controls.

We next studied the frequency of individual KIR genes in the couples with RM and compared them with controls (Fig. 3). Importantly, the frequencies of individual KIR genes were the same in our control population as that reported for two other Caucasian English populations (Norman *et al.*, 2001; Cook *et al.*, 2003). Furthermore, as expected, the male partners of RM couples had similar KIR gene frequencies as these controls. The frequencies of the inhibitory KIR for HLA-C (2DL1, 2DL2 and 2DL3) were similar in affected couples and controls. In contrast, the frequencies of all KIR genes on the B haplotype telomeric to 2DL4 were significantly reduced in women with RM. Strikingly, the most significant protection against RM was conferred by KIR2DS1, the activating receptor for C2 allotypes ($P = 0.00035$, OR 2.63, CI 1.54–4.49).

Discussion

These results further support the idea that reproductive performance is modulated by the response of maternal NK cells to fetal trophoblast through KIR–HLA interactions. We found an association of the lack of activating KIR in the affected woman (KIR AA genotype) and an increased HLA-C2 group frequency in the RM couples compared with control genotype frequencies. Although RM is a heterogeneous condition, we have only investigated the couples where the women have been exhaustively screened for known causes of RM and there has been no change of partner. In these couples defective placentation is the likely pathogenesis, an aetiology shared with pre-eclampsia (Norwitz, 2006). Defective placentation has been described in a significant proportion of cases of early pregnancy loss (Khong *et al.*, 1987). This involves reduced trophoblast invasion into both the decidua and spiral arteries. The loose plugs of endovascular trophoblast that normally limit the maternal circulation to the placenta may be incomplete (Hustin *et al.*, 1990). This leads to the premature entry of maternal blood at high pressure into the intervillous space resulting in damage and fetal loss (Jauniaux and Burton, 2005). In pregnancies that do continue, the destruction of the arterial media by the interstitial trophoblast that modifies the arteries is inadequate, so that there is insufficient blood flow to the feto-placental unit. Defective placentation is well recognized as the underlying primary defect in pre-eclampsia as well as many cases of intrauterine growth restriction and stillbirth (Pijnenborg *et al.*, 2003, 2006). There is, therefore, a spectrum of pregnancy outcomes resulting from poor placentation. Thus, in almost half the couples who go on to have a living child, premature delivery, low birthweight or pre-eclampsia occur in keeping with a common underlying pathogenesis (Clifford *et al.*, 1997; Weintraub *et al.*, 2005).

We found that pre-eclampsia was associated with an increased risk with the same type of KIR/HLA combination as reported here for RM; the KIR AA genotype frequency was increased in the mother when there was an HLA-C2 group in the fetus (Hiby *et al.*, 2004). A genetic contribution from the male partner is known to be important in pre-eclampsia and low birthweight, but this is the first time it has been reported in RM (Lie *et al.*, 1998; Esplin *et al.*, 2001; Skjaerven *et al.*, 2002). Our findings are in keeping with the change of partner effect known to be important in pre-eclampsia and also reported in RM (Vatten and Skjaerven, 2003; Maconochie *et al.*, 2007). Any genetic study such as this should be replicated using the same stringent screening of the couples in other cohorts. It would also be important to look at different ethnic groups as both KIR and HLA-C genotypes have wide geographical variation. There have been other studies that have analysed KIR genotypes in women with RM but the father's genetic contribution was not usually considered. Like our study, reports by two other groups also show an increased frequency of the inhibitory KIR AA genotype (Witt *et al.*, 2004; Flores *et al.*, 2007). Witt *et al.* also show decreased frequency of the telomeric, but not centromeric, B haplotype activating KIR, although this did not reach significance. Flores *et al.* only detail a lack of

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KIR2DL2, a signature for B haplotypes, with no data on the activating receptors. In contrast to our study, Varla-Leftherioti *et al*. (2003) reported that their 26 RM patients lacked inhibitory KIR receptors. This high frequency of KIR2DL1 and KIR2DL2-negative samples in their study may be because the primers used for the KIR genotyping do not amplify certain alleles of these two inhibitory KIR (Uhrberg *et al*., 1997). Lastly, the report on a Chinese Han RM population (Wang *et al*., 2007) is also in contrast to our findings as it describes an increased frequency of the activating KIR receptors but their control population has activating KIR frequencies far higher than those reported for any Chinese population including the Chinese Han (Jiang *et al*., 2005). Wang *et al*. (2007) do, however, show that women in affected couples who both carry an HLA-C2 allele significantly lack KIR2DS1.

The importance of the HLA-C1 and C2 groups alone has been investigated previously (Christiansen *et al*., 1997), with small study groups of 30 affected and 30 control couples, and no association of RM with the HLA-C1 or C2 NK binding motifs was seen and KIR were not genotyped. We cannot compare our study to this one as we have compared affected couples with controls that are mothers only. We have not had access to normal couples as controls. Eventually, it will be of interest to see if couples where the female partner has the KIR AA genotype (and in particular lacks KIR2DS1) and the male is HLA-C2 homozygous have a poorer prognosis than those where the male partner is HLA-C1 homozygous. If confirmed, our findings could lead to certain couples being advised in future to seek sperm from an HLA-C1 homozygous male.

We have not typed in this study for alleles of KIR2DL4 and these are in linkage disequilibrium to other KIR A or B haplotype genes (Witt *et al*., 2000, 2002). No association was found in a previous study of RM with null alleles of KIR2DL4, and indeed normal pregnancy occurs with the null KIR2DL4 allele and in the absence of the KIR2DL4 gene altogether (Gomez-Lozano *et al*., 2003; Witt *et al*., 2004). HLA-G is the ligand for KIR2DL4. Variants of HLA-G have been studied in several reproductive disorders including RM, but the results have been conflicting. Large numbers of patients will be needed to analyse any additional contribution of KIR2DL4/HLA-G interaction in women alongside the KIR2D/HLA-C interaction.

In RM, we have been able to show that the KIR gene on the B haplotype that is likely to be protective is KIR2DS1 because the lack of this gene in RM women was highly significant. In addition, this finding makes biological sense because KIR2DS1 is the activating receptor for C2 groups and so the functional effect would be to overcome the strong inhibition mediated by a C2-KIR2DL1 interaction (Stewart *et al*., 2005). Our findings do help to refute the pervasive idea that the function of trophoblast HLA class I molecules on the invading trophoblast is to inhibit killing by the NK cells. Many researchers working with the relevant primary cell populations have found that uNK do not kill trophoblast but do produce a variety of cytokines and chemokines that modulate invasion and homing to the spiral arteries (Hanna *et al*., 2006; Lash *et al*., 2006). Importantly, levels of angiogenic factors and chemokines produced by uNK cells are reduced following KIR2DL1 binding to C2 showing that KIR/HLA-C interactions can regulate factors known to influence reproductive performance. A correct balance of activation and not too much inhibition of these physiological functions of the uNK is therefore an essential part of successful implantation. Overall, our findings underscore the importance of the innate immune system in reproductive success.

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Authors' roles

S.E.H. was involved in the design of the study, genotyped the samples and analysed the results.

L.R. helped write the proposal and provided clinical input.

L.F. extracted DNA and helped with the genotyping and analysis.

W.L. selected the patients, recorded the clinical data and took the blood samples.

M.C. provided intellectual input, revised the paper and provided help with genotyping and statistics.

A.M. designed the study, took part in interpretation of data and wrote the paper.

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