Polymorphisms of endocrine gland-derived vascular endothelial growth factor gene and its receptor genes are associated with recurrent pregnancy loss

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BACKGROUND: Endocrine gland-derived vascular endothelial growth factor (EG-VEGF) and its receptor genes [prokineticin receptor 1 (PKR1) and prokineticin receptor 2 (PKR2)] have been identified in the last decade and their expression is restricted to the steroidogenic glands (ovary, testis, adrenal gland and placenta). Their expression patterns also suggest a close relationship to early pregnancy. However, little information is available regarding the role of EG-VEGF and its receptors (PKR1 and PKR2) in recurrent pregnancy loss (RPL). This study was conducted to investigate the association between polymorphisms of EG-VEGF and its receptor genes (PKR1 and PKR2) and idiopathic RPL.

METHODS: In this case–control study, 115 women with a history of idiopathic RPL and 170 controls were included. A total of 11 tag single nucleotide polymorphisms (SNPs) selected from EG-VEGF, PKR1 and PKR2 were genotyped. We further used multifactor dimensionality reduction (MDR) analysis to choose a best model and evaluate gene–gene interactions.

RESULTS: Two tag SNPs of PKR1 (rs4627609, rs6731838) and one tag SNP of PKR2 (rs6053283) were significantly associated with idiopathic RPL (P<0.05). The frequencies of haplotypes C-G and T-A of PKR1 and haplotype A-G-C-G-G of PKR2 were significantly increased in women with idiopathic RPL (P<0.05); MDR tests revealed gene–gene interactions between three loci [EG-VEGF (rs7513898), PKR1 (rs6731838), PKR2 (rs6053283)] based on the association model (P=0.008). The adjusted odds ratio of high- and low-risk genotype combinations in the three-locus model was 3.94 (95% confidence interval: 2.38–6.52).

CONCLUSIONS: EG-VEGF receptor (PKR1, PKR2) gene polymorphisms and haplotypes were associated with idiopathic RPL. These three genes (EG-VEGF, PKR1 and PKR2) jointly contribute to RPL in the Taiwanese Han population.

Key words: endocrine gland-derived VEGF / prokineticin receptors 1 and 2 / recurrent pregnancy loss / tag SNP / gene–gene interaction

Introduction

Recurrent pregnancy loss (RPL) is the occurrence of repeated pregnancies that end in miscarriage, usually before 20 weeks of gestation. RPL affects ~1–5% of women who conceive (Baek et al., 2007). Various factors that contribute to RPL have been identified, including uterine anomaly, chromosomal abnormalities, endocrine dysfunction, thrombophilia, immune disorders, lifestyle factors and maternal infections (Regan et al., 1989). However, in up to 50% of patients who experience RPL, the underlying causes remain undetermined (Li et al., 2002).

Endocrine gland-derived vascular endothelial growth factor (EG-VEGF), also known as prokineticin 1 (PK1), belongs to the prokineticin family. It induces tissue-specific proliferation, migration and fenestration in capillary endothelial cells, and its expression is mainly restricted to the steroidogenic glands (ovary, testis, adrenal gland and placenta).
(LeCouter et al., 2001). EG-VEGF acts through the G-protein-coupled receptors prokineticin receptor 1 (PKR1) and prokineticin receptor 2 (PKR2), and is likely to be involved in male reproductive tissues (testis and prostate) (LeCouter et al., 2003; Pasquali et al., 2006) and female reproductive tissues (ovary, uterus and various tissues during pregnancy) (Ferrara et al., 2003; Fraser et al., 2005; Hoffmann et al., 2006).

The expression pattern of EG-VEGF, PKR1 and PKR2 has been characterized in human endometrium (Battersby et al., 2004; Evans et al., 2008). The expression levels of endometrial EG-VEGF and PKR1 peak during the secretory phase of the menstruation cycle (Battersby et al., 2004; Evans et al., 2008). Their expression levels are further elevated during the first trimester of pregnancy in humans, reaching a peak at 8–10 weeks of gestation, and decreasing thereafter (Hoffmann et al., 2006; Evans et al., 2008). Their expressions in early gestational tissue are mainly localized to syncytiotrophoblast and cytotrophoblast layers (Hoffmann et al., 2006). In mouse placenta, EG-VEGF and its receptors are expressed throughout gestation, but EG-VEGF and PKR1 expression is most abundant during early gestation (9.5–10.5 days post conception) (Hoffmann et al., 2007). Microarray analysis also suggested that EG-VEGF may be involved in regulating the endometrial expression of multiple cytokines and implantation (Evans et al., 2008; Haouzi et al., 2009).

Considering the potential roles of EG-VEGF in regulating human early pregnancy, we hypothesized that polymorphisms of EG-VEGF and its receptors are implicated in implantation failure. This study was conducted to investigate the association between polymorphisms of EG-VEGF and its receptors and idiopathic RPL. For the first time, we show that the EG-VEGF system confers susceptibility to RPL.

Materials and Methods

Subjects

The present study was approved by the Institutional Review Board of National Cheng Kung University Hospital (Tainan, Taiwan, Republic of China), and informed consent was obtained from all patients and controls in this study. A total of 115 women who had experienced at least two consecutive spontaneous miscarriages (SMs) were recruited from outpatient clinics of our hospital during the period from January 2004 through 20 January 2010. All women had conceived naturally without the aid of assisted reproductive technologies (ARTs). SM included both embryonic and anembryonic losses before 12 weeks of gestational age, which were determined by ultrasound dating and/or combined with the last menstrual period. Biochemical pregnancy was excluded from the study. All subjects had undergone a comprehensive examination as described in our previous publications, including a detailed history, a physical examination, chromosome analysis of peripheral blood lymphocytes and transvaginal three-dimensional ultrasound (to detect uterine anomalies and endometrial defects). They were also checked for 75 g oral glucose tolerance tests, thyroid functions (T3, T4 and thyroid-stimulating hormone), anti-cardiolipin antibodies (IgG, IgM and β2-glycoprotein), lupus anticoagulant, anti-thrombin III, protein S, protein C and endocrinology profiles on Day 3 of the menstrual cycle FSH, LH, prolactin and testosterone (Kuo, 2002; Kuo and Guo, 2004; Kuo et al., 2008). Women with any identifiable cause of RPL (e.g. submucosal myoma, uterine anomalies, balanced chromosomal rearrangements, etc.) were also excluded from the study. We also recruited 170 women from our delivery room as control subjects; they had delivered at least one full-term healthy baby without the aid of ART and had not experienced miscarriage or pregnancy complications (Kuo, 2002; Kuo and Guo, 2004; Kuo et al., 2008).

Genotyping

We explored the tag single nucleotide polymorphisms (SNP) of the EG-VEGF, PKR1 and PKR2 genes on the website (www.hapmap.org). A total of 11 HapMap tag SNPs, for population (Chinese Hans in Beijing), were selected to encompass the entire haplotype block structure of these three genes using the algorithm Tagger-pair-wise Tagging (Filter: minor allele frequency greater than to equal to 0.10, r2 greater than equal to 0.95). Four, two and five tag SNPs were selected for the EG-VEGF, PKR1 and PKR2 genes, respectively (Fig. 1).

Genomic DNA was extracted from lymphocyte using a Puregene DNA isolation kit (Gentra, Minneapolis, MN, USA). The SNPs were detected by primer extension analysis using end-point TaqMan assays (Applied Biosystems, Warrington, UK) in 96-well arrays, and genotypes were subsequently read on a 7900 Sequence Detector (Applied Biosystems). Reactions were carried out using standard conditions supplied by the company.

Statistical analysis

Tests for comparing clinical information between two groups (age, number of successful pregnancies) and association with single markers and haplotypes between RPL patients and normal controls were performed using a χ2 test or Fisher’s exact test. A P-value of <0.05 was considered statistically significant. The relative risk of RPL was estimated from logistic odds ratios (OR) with a 95% confidence interval (CI) in multivariate analysis after maternal age adjustment. The Hardy–Weinberg equilibrium was calculated in accordance with standard procedures, using χ2 analysis. Tests for haplotype association with RPL were performed using SNPAlalyze 7.0. Pro software (DYNACOM Co., Ltd, Yokohama, Japan), and statistically significant P-values were corrected for multiple testing using the permutation test. The linkage equilibrium (LD) coefficient (D’) between each pair of SNPs was analyzed by Haploview 4.1 (Daly Lab at the Broad Institute, Cambridge, USA).

Under the hypothesis that more than one gene polymorphism carrying risk alleles may increase the risk for RPL, gene–gene interactions were explored. We analyzed gene–gene interactions among 11 loci of these 3 genes using the MDR method (MDR software, version 2.0) (Hahn et al., 2003; Ritchie et al., 2003; Pan, 2010). High- and low-risk statuses were determined on the basis of the work of Moore et al. (Ritchie et al., 2001; Hahn et al., 2003). In brief, boxes were labeled as high risk if the ratio of cases to controls met or exceeded the threshold of 1.0 and low risk if the threshold was not exceeded. Logistic regression analysis (SAS software, version 9.2, SAS Inc., Cary, NC, USA) and χ2 tests were performed to confirm the results from MDR analyses. A P-value of less than 0.05 was considered statistically significant.

Results

All the patients and the controls were of Taiwanese Han ethnicity and their ages at enrollment were 30.95 ± 5.04 (mean ± SD) and 29.76 ± 4.75 years, respectively. The time interval between enrollment and previous pregnancy loss was <6 months. The number of previous pregnancy losses was 2.61 ± 0.99 (mean ± SD) in the study group. The number of women who experienced 2 and more than 2 consecutive pregnancy losses was 63 and 52, respectively.
We selected four tag SNPs for the EG-VEGF, two for the PKR1 and five for the PKR2 gene for genotyping. These SNPs and the LD block of the three genes are shown in Fig. 1. All three genes were highly linked in inheritance (LD coefficient more than 0.9). The genotypic and allelic frequencies of EG-VEGF, PKR1 and PKR2 in the women with RPL and the control subjects are shown in Tables I and II. The genotypic frequencies of two tag SNPs of PKR1 (rs4627609, rs6731838) and one tag SNP of PKR2 (rs6053283) showed significant differences between the patients and controls ($P < 0.05$). The risk alleles (both A alleles) of rs6731838 (PKR1) and rs6053283 (PKR2) were significantly associated with RPL ($P < 0.05$).

In the haplotype analysis (Table III), we selected three and four polymorphisms of the EG-VEGF gene, two polymorphisms of the PKR1 gene and five polymorphisms of the PKR2 gene after setting the threshold of the LD coefficient ($D' > 0.95$). Although the haplotypic frequency of EG-VEGF did not show a significant difference between the patients and controls ($P < 0.05$). The risk alleles (both A alleles) of rs6731838 (PKR1) and rs6053283 (PKR2) were significantly associated with RPL ($P < 0.05$).

Gene–gene interactions among 11 loci of these three genes were analyzed using the MDR method. A three-locus model, which included the polymorphisms of EG-VEGF (rs7513898), PKR1 (rs6731838) and PKR2 (rs6053283), was regarded as the best fit model with an accuracy of 63.08% ($P = 0.008$) and a maximum cross-validation consistency of 9 out of 10 (Table IV). These results are consistent with those obtained from logistic regression analyses. In this model, the age-adjusted OR for the high-risk to the low-risk groups was 3.94 (95% CI 2.38–6.52; $P < 0.0001$). Further, based on the pattern of high-and low-risk genotypes (Fig. 2), this three-locus model showed evidence of gene–gene interaction.

### Discussion

In the present study, we investigated the association between the EG-VEGF system and the occurrence of idiopathic RPL by using tag SNPs. For the first time, we demonstrated that polymorphisms and haplotypes of PKR1 and PKR2 are significantly associated with idiopathic RPL. In addition, MDR analysis showed significant genetic interactions between three loci (EG-VEGF, PKR1 and PKR2) and these three loci jointly confer susceptibility to RPL.

Prokineticin-1 (EG-VEGF) mediates tissue-specific angiogenesis (LeCouter et al., 2001), immune regulation (Dorsch et al., 2005), and modulation of inflammatory responses (Denison et al., 2008). All these processes play important roles in endometrial receptivity, embryo implantation and placentation. EG-VEGF and its receptors (PKR1, PKR2) are expressed in human placenta of the first and third trimester period, and are localized to syncytiotrophoblast, cytotrophoblast, fetal endothelium and macrophages (Hofbauer cells) (Hoffmann et al., 2006; Denison et al., 2008). The expression pattern of the EG-VEGF system also suggests the important roles it plays in human early pregnancy, when vascularization of the chorionic villi and embryonic development is essential for successful pregnancy. However, the mechanism by which the EG-VEGF system regulates human early pregnancy is largely unknown.

Angiogenesis is critical in the regulation of placental vessel formation and maternal vascular adaptation. The peak expression of EG-VEGF and PKR1 is correlated with the hypoxic period during placental development, and hypoxia-inducible factor Iα (HIF-1α) has been proposed to be involved in regulating EG-VEGF, considering the presence of the hypoxia-response element in the promoter region of EG-VEGF (LeCouter et al., 2001). In addition to HIF-1α, the dynamic expression...
of EG-VEGF in pregnancy may also be regulated by estrogen, progesterone and human chorionic gonadotrophin (Battersby et al., 2004; Evans et al., 2009; Shaw et al., 2010), which are all dramatically up-regulated during early pregnancy and may also regulate the process of angiogenesis. Polymorphisms of the VEGF gene have been shown to be associated with RPL (Galazios et al., 1995; Lim et al., 1997; Robb et al., 2001). Both are expressed in human placenta during the first trimester of pregnancy, whereas they have a distinct spatio-temporal pattern of expression in the mammalian placenta (Hoffmann et al., 2006, 2007). VEGF expression was restricted to the cytotrophoblasts and extravillous trophoblasts, while EG-VEGF was mainly localized to the syncytiotrophoblast layer and cytotrophoblasts at advanced gestation (Hoffmann et al., 2006). Given their distinctive expression patterns, EG-VEGF and VEGF may play complementary roles during the first trimester of human pregnancy.

EG-VEGF has been shown to be up-regulated during the implantation window (Haouzi et al., 2009). In the cell model, EG-VEGF was shown to stimulate a group of genes, including IL-1β, IL-6, IL-8, LIF and COX-2 (Evans et al., 2008). EG-VEGF also facilitates embryo implantation through induction of LIF expression (Denison et al., 2008). LIF, COX-2, IL-6 and IL-11 have all been shown to play important roles in embryo implantation and decidualization (Stewart et al., 1992; Tabibzadeh et al., 1995; Lim et al., 1997; Robb et al., 1998). Recently, EG-VEGF was shown to be a uterine receptivity marker (Haouzi et al., 2009). All of these data unanioumly indicate the important roles of the EG-VEGF system in the establishment of early pregnancy. EG-VEGF and PKR1 may induce their target genes by phosphorylation of their downstream target extracellular signal-regulated kinase 1/2 through cross-talk with the epidermal growth factor receptor system (Evans et al., 2008), or by activation of mitogen-activated protein kinase and the PI3 kinase/Akt pathway (Lin et al., 2002). Given the complex signaling pathways of the EG-VEGF system, it would be worthwhile to study how specific PKR1/PKR2 haplotypes affect signaling pathways in early pregnancy.

The EG-VEGF system consists of three genes: EG-VEGF, PKR1 and PKR2. Mutations of the PKR2 gene have been reported in hypogonadotropic hypogonadism. Both male and female patients with Kallmann syndrome carrying biallelic PKR2 mutations had a severe reproductive disorder, whereas the reproductive phenotype of mono-allelic mutation of PKR2 was more variable (Monnier et al., 2009; Sarfati et al., 2010). Besides the ligand, the polymorphisms of the receptor (PKR2) gene have also been shown to be associated with mood disorder in the Japanese population (Kishi et al., 2009). Other pathological conditions which have been linked to the EG-VEGF signaling system include polycystic ovarian syndrome (PCOS) (LeCouter et al., 2001; Ferrara et al., 2003), ectopic pregnancy (Shaw et al., 2010), endometriosis (Tiberi et al., 2010) and pre-eclampsia (Hoffmann et al., 2006, 2007; Maldonado-Pérez et al., 2007). In patients with endometriosis and PCOS, the expression of EG-VEGF was higher in ectopic endometrial and polycystic ovarian tissue, suggesting the role of EG-VEGF

### Table I Allele and genotype frequencies of EG-VEGF polymorphisms.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Allele/genotype</th>
<th>Case (n = 115)</th>
<th>Control (n = 170)</th>
<th>P-value</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs3795828</td>
<td>G</td>
<td>157 (68.3%)</td>
<td>242 (71.2%)</td>
<td>0.46</td>
<td>1.148 (0.798–1.652)</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>73 (31.7%)</td>
<td>98 (28.8%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>53 (46.1%)</td>
<td>89 (52.4%)</td>
<td>0.30</td>
<td>1.285 (0.800–2.066)</td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>51 (44.3%)</td>
<td>64 (37.6%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>11 (9.6%)</td>
<td>17 (10.0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs12409155</td>
<td>C</td>
<td>165 (71.7%)</td>
<td>251 (73.8%)</td>
<td>0.58</td>
<td>0.900 (0.618–1.310)</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>65 (28.3%)</td>
<td>89 (26.2%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>60 (52.2%)</td>
<td>88 (51.8%)</td>
<td>0.95</td>
<td>0.984 (0.613–1.580)</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>45 (39.1%)</td>
<td>75 (44.1%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>10 (8.7%)</td>
<td>7 (4.1%)</td>
<td></td>
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</tr>
<tr>
<td>rs7513898</td>
<td>A</td>
<td>137 (59.6%)</td>
<td>185 (54.4%)</td>
<td>0.22</td>
<td>1.234 (0.879–1.732)</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>93 (40.4%)</td>
<td>155 (45.6%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>42 (36.5%)</td>
<td>48 (28.2%)</td>
<td>0.14</td>
<td>0.684 (0.413–1.134)</td>
</tr>
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<td></td>
<td>AG</td>
<td>53 (46.1%)</td>
<td>89 (52.4%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>20 (17.4%)</td>
<td>33 (19.4%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs7514102</td>
<td>A</td>
<td>138 (60.0%)</td>
<td>190 (55.9%)</td>
<td>0.33</td>
<td>1.184 (0.843–1.663)</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>92 (40.0%)</td>
<td>150 (44.1%)</td>
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<tr>
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<td>AA</td>
<td>42 (36.5%)</td>
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<td>0.35</td>
<td>0.787 (0.478–1.297)</td>
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<td></td>
<td>AG</td>
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<td>84 (49.4%)</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>GG</td>
<td>19 (16.5%)</td>
<td>33 (19.4%)</td>
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</tbody>
</table>
in the angiogenesis of these two pathologic conditions (Ferrara et al., 2003; Shaw et al., 2010). On the other hand, the deficient adaptation of the materno-placental unit in the first trimester and the relatively hypoxic placenta in later gestation all suggested that EG-VEGF and PKR1 might have a role in the development of pre-eclampsia (Hoffmann et al., 2006, 2007; Maldonado-Pérez et al., 2007).

In this study, a tag SNP was used for the association study. By using tag SNPs which represent a region of the genome, the entire LD block could be investigated far more efficiently. We found that genetic variants of PKR1 and PKR2 confer susceptibility to RPL. We went further to explore gene–gene interactions and to test if a subject carrying more than one risk allele would be at increased risk of experiencing RPL. Traditionally, a simple genetic model (dominant, recessive or co-dominant model) is used to test the association between genetic variants and common diseases. However, most common diseases could not be explained by a simple genetic model (Ritchie et al., 2001; Cho et al., 2004; Williams et al., 2004). MDR provides a model-free, non-parametric data reduction method for detecting multilocus

### Table II: Allele and genotype frequencies of PKR1 and PKR2 polymorphisms.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Allele/genotype</th>
<th>Case (n = 115)</th>
<th>Control (n = 170)</th>
<th>P-value</th>
<th>OR (95% CI)</th>
</tr>
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<td>PKR1</td>
<td></td>
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</tr>
<tr>
<td>rs4627609</td>
<td>T</td>
<td>154 (67.0%)</td>
<td>201 (59.1%)</td>
<td>0.06</td>
<td>0.714 (0.503–1.012)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>76 (33.0%)</td>
<td>139 (40.9%)</td>
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<tr>
<td></td>
<td>TT</td>
<td>45 (39.1%)</td>
<td>54 (31.8%)</td>
<td>0.023</td>
<td>0.352 (0.139–0.894)</td>
</tr>
<tr>
<td></td>
<td>TC</td>
<td>64 (55.7%)</td>
<td>93 (54.7%)</td>
<td></td>
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<tr>
<td></td>
<td>CC</td>
<td>6 (5.2%)</td>
<td>23 (13.5%)</td>
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<tr>
<td>rs6731838</td>
<td>G</td>
<td>136 (59.1%)</td>
<td>233 (68.5%)</td>
<td>0.02</td>
<td>1.505 (1.062–2.133)</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>94 (40.9%)</td>
<td>107 (31.5%)</td>
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<tr>
<td></td>
<td>GG</td>
<td>34 (29.6%)</td>
<td>77 (45.3%)</td>
<td>0.008</td>
<td>1.973 (1.194–3.257)</td>
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<td></td>
<td>GA</td>
<td>68 (59.1%)</td>
<td>79 (46.5%)</td>
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<td>AA</td>
<td>13 (11.3%)</td>
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<td>PKR2</td>
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<td>rs3746684</td>
<td>A</td>
<td>122 (53.0%)</td>
<td>196 (57.6%)</td>
<td>0.28</td>
<td>0.830 (0.593–1.162)</td>
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<tr>
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<td>G</td>
<td>108 (47.0%)</td>
<td>144 (42.4%)</td>
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<td>AA</td>
<td>37 (32.2%)</td>
<td>57 (33.5%)</td>
<td>0.81</td>
<td>1.063 (0.642–1.761)</td>
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<td>48 (41.7%)</td>
<td>82 (48.2%)</td>
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<td>GG</td>
<td>30 (26.1%)</td>
<td>31 (18.2%)</td>
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<tr>
<td>rs6053283</td>
<td>G</td>
<td>192 (83.3%)</td>
<td>302 (88.8%)</td>
<td>0.07</td>
<td>1.573 (0.969–2.554)</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>38 (16.5%)</td>
<td>38 (11.2%)</td>
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<td></td>
<td>GG</td>
<td>81 (70.4%)</td>
<td>137 (80.6%)</td>
<td>0.047</td>
<td>1.743 (1.003–3.027)</td>
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<td></td>
<td>GA</td>
<td>30 (26.1%)</td>
<td>28 (16.5%)</td>
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<tr>
<td></td>
<td>AA</td>
<td>4 (3.5%)</td>
<td>5 (2.9%)</td>
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<tr>
<td>rs6053286</td>
<td>C</td>
<td>168 (73.0%)</td>
<td>246 (72.4%)</td>
<td>0.86</td>
<td>1.035 (0.711–1.508)</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>62 (27.0%)</td>
<td>94 (27.6%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>62 (53.9%)</td>
<td>89 (52.4%)</td>
<td>0.80</td>
<td>0.939 (0.585–1.509)</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>44 (38.3%)</td>
<td>68 (40.0%)</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>TT</td>
<td>9 (7.8%)</td>
<td>13 (7.6%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs6085088</td>
<td>G</td>
<td>130 (56.5%)</td>
<td>207 (60.9%)</td>
<td>0.30</td>
<td>1.197 (0.852–1.682)</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>100 (43.5%)</td>
<td>133 (39.1%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>39 (33.9%)</td>
<td>65 (38.2%)</td>
<td>0.46</td>
<td>1.206 (0.736–1.978)</td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>52 (45.2%)</td>
<td>77 (45.3%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>24 (20.9%)</td>
<td>28 (16.5%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs8116536</td>
<td>G</td>
<td>127 (55.2%)</td>
<td>198 (58.2%)</td>
<td>0.48</td>
<td>1.131 (0.807–1.585)</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>103 (44.8%)</td>
<td>142 (41.8%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>41 (35.7%)</td>
<td>63 (37.1%)</td>
<td>0.81</td>
<td>1.063 (0.649–1.739)</td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>45 (39.1%)</td>
<td>72 (42.4%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>29 (25.2%)</td>
<td>35 (20.6%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Bold indicates statistical significance.
genotype combinations that predict disease risk for common, complex diseases. MDR was also demonstrated to have good power to identify high-order gene–gene interactions (Ritchie et al., 2003). It has been proven to be maximally efficient at discriminating between clinical end-points using multilocus genotype data (Hahn and Moore, 2004). In this study, MDR was applied to explore RPL susceptibility in a Taiwanese Han population. We found women with specific genotype combinations are at increased risk of experiencing RPL. Although polymorphisms of EG-VEGF per se did not show significant differences between patients and control subjects, the MDR test showed it interacts with PKR1 and PKR2, suggesting EG-VEGF as a modifier in determining the pregnancy outcome.

Although the literature already provides the functional relevance of the EG-VEGF system in the female reproductive function, its role in SM remains to be explored. In this study, we provided the first evidence to show interactions between three loci (EG-VEGF, PKR1 and PKR2). Moreover, these three loci jointly confer susceptibility to RPL in the Taiwanese Han population. The weakness of our study includes a limited sample size and lack of functional validation of the genetic variants. The relatively small sample size attenuated the power of

<table>
<thead>
<tr>
<th>Table III</th>
<th>Haplotype analysis of EG-VEGF and PKR1, PKR2.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNP marker</td>
<td>Haplotype</td>
</tr>
<tr>
<td>EG-VEGF</td>
<td></td>
</tr>
<tr>
<td>rs3795828</td>
<td>G-C-G-G</td>
</tr>
<tr>
<td>rs12409155</td>
<td>A-C-A-A</td>
</tr>
<tr>
<td>rs7513898</td>
<td>G-T-A-A</td>
</tr>
<tr>
<td>rs7514102</td>
<td>G-C-G-A</td>
</tr>
<tr>
<td>PKR1</td>
<td>C-G</td>
</tr>
<tr>
<td>rs6731838</td>
<td>T-A</td>
</tr>
<tr>
<td>PKR2</td>
<td></td>
</tr>
<tr>
<td>rs3746684</td>
<td>A-G-C-G-A</td>
</tr>
<tr>
<td>rs6053283</td>
<td>G-G-T-A-G</td>
</tr>
<tr>
<td>rs6053286</td>
<td>G-A-C-A-G</td>
</tr>
<tr>
<td>rs6085088</td>
<td>A-G-C-G-G</td>
</tr>
<tr>
<td>rs8116536</td>
<td>G-G-C-G-G</td>
</tr>
</tbody>
</table>

Bold indicates statistical significance.

\(^a\)Global permutation P-value.

\(^b\)Permutation P-value.

<table>
<thead>
<tr>
<th>Table IV</th>
<th>Multi-locus interaction model for RPL by means of MDR analysis.</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of loci</td>
<td>Best model</td>
</tr>
<tr>
<td>1</td>
<td>PKR1 (rs6731838)</td>
</tr>
<tr>
<td>2</td>
<td>PKR1 (rs6731838), PKR2 (rs6053283)</td>
</tr>
<tr>
<td>3</td>
<td>EG-VEGF (rs7513898), PKR1 (rs6731838), PKR2 (rs6053283)</td>
</tr>
<tr>
<td>4</td>
<td>EG-VEGF (rs7513898), PKR1 (rs6731838), PKR2 (rs6053283), PKR2 (rs8116536)</td>
</tr>
</tbody>
</table>

Bold indicates statistical significance.

\(^a\)Permutation P-value.
the statistical significance. Among three tag-SNPs [two for PKR1 (rs4627609, rs6731838) and one for PKR2 (rs6053283)], the power ranged from 62 to 75% with borderline P-values (between 0.008 and 0.047). MDR was introduced to improve the power in this regard. The tag-SNPs identified in this study may not be the ones with functional significance. It could be the nearby polymorphisms that really affect gene functions and interfere with implantation or placentation. Further studies are required to replicate our findings in different ethnic groups with a large sample size. It is also necessary to validate the function of different haplotypes for the individual gene. Considering that the OR of RPL for high-risk genotypes is around 3.94, other factors must be implicated in the pathogenesis of RPL.

Authors’ roles

M.-T.S. contributed toward the design and execution of the study and the drafting of the manuscript; S.-H.L. and Y.-C.C. contributed toward statistical analysis; M.-T.S., I.-W.L., C.-C.H., H.-A.P. and P.-L.K. were involved with patient collection and P.-L.K. was involved with the critical discussion and the correspondence.

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References


