Prokineticin receptor variants (PROKR1-I379V and PROKR2-V331M) are protective genotypes in human early pregnancy

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Abstract

Endocrine gland-derived vascular endothelial growth factor (EG-VEGF) and its receptor genes (PROKR1 (PKR1) and PROKR2 (PKR2)) play an important role in human early pregnancy. We have previously shown that PROKR1 and PROKR2 are associated with recurrent miscarriage (RM) using the tag-SNP method. In this study, we aimed to identify PROKR1 and PROKR2 variants in idiopathic RM patients by genotyping of the entire coding regions. Peripheral blood DNA samples of 100 RM women and 100 controls were subjected to sequence the entire exons of PROKR1 and PROKR2. Significant non-synonymous variant genotypes present in the original 200 samples were further confirmed in the extended samples of 144 RM patients and 153 controls. Genetic variants that were over- or under-represented in the patients were ectopically expressed in HEK293 and JAR cells to investigate their effects on intracellular calcium influx, cell proliferation, cell invasion, cell–cell adhesion, and tube organization. We found that the allele and genotype frequencies of PROKR1 (I379V) and PROKR2 (V331M) were significantly increased in the normal control groups compared with idiopathic RM women (P<0.05). PROKR1 (I379V) and PROKR2 (V331M) decreased intracellular calcium influx but increased cell invasiveness (P<0.05), whereas cell proliferation, cell–cell adhesion, and tube organization were not significantly affected. In conclusion, PROKR1 (I379V) and PROKR2 (V331M) variants conferred lower risk for RM and may play protective roles in early pregnancy by altering calcium signaling and facilitating cell invasiveness.

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Introduction

Recurrent miscarriage (RM) is the occurrence of repeated abortion in early gestation, affecting about 1–5% of women who conceive (Baek et al. 2007). RM is a challenging clinical condition notable for a multifactorial etiology, and that up to 50% of cases remain undetermined even after detailed clinical examination (Li et al. 2002). In patients with idiopathic RM, gene polymorphisms have been proposed as susceptibility factors that increase the risk of miscarriage when compared with otherwise healthy women (Su et al. 2011a, 2011b).

Endocrine gland-derived vascular endothelial growth factor (EG-VEGF), also known as prokineticin 1 (PK1), was described to have a wide range of functions including tissue-specific angiogenesis, modulation of inflammatory responses, and regulation of hematopoiesis (LeCouter et al. 2001, Ngan & Tam 2008). Its expression is mainly restricted to the steroidogenic glands (ovary, testis, adrenal gland, and placenta) and is closely involved in angiogenesis and physiological functions of male reproductive (testis and prostate) (LeCouter et al. 2003, Pasquali et al. 2006) and female reproductive systems (ovary, uterus, and placenta) (Ferrara et al. 2003, Fraser et al. 2005, Hoffmann et al. 2006). Endometrial EG-VEGF and PROKR1 (PKR1) expressions are the highest during luteal phase of menstruation and further elevated after pregnancy reaching a peak at 8–10 weeks of gestation and decreased thereafter (Hoffmann et al. 2006, 2009, Evans et al. 2008, 2009). Their expression in early gestational tissues is mainly localized in syncytiotrophoblast and cytotrophoblast layers (Hoffmann et al. 2006). Recently, EG-VEGF has been regarded as a uterine receptivity marker and was shown to regulate a group of genes that was critical for embryo implantation and pregnancy maintenance (Denison et al. 2008, Evans et al. 2008, Haozui et al. 2009, Gorowiec et al. 2011).

EG-VEGF acts through activation of two G-protein-coupled receptors (GPCRs), prokineticin receptor 1 (PROKR1) and prokineticin receptor 2 (PROKR2...
PROKR1 and PROKR2 are encoded on different human chromosome regions 2p13.1 and 20p12.3 respectively. They share an 85% amino acid identity and diverge mainly in their N-terminal sequences (Masuda et al. 2002, Soga et al. 2002). The structures of GPCRs comprised an extracellular N-terminal domain, 7-transmembrane domains, and an intracellular C-terminal domain. They can sense molecules outside the cell and activate intracellular signal transduction pathways through modification of the transmembrane structure and coupling to different G proteins. PROKR1 and PROKR2 are differentially and temporally expressed in different organs of female reproductive tract, including the ovaries, endometrium, and placenta (Battersby et al. 2004, Evans et al. 2008). Although they were believed to have important and different effects on human early pregnancy, their exact roles in reproduction remain to be characterized.

In our previous study, we had found that gene polymorphisms/haplotypes of PROKR1 and PROKR2 were significantly associated with RM using tag-SNP method (Su et al. 2010). However, the loci that are involved in embryo implantation/survival are still unknown. The aims of this study were set to identify functional polymorphisms of PROKR1 and PROKR2 in RM patients by genotyping of the entire coding regions. We found that PROKR1 (I379V) and PROKR2 (V331M) were associated with human pregnancy and may have protective roles in early pregnancy by altering calcium signaling and facilitating cell invasiveness. The current study provides potential clinical application and explanations for the roles of prokineticin receptors in human early pregnancy.

Materials and methods

Subjects
This study was approved by the Institutional Review Board of National Cheng Kung University Hospital (Tainan, Taiwan, Republic of China), and informed consents were obtained from all patients and controls in this study. A total of 100 women who had experienced at least two consecutive spontaneous abortions (SA) were recruited from outpatient clinics of our hospital. All women had conceived naturally without the aid of ART. SA includes both embryonic and anembryonic losses before 12 weeks of gestational age, which was 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 (Kuo & Guo 2004, Kuo et al. 2008, Su et al. 2010). Women with any identifiable cause of RM were excluded from the study. We also recruited 100 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. In order to confirm the significance of the protective genotypes found in the original 200 subjects (100 RM patients and 100 controls), we further extended sample sizes to 144 RM patients and 153 controls using the same criteria.

Detection of PROKR1 and PROKR2 variants
Genomic DNA was extracted from peripheral blood leukocyte using a Puregene DNA isolation kit (Genta, Minneapolis, MN, USA). The PROKR1 and PROKR2 genes are located on 2p13.1 (GenBank accession no. NM_138964.2) and 20p12.3 (NM_144773.2) respectively, and both of them consist of two exons. Each DNA sample was amplified using a Thermocycler (Px2 thermal cycler, Thermo Hybaid, Ashford, UK) and the primers were shown in Supplementary Table 1, see section on supplementary data given at the end of this article. DNA sequences were determined with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems). All sequence variants were found on both strands and confirmed in a separate PCR. The nucleotide changes were assessed for their presence in the National Center for Biotechnology Information database of single-nucleotide polymorphisms and the 1000 Genome Project database, and among control alleles.

Generation of variant PROKR1- and PROKR2-expressing plasmids and transfection experiments
Variant sequence of PROKR1 (I379V) and PROKR2 (V331M) was introduced into the WT Myc-DDK-tagged cDNA in a pCMV6-Entry vector (Origene Technologies, Inc., Rockville, MD, USA), which encodes the entire coding regions of human cDNA (GenBank NM_138964.2 and NM_144773.2) of PROKR1 and PROKR2, using Stratagene’s Quick Change II Site-directed Mutagenesis Kit (La Jolla, CA, USA). All constructs were verified by nucleotide sequencing. PROKR1 and PROKR2 constructs were propagated in JM109 Escherichia coli cells. The established constructs were transfected into HEK293 and JAR cell lines by Lipofectamine 2000 (Invitrogen), and their expressions were confirmed by western blotting. We further verified expression of PROKR1 and PROKR2 protein on the cell membrane by ProteoJET membrane protein extraction kit (Fermentas AB, Stockholm, Sweden; Fermentas St Leon-Rot, Germany) and flow cytometry using rabbit polyclone anti-human PKR1 and PKR2 antibodies (Abcam, Cambridge, UK) (data not shown).

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**Intracellular calcium influx assay**

Twenty-four hours after transfection, cells were harvested from plates using EDTA-trypsin and washed with HBSS-based buffer (20 mM HEPES, 1 mM MgSO4, 3.3 mM Na2CO3, 1.3 mM CaCl2, and 2.5 mM probenecid, pH 7.4) supplemented with 0.1% BSA. Cells were loaded with 4 μM calcium indicator Fluo-4 AM (Molecular Probes, Eugene, OR, USA) for 1 h at 37 °C. After washing twice, cells were resuspended to a concentration of 1×10⁶ cells/ml. The green fluorescence emission of Fluo-4 was analyzed using FACSCalibur flow cytometry (BD Immunocytometry System, Franklin Lakes, NJ, USA) as described previously (June & Moore 2004, Desmeules et al. 2009). Following the establishment of a green fluorescence Ca2⁺ baseline, the indicated concentration of EG-VEGF (Peprotech, London, UK) (0.05, 0.5, 5, 50, or 500 nM) was added to the cell suspension for the detection of green fluorescence fluctuation. The experiment was performed at least three times in triplicate.

**Cell proliferation assay**

The effects of PROKR1 and PROKR2 variants on cell proliferation were assessed when compared with those from their WTs. Cell lines were grown at 10,000 cells/well in a 24-well plate in DMEM medium containing 10% FBS for 24 h. Before the assay, the cells were starved in DMEM medium containing 4% FBS for 24 h and then treated with 0 or 5 nM of EG-VEGF for 24–48 h. For HEK293 cells, the total cell number was determined with a hemocytometer after trypsinization. For JAR cells, cell numbers were determined by WST assay. After adding 10 μl of cell proliferation reagent WST-1 (Roche) in each well for 30 min at 37 °C in a CO2 incubator, the absorbance at 450 nm, reflecting the activity of calcium influx in the cell suspension for the detection of green fluorescence fluctuation. The experiment was performed at least three times.

**Cell–cell adhesion assay**

In order to evaluate whether PKR variants affect cell–cell adhesion process of embryo implantation, we explored it between RL95-2- and PKR-transfected JAR cells. RL95-2 (1×10⁵ cells) were seeded in 96-well plates and allowed to reach confluence. PKR-transfected JAR cells were labeled with Calcein AM (Vybrant Cell Adhesion Assay Kit (V-13181), Molecular Probes) at 37 °C for 30 min in serum-free RPMI 1640 medium, then washed with PBS to remove free dye, and resuspended in the RPMI 1640 medium containing 2% FBS. Labeled JAR cells (2×10⁵ cells) were added to each RL95-2-containing well in the absence or presence of EG-VEGF treatment (0 or 5 nM) and incubated for 1 h. Non-adherent cells were removed by two gentle washes with PBS. The degree of JAR cell adhesion to RL95-2 was measured by a microplate fluorometer (Fluoroskan Ascent, Thermo) at an emission of 517 nm and absorption of 494 nm after the cells were lysed with DMSO. The experiment was performed at least three times in triplicate.

**Cell invasion assay**

To investigate the effects of PROKR1 and PROKR2 variants on cell invasion, the transfected HEK293 or JAR cells (10⁶ cells/well) were trypsinized and resuspended in serum-free medium and placed in the upper chamber coated with Matrigel (1 μg/μl at 37 °C for 1 h; BD Biosciences, San Diego, CA, USA) in trans-well plates (millicell cell culture insert; 8 μm pore size; Millipore, Billerica, MA, USA) with or without EG-VEGF (5 nM) as described (Singh et al. 2011). DMEM containing 10% FBS was placed in the lower chamber. The cells were incubated for 24 h at humidified atmosphere with 95% air and 5% CO2 at 37 °C. Invaded cells on the bottom side of the membrane were fixed with 100% cold methanol and stained with Giemsa’s azur eosin methylene blue solution (Merck) and counted under a light microscope at ×200. The data were expressed as the averages of three independent experiments.

**Tube formation assay**

The effects of PROKR1 and PROKR2 variants on tube organization were assessed by growing transfected cells on Matrigel. Approximately 150 μl ice-cold Matrigel (BD Biosciences) was layered into each well of 24-well plates. The Matrigel were allowed to completely solidify at 37 °C for 1 h. Cells (10⁵ cells/well) were incubated at 37 °C in an atmosphere of humidified 95% air/5% CO2. Invaded cells for 24 h. EG-VEGF treatment was applied at the time of seeding. Hourly observations were made under an inverted photomicroscope to document the developmental stages. Tubal formation was assessed by measuring tubal length in four quadrants of high-power filed in each well (Brouillet et al. 2010). Each assay was done in triplicate and each experiment was repeated at least three times.

**Statistical analysis**

Tests for comparing allele and genotype frequencies between RM 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. All values of the experimental assays were expressed as mean ± s.e.m. Differences between the groups were compared using the unpaired two-tailed t-test and a P value of <0.05 was considered statistically significant. The activity of calcium influx in
the variants at each dose was expressed as a percentage of the maximum WT response, and comparisons to WT activity were made using a one-way ANOVA analysis with the Dunnett’s multiple comparison test.

**Results**

**Variations of PROKR1 and PROKR2 in RM women and normal controls**

All the patients and controls were of Taiwanese Han ethnicity and their ages at enrollment were 30.9 ± 4.5 (mean ± s.d.) and 29.9 ± 4.8 years respectively. The time interval between enrollment and previous miscarriage was < 6 months. The number of previous miscarriages was 2.6 ± 0.9 (mean ± s.d.) in the study group. The number of women who experienced two and more consecutive miscarriages was 55 and 45 respectively.

We identified seven variants (S40G, G77S, and G93L in exon 1; P169P, A194S, F314F, and I379V in exon 2) in the PROKR1 coding region and ten variants (A51T, F50F, L127R, V131I, and Y140C in exon 1; and L155L, T195T, V331M, N370N, and T374T in exon 2) in the PROKR2 coding region of these 100 RM women and 100 normal controls (Tables 1 and 2). Among these variants, allele and genotype frequencies of PROKR1 (F314F and I379V) and PROKR2 (V331M, N370N, and T374T) were significantly higher in the control group than in the RM group (P < 0.05). Of note, three nearby variants of PROKR2 (V331M, N370N, and T374T) found in the same four normal controls were in complete linkage and formed a specific haplotype (A-T-A) that protected women from the risk of RM. These data suggest that PROKR1 (I379V) and PROKR2 (V331M) had a less risk to develop RM and may play protective roles in human early pregnancy.

Among those genotype frequencies that were significantly different between case and control groups (PROKR1 (F314F and I379V) and PROKR2 (V331M, N370N, and T374T)), only PROKR1 (I379V) and PROKR2 (V331M) were non-synonymous variants. Although PROKR1 (I379V) and PROKR2 (V331M) variants had achieved statistical significance in 200 samples (P = 0.032 and 0.043 respectively), we further increased sample sizes to test the two variants in order to increase statistic power. Both PROKR1 (I379V) and PROKR2 (V331M) were more significantly different after extending sample sizes to 144 and 153 from RM patients and controls respectively (P < 0.001 and P = 0.002 respectively; Supplementary Table 2, see section on supplementary data given at the end of this article). Considering that PROKR1 (I379V) and PROKR2 (V331M) led to amino acid changes and their locations in the intracellular C-terminal domain of the G protein-coupled receptors (Fig. 1), we expected that these alterations might change downstream signaling pathways. Therefore, in the following experiments, we focused on these two non-synonymous PKR variants for further functional studies.

**PROKR1 (I379V) and PROKR2 (V331M) decreased intracellular calcium influx**

Calcium signaling was shown to be a critical function index of GPCRs in several pathological conditions.

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**Table 1** Polymorphisms of PROKR1 gene in RPL patients and normal controls.

<table>
<thead>
<tr>
<th>Chromosome location, gene polymorphism, amino acid position, and change</th>
<th>Allele frequency</th>
<th>Genotype frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Allele</td>
<td>Case (n=200)</td>
</tr>
<tr>
<td>68873071-exon 1, rs7570797 (A→G), S40G</td>
<td>G</td>
<td>6 (3%)</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>194 (97%)</td>
</tr>
<tr>
<td>68873182-exon 1, G→A, G77S</td>
<td>A</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>200 (100%)</td>
</tr>
<tr>
<td>68873232-exon 1, G→A, L93L</td>
<td>A</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>200 (100%)</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>0</td>
</tr>
<tr>
<td>68882033-exon 2, G→A, P169P</td>
<td>A</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>200 (100%)</td>
</tr>
<tr>
<td>68882106-exon 2, G→T, A194S</td>
<td>T</td>
<td>1 (0.5%)</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>199 (99.5%)</td>
</tr>
<tr>
<td>68882468-exon 2, rs6723313 (T→C), F314F</td>
<td>T</td>
<td>138 (69%)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>62 (31%)</td>
</tr>
<tr>
<td>68882661-exon 2, rs34715748 (A→G), I379V</td>
<td>A</td>
<td>190 (95%)</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>10 (5%)</td>
</tr>
</tbody>
</table>

Significant P values are shown in bold.
Table 2 Polymorphisms of PROKR2 gene in RPL patients and normal controls.

<table>
<thead>
<tr>
<th>Chromosome location, gene polymorphism, amino acid position, and change</th>
<th>Allele frequency</th>
<th>Genotype frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>5294866-exon 1, C→T</td>
<td>C 199 (99.5%)</td>
<td>CC 99 (99%)</td>
</tr>
<tr>
<td></td>
<td>T 1 (0.5%)</td>
<td>CT 1 (1%)</td>
</tr>
<tr>
<td>5294865-exon 1, G→A</td>
<td>G 196 (98%)</td>
<td>GG 96 (96%)</td>
</tr>
<tr>
<td></td>
<td>A 4 (2%)</td>
<td>GA 4 (4%)</td>
</tr>
<tr>
<td>5294635-exon 1, C→G</td>
<td>C 199 (99.5%)</td>
<td>CC 99 (99%)</td>
</tr>
<tr>
<td></td>
<td>G 1 (0.5%)</td>
<td>CG 1 (1%)</td>
</tr>
<tr>
<td>5294866-exon 1, G→A</td>
<td>G 200 (100%)</td>
<td>GG 100 (100%)</td>
</tr>
<tr>
<td></td>
<td>A 0</td>
<td>GA 0</td>
</tr>
<tr>
<td>5294635-exon 1, C→G</td>
<td>C 108 (54%)</td>
<td>CC 54 (54%)</td>
</tr>
<tr>
<td></td>
<td>G 198 (99.5%)</td>
<td>CG 198 (99.5%)</td>
</tr>
</tbody>
</table>

Significant P values are shown in bold.

(Cole et al. 2008, McCoy et al. 2012). Given the important roles of PROKR1 and PROKR2 in response to EG-VEGF during early pregnancy, we investigated the impacts of two PKR variants (PROKR1 I379V and PROKR2 V331M) on EG-VEGF-induced intracellular calcium mobilization using a fluorescence-based assay. First, we confirmed that ectopic PKRs were expressed in HEK293 and JAR cell lines using western blotting. These two proteins were predominantly residing on the cell membrane instead of the cytosol (data not shown). EG-VEGF, the ligand for PKRs, activated calcium mobilization of PROKR1 and PROKR2 with EC50 <5 nM and generated a dose–response curve of intracellular calcium influx in HEK293 and JAR cells (Fig. 2A and B respectively). However, PROKR1-I379V and PROKR2-V331M decreased calcium influx activities by around 30–50% compared with their wild-type (WT) PKRs (P <0.05), indicating that one single amino acid substitution in the PROKR1 or PROKR2 was able to alter downstream signaling. There was no cell-type specificity as similar effects of variants on calcium mobilization were observed in HEK293 (Fig. 2A) and JAR cells (Fig. 2B).

PROKR1 (I379V) and PROKR2 (V331M) did not alter cell proliferation and tube organization

Cell proliferation and angiogenesis are critical in the stages of implantation, embryogenesis, and placentation. We examined whether these two variants altered the abilities of cell proliferation and tube organization, an index of angiogenic activity, following the ectopic expression of either receptors in cells. The effects of EG-VEGF on PKR-overexpressing HEK293 and JAR cell proliferation were evaluated using doubling-time and WST assays respectively. Both cell proliferation methods showed similar results in these two cell lines. When comparing variant and WT PKRs, the cell numbers of PROKR1- or PROKR2-overexpressing HEK293 and JAR cells were not significantly different after 2 days of cell culture in the absence or presence of EG-VEGF (5 nM). The result showed that EG-VEGF did not have any effect on cell proliferation in both cell lines (Supplementary Figure 1, see section on supplementary data given at the end of this article).

To evaluate the angiogenic effect of EG-VEGF-induced tubal organization on variant PKRs-expressing cells, we measured capillary tube formation by calculating branching length between two nodes in the absence or
presence of EG-VEGF (5 nM) at different time intervals (3–22 h). After 3 h of incubation on the Matrigel, HEK293 and JAR cells (data not shown) rapidly reorganized and subsequently formed tube-like structures on Matrigel. Average tubal length was measured in each group. EG-VEGF at 5 nM had no stimulatory effect on tube formation at all time intervals (Supplementary Figure 2, see section on supplementary data given at the end of this article). When compared with their WT counterparts, both variant receptors

Figure 1 Identified PKR variants on the secondary structures of (A) PROKR1 and (B) PROKR2. Schematic of PROKR1 and PROKR2 membrane proteins was predicted using the SOSUI secondary structure prediction program (http://bp.nuap.nagoya-u.ac.jp/sosui/). PROKR1 and PROKR2 variants identified in RM patients and normal controls are shown on the structures of PKRs. Non-synonymous PROKR1-I379V and PROKR2-V331M variants are both located in the intracellular C-terminal domains (asterisks).

Figure 2 Effects of EG-VEGF-induced calcium influx for PKR variants and their wild-type counterparts. (A) HEK293 and (B) JAR cells were transiently transfected with either WT or variant PKR construct (left: PROKR1; right: PROKR2), and fluorescent signals were monitored at increasing dose of EG-VEGF after treating with a calcium indicator (Fluo-4). Both PKR variants (I379V and V331M) had decreased abilities to activate intracellular calcium mobilization compared with their wild-type PKRs. The statistical significance is indicated by asterisks (P< 0.05, one-way ANOVA with Dunnett’s multiple comparison test). EG-VEGF-induced calcium influx in a dose-dependant manner. The phenomenon was similar in (A) HEK293 and (B) JAR cells.
behaved similarly without any effect on tube formation in HEK293 or JAR cells.

**PROKR1 (I379V) and PROKR2 (V331M) enhanced cell invasion abilities but did not affect cell–cell adhesion**

During the stages of embryo implantation and placental development, trophoblast cell adhesion and further invasion are critical for successful maintenance of normal pregnancy. We performed cell–cell adhesion between RL-95-2 and PKR-transfected JAR cells and found that PROKR1 (I379V) and PROKR2 (V331M) variants have no significant influence on cell adhesion compared with their WTs and EG-VEGF did not have an effect on cell–cell adhesion (Supplementary Figure 3, see section on supplementary data given at the end of this article). We further evaluated cell invasion ability in a trans-well system and found that EG-VEGF facilitated cell invasion of PKR-expressing HEK293 and JAR cells (Fig. 3A, P<0.05 and Fig. 3B, P<0.02, respectively). In addition, PROKR1 (I379V) and PROKR2 (V331M) significantly increased cell invasiveness compared with their WT PKR-expressing HEK293 (Fig. 3A) and JAR cells (Fig. 3B) (both P<0.05).

**Discussion**

Our previous study showed the association of PROKR1 and PROKR2 polymorphisms and haplotypes with idiopathic RM using tag-SNP method. Although this method efficiently covers the whole gene region, including introns, we still had no idea on the exact loci that confer the susceptibility for RM. In this study, we analyzed the entire coding sequences of PROKR1 and PROKR2 genes in both RM patients and parous control subjects and identified one non-synonymous variant from PROKR1 and PROKR2 respectively. In addition, these two SNPs were over-represented in the controls. We further demonstrated that PROKR1-I379V and PROKR2-V331M affected cell biological functions by altering intracellular calcium mobilization and facilitating cell invasiveness. These data provide evidence for protective roles of these PROKR1 and PROKR2 variants in human early pregnancy.

PROKR1-I379V and PROKR2-V331M are located in the intracellular C-terminal domains of GPCRs, and the change of amino acid may alter the process of G-protein (Gq, Gs, and Gi) coupling and intracellular calcium influx (Masuda et al. 2002, Abreu et al. 2008, Levit et al. 2011, McCoy et al. 2012). Intracellular Ca$^{2+}$ is an
important second messenger, and the downstream effects of calcium mobilization are likely to be numerous and may include activation of various secretory and signaling pathways (Petit & Bélisle 1995, Karl et al. 1997, Tinel et al. 2000). Ca\(^{2+}\) is closely moderated cell migration and invasion via Ca\(^{2+}\)-dependant effectors, such as calcineurin, calmodulin, actin, myosin, and integrins (Prevarskaya et al. 2011), and also critical in mediating egg activation and successful preimplantation embryo development (Miao & Williams 2012). EG-VEGF and PKRs were reported to exert their biological functions by phosphorylation of their downstream target ERK1/2, through cross talk with the EGFR system (Evans et al. 2008), or by activation of the PI3 kinase/Akt pathway (Lin et al. 2002) and calcium/cAMP mobilization (Brouillet et al. 2012). EG-VEGF also modulates IL8, IL11, and DKK1 expression via the calcineurin/NFAT signaling pathway (Cook et al. 2010, Macdonald et al. 2011). Most of the above cytokines and pathways were closely related to endometrial decidualization and embryo implantation (Evans et al. 2008, 2009, Macdonald et al. 2011, Brouillet et al. 2012).

Implantation is a complex process of embryonic attachment to the endometrium and subsequent invasion into the stroma of the uterine wall. Sufficient trophoblast invasion is not only for an adequate anchoring but also for a invading maternal vasculature to assess oxygen and nutrients. In 8–12 weeks of human gestation, the peak expression of EG-VEGF and PROKR1 in endometrium coincides with a hypoxic period (Hoffmann et al. 2006, Evans et al. 2008). Adequate trophoblast invasion is needed to help an embryo to obtain sufficient oxygen and nutrition before the establishment of extensive vasculogenesis. EG-VEGF regulates trophoblast invasion during early pregnancy, and the mechanisms were suggested to involve oxygen tension, human chorionic gonadotropin, and activation of metalloproteinases MMP-2 and MMP-9 (Brouillet et al. 2012). In this study, PROKR1-I379V and PROKR2-V331M did not have influence on cell–cell adhesion (attachment) but facilitate cell invasiveness. We speculated that these two genotypes may exert their protective effects on early pregnancy by enhancing fetal extravillous cytrophoblast cells (EVT) to invade into the inner decidua and promoting invasion of decidual stromal cells (DSCs) in the process of implantation. Moreover, cell invasiveness effects were not only seen in a trophoblast cell line (JAR cells) but also seen in HEK293 cells, which is a kidney embryonic cell line. EG-VEGF, PROKR1, and PROKR2 were recently shown to play critical roles in inflammatory and immune responses in human pregnancy (Dorsch et al. 2005, Gorowiec et al. 2011, Shaw et al. 2010). EG-VEGF and its receptors are up-regulated in myometrium and placenta and may act as mediators for initiating term or preterm parturition to regulate a group of genes, mainly chemokines and cytokines, geared toward a pro-inflammatory response (Catalano et al. 2010, Gorowiec et al. 2011). In the patients of Chlamydia trachomatis infection, increased tubal expression of PROKR2 via TLR2-mediated NfkB activation was proposed to predispose to tubal implantation (Shaw et al. 2010). Therefore, we also speculated that these two genotypes may help immune cells that expressed EG-VEGF or PKRs to migrate from peripheral circulation to uterus, predisposing a pro-inflammatory environment for embryo implantation, leading to a beneficial site for a successful pregnancy.

Several PROKR2 mutations are reported to be related to Kallmann syndrome (KS) and idiopathic hypogonadotropic hypogonadism (IHH) (Dodé et al. 2006, Pitteloud et al. 2007, Abreu et al. 2008, Cole et al. 2008, Leroy et al. 2008, Canto et al. 2009, Sarfati et al. 2010). Most of these mutations have various functional effects, including altering calcium mobilization, MAPK activation, or receptor trafficking (Abreu et al. 2010, Peng et al. 2011). Many of these PROKR2 mutations were, however, also found in clinically unaffected individuals, and PROKR2-V331M was one of them. Previously, PROKR2-V331M was reported to be a pathological mutation in the studies of KS and IHH, and the frequency of V331 mutation in these patients is around 0.5% (Dodé et al. 2006, Cole et al. 2008). In these studies, several V331M carriers in the same families did not display KS phenotype, suggesting that V331M may be a low-penetrance mutation or not a causing mutation for KS (Dodé et al. 2006, Cole et al. 2008, Abreu et al. 2010). All women carrying V331M in our study were in normal control group and had delivered at least one full-term healthy baby without the aid of assisted reproductive technologies (ART) and had not experienced miscarriage or pregnancy complications, indicating that they had no KS-related phenotype at all. We also found V331M in the database of 1000 Genomes Project, which provide human genetic variations by deep sequencing in different ethnic populations. Therefore, PROKR2-V331M is more like a variation in the general population and our study suggests that it could be a protective genotype that confers a lower risk for having RM.

There are some limitations in this study. First, conceptus or decidual tissues were not available to confirm the variant genotypes owing to retrospective study design. Ideally, we should isolate the tissue carrying PROKR1-I379V or PROKR2-V331M from primary culture for use in experiments. Secondly, JAR and RL95-2 are cancer cell lines and may not be the best cell model for investigation. Although PKR variant-related cell functions have no differences in JAR and HEK293 cells, better cell lines would be those derived from decidual or trophoblast tissue. Thirdly, the relationship of the decreased calcium influx and cell invasion or other cell activity in early pregnancy is not clear. Further studies are required to evaluate and define the specific effect of decreased calcium influx on uterine receptivity.
or embryonic implantation. Fourthly, if the protective effect of \textit{PROKR1-I379V} or \textit{PROKR2-V331M} in human early pregnancy is through increasing cell invasion capability, the target cell types, such as trophoblasts, macrophages/monocytes, DSCs, or T cells, are needed to clarify.

Upregulation of \textit{EG-VEGF} together with its regulatory effects on implantation-related genes during implantation window suggests a role of \textit{EG-VEGF} in early pregnancy (Evans et al. 2008, Haouzi et al. 2009). \textit{EG-VEGF} and \textit{PKRs} can regulate angiogenic process in microvascular endothelial cells from placenta (Brouillet et al. 2010) and trophoblast invasion in human gestational tissue (Hoffmann et al., 2006, 2009). Moreover, their expression in monocytes and macrophages may also be involved in immune adaptation and inflammatory process in uterine environment, which would facilitate a successful implantation (Dekel et al. 2010, Paulesu et al. 2010, Mor et al. 2011). In this study, we identified that two non-synonymous \textit{PKR} variants (\textit{PROKR1-I379V} and \textit{PROKR2-V331M}) were protective genotypes in human early pregnancy, using sequence of the entire coding regions from RM patients. They were shown to decrease calcium influx but enhance cell invasiveness in our \textit{in vitro} studies. We speculate that the \textit{PKR} genotypes may play protective roles through the fetal effect (trophoblast cells) and maternal effect (DSCs) by enhancing their cell invasion abilities in the maternal–fetal interface. They may also be involved in differentially regulating a group of cytokines and chemokines that attract the migration of monocytes/macrophages to the uterus, forming a pro-inflammatory environment that facilitate implantation. However, more studies are warranted to address these questions.

In conclusion, our findings extend our previous work via fine mapping the \textit{PROKR1} and \textit{PROKR2} coding regions to explore the variant loci associated with RM risk. We found that \textit{I379V} of \textit{PROKR1} and \textit{V331M} of \textit{PROKR2} may play their protective roles by altering intracellular calcium signaling and increasing cell invasiveness. This study corroborates clinical relevance of \textit{EG-VEGF} system in human early pregnancy and potential therapeutic targets for idiopathic RM patients in future personalized medicine.

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**Author contribution statement**
M-T Su involved in the study design, execution, and manuscript drafting. S-H Lin and Y-C Chen involved in statistical analysis. M-T Su and P-L Kuo involved in patient collection. L-W Wu and P-L Kuo involved in critical discussion and correspondence.

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**References**

**Supplementary data**
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**Declaration of interest**
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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