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GENETICS

Gene-gene interactions and gene polymorphisms of VEGFA and EG-VEGF gene systems in recurrent pregnancy loss

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Abstract
Purpose Both vascular endothelial growth factor A (VEGFA) and endocrine gland-derived vascular endothelial growth factor (*EG-VEGF*) systems play major roles in angiogenesis. A body of evidence suggests VEGFs regulate critical processes during pregnancy and have been associated with recurrent pregnancy loss (RPL). However, little information is available regarding the interaction of these two major major angiogenesis-related systems in early human pregnancy. This study was conducted to investigate the association of gene polymorphisms and gene-gene interaction among genes in VEGFA and *EG-VEGF* systems and idiopathic RPL.
Methods A total of 98 women with history of idiopathic RPL and 142 controls were included, and 5 functional SNPs selected from VEGFA, KDR, *EG-VEGF (PROK1)*, *PROKR1* and *PROKR2* were genotyped. We used multifactor dimensionality reduction (MDR) analysis to choose a best model and evaluate gene-gene interactions. Ingenuity pathways analysis (IPA) was introduced to explore possible complex interactions.
Results Two receptor gene polymorphisms [KDR (Q472H) and PROKR2 (V331M)] were significantly associated with idiopathic RPL ($P<0.01$). The MDR test revealed that the KDR (Q472H) polymorphism was the best loci to be associated with RPL ($P=0.02$). IPA revealed EG-VEGF and VEGFA systems shared several canonical signaling pathways that may contribute to gene-gene interactions, including the Akt, IL-8, EGFR, MAPK, SRC, VHL, HIF-1A and STAT3 signaling pathways.
Conclusion Two receptor gene polymorphisms [KDR (Q472H) and PROKR2 (V331M)] were significantly associated with idiopathic RPL. EG-VEGF and VEGFA systems shared several canonical signaling pathways that may contribute to gene-gene interactions, including the Akt, IL-8, EGFR, MAPK, SRC, VHL, HIF-1A and STAT3.

Keywords VEGFA · EG-VEGF (PROK1) · Recurrent pregnancy loss · Gene-gene interaction · Ingenuity pathways analysis

Capsule Gene polymorphisms of *VEGFA* and *EG-VEGF* systems are associated with idiopathic recurrent pregnancy loss and they may jointly contribute to miscarriages through several shared canonical pathways.

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Introduction

Angiogenesis and a sufficient blood supply are critical for a successful early pregnancy for endometrial preparation, implantation, and placental and fetal vasculature development [1, 2]. An inadequate blood supply and angiogenesis defect may contribute to several adverse pregnancy outcomes, such as infertility, miscarriage, intrauterine fetal distress/growth restriction, preeclampsia, and even intrauterine fetal death [1, 3]. In patients with idiopathic recurrent pregnancy losss (RPL), angiogenesis-related gene polymorphisms have been

proposed as susceptibility factors that increase the risk of miscarriages compared with otherwise healthy women [4].

Vascular endothelial growth factor A (VEGFA) is a potent angiogenic factor and is a survival factor for endothelial cells during physiological and tumor angiogenesis, and functions in vasodilatation, vascular permeability and anti-apoptosis [5]. Establishment of pregnancy requires extensive angiogenesis both in the chorionic villi and during embryogenesis. VEGF and its receptors (VEGFR1/Flt-1, VEGFR2/KDR/Flk-1) play essential roles in oocyte maturation, embryo implantation, fetal development and placentation [6, 7]. Mice lacking the expression of VEGFA or either of the 2 receptors died in utero due to inadequate vascular formation [8, 9]. Aberrant placental VEGFA expression and vascular abnormalities may result in adverse pregnancy outcomes, including pregnancy loss, intrauterine fetal death, intrauterine fetal growth restriction and pre-eclampsia [10, 11].

Endocrine gland-derived vascular endothelial growth factor (EG-VEGF), also known as prokineticin 1 (PROK1), is a tissue-specific angiogenic factor. Its expression is restricted mainly to the steroidogenic glands, especially to the tissues of the ovary, testis, adrenal gland, and placenta, and it induces cell proliferation, migration, and fenestration in capillary endothelial cells [12]. EG-VEGF acts through the G-protein coupled receptors prokineticin receptor 1 (PROKR1) and prokineticin receptor 2 (PROKR2), which are involved in the regulation of male and female reproduction [13–16]. EG-VEGF was suggested to be involved in embryo implantation and third trimester parturition through regulating multiple inflammatory-related cytokines in the endometrium and myometrium [17, 18], and was regarded as a key endocrine factor in placental development [19].

In view of the importance of angiogenesis in human pregnancy and the fact that little information is available regarding the interaction between VEGFA and EG-VEGF systems in early gestation, a study was conducted to investigate the association of gene polymorphisms and gene-gene interaction among genes in the 2 major angiogenesis-related systems and idiopathic RPL. The second aim was to explore the possible complex pathways linking these 2 systems, using ingenuity pathways analysis (IPA).

Materials and methods

Subjects

The present study was approved by the Institutional Review Board of National Cheng Kung University Hospital, and informed consent was obtained from all patients and controls in this study. Ninety-eight women who had experienced at least 2 consecutive spontaneous abortions (SA) were recruited from outpatient clinics of our hospital. All women had conceived naturally without the aid of assisted reproductive technologies (ART). SA included both embryonic and anembryonic losses before 12 weeks of gestational age. Cases of biochemical pregnancy were excluded from the study. All subjects had undergone a comprehensive examination as described in our previous publications, including a detailed history, physical examination, chromosome analysis of peripheral blood lymphocytes, and trans-vaginal 3-dimensional ultrasound to detect uterine anomalies and endometrial defects. They also underwent the 75-g oral glucose tolerance test, and were checked for thyroid functions (T3, T4, and TSH), anti-cardiolipin antibodies (IgG, IgM and β-glycoprotein), lupus anticoagulant, anti-thrombin III, protein S, protein C, and endocrinology profiles on day 3 of the menstrual cycle [follicle-stimulating hormone (FSH), luteinizing hormone (LH), prolactin (PRL), and testosterone (T)] [20, 21]. Women with any identifiable cause of RPL, history of infertility, or gynecological disorders which might interfere with implantation or placentation were excluded from the study. We also recruited 142 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.

Genotyping

Five functional SNPs were selected from VEGFA and EG-VEGF gene systems (Table 1) based on our previous studies [20, 21]: VEGFA (−1154G/A, rs1570360), KDR (Q472H, rs1870377), EG-VEGF (V67I, rs7514102), PROKR1 (I379V, rs34715748) and PROKR2 (V331M, rs117106081). VEGFA (−1154G/A, rs1570360) and KDR (Q472H, rs1870377) were detected by primer extension analysis using end-point TaqMan assays (Applied Biosystems, Warrington, UK). EG-VEGF (V67I), PROKR1 (I379V) and PROKR2 (V331M) were detected by the Sanger sequence method [21].

Statistical analysis

Comparisons of clinical information between the 2 groups (age, number of successful pregnancies) and the association with single markers between RPL patients and normal controls were performed using a χ^2 test or Fisher's exact test. A P value of <0.05 was considered statistically significant. In comparing genotype/allele frequencies between 2 groups, stringent statistics was introduced using the Bonferroni–Dunn method for multiple testing corrections and a P value of <0.01 was considered statistically significant. The relative risk of habitual abortion was estimated from logistic odds ratios (OR) with a 95 % confidence interval (CI) in multivariate analysis. Hardy-Weinberg equilibrium was calculated in accordance with standard procedures using χ^2 analysis.

Table 1 Genes and variants analyzed in this study

Gene name	Chromosome	Functional loci	Single nucleotide polymorphism (rs#)
Vascular Endothelial Growth factor A (VEGFA)	6p12	−1154 G > A	rs1570360 G/A
Kinase Insert Domain Receptor (KDR)	4q11-q12	Q472H	rs1870377 A/T
Prokineticin 1 (EG-VEGF)	1p21	V67I	rs7514102 G/A
Prokineticin Receptor 1 (PROKR1)	2p13.1	I379V	rs34715748 A/G
Prokineticin Receptor 2 (PROKR2)	20p12.3	V331M	rs117106081 G/A

Individuals that carried more than one risk allele or genotype may have a higher risk of RPL; therefore, gene-gene interactions were explored. We analyzed gene-gene interactions among 5 polymorphisms using the multifactor dimensionality reduction (MDR) method (MDR software, version 2.0) [22], and a *P* value of less than 0.05 was considered statistically significant.

Ingenuity pathway analysis (IPA)

We also used the commercial software Ingenuity Pathway Analysis (Ingenuity® Systems; IPA) (http://www.ingenuity.com/) to identify enriched pathways and functional themes, as reported previously [23]. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base (IPKB). The IPKB, containing a large network of curated molecular interactions and pathways, was searched to find the shortest paths enriched in genes of interest (VEGFA, KDR, PROK1, PROKR1, and PROKR2). Graphical representations of these sub-networks, containing direct and indirect molecular relationships, were generated.

Results

All the patients and controls were of Taiwanese Han descent and their ages at enrollment were, respectively, 30.95± 4.05 years (mean ± standard deviation [SD]) and 29.80± 4.49 years, which was not significantly different. The time interval between enrollment and previous miscarriage was less than 6 months. The number of previous pregnancy losses in the study group was 2.61±0.99 (mean ± SD); 56 and 42 women, respectively, experienced 2 and >2 consecutive pregnancy losses. We selected one polymorphism each from the VEGFA, KDR, EG-VEGF, PROKR1 and PROKR2 genes to genotype in each subject (Table 1). The genotypic and allelic frequencies of VEGFA (−1154G > A), KDR (Q472H), EG-VEGF (V67I), PROKR1 (I379V) and PROKR2 (V331M) in the women with RPL and the control subjects are shown in Table 2. The genotypic frequency of KDR (Q472H) and PROKR2 (V331M) was significantly different between patients and controls using stringent statistical method for multiple testing ($P<0.01$). The protective allele of PROKR2 (A allele) was significantly associated with RPL ($P<0.01$).

Gene-gene interactions were analyzed among 5 polymorphisms of these 5 genes using the MDR method. The KDR (Q472H) polymorphism was regarded as the best fit model, with an accuracy of 60.0 %, to be associated with RPL ($P=0.02$), and a maximum cross-validation (CV) consistency of 10 out of 10 (Table 3). KDR (Q472H) and PROKR2 (V331M) were shown to be the best 2-locus models with an accuracy of 53.4 %; however, gene-gene interactions were not significant in these 2 loci ($P=0.24$, Table 3).

IPA analysis showed EG-VEGF and VEGFA systems shared several enriched pathways, and gene-gene interactions were generated with functional themes, such as Akt, IL-8, EGFR, MAPK, SRC, VHL, HIF-1A and STAT3 (Fig. 1). Most IPA-identified "hubs" were canonical signaling pathways that directly or indirectly regulated cell growth, proliferation, apoptosis, migration, cell adhesion, and immune and inflammation responses in both the VEGFA and EG-VEGF systems.

Discussion

In the present study, we investigated the genetic association between ligands and receptor gene polymorphisms of EG-VEGF and VEGFA systems and the occurrence of idiopathic recurrent pregnancy loss (RPL). We demonstrated that PROKR2 (V331M) and KDR (Q472H) were significantly associated with idiopathic RPL, and KDR (Q472H) was the best locus to confer susceptibility in the study. Although PROKR2 (V331M) and KDR (Q472H) failed to show gene-gene interactions by statistical analysis, IPA revealed several shared signaling pathways (Akt, IL-8, EGFR, MAPK, SRC, VHL, HIF-1A and STAT3) existed among 5 genes in EG-VEGF and VEGFA systems, suggesting their functional interactions may occur through these canonical hubs and pathways.

Although EG-VEGF is not structurally related to the VEGF family, the biological activities of the 2 molecules are indistinguishable. These 2 major angiogenesis systems were both regulated by oxygen tension with a hypoxia-inducible factor-

Table 2 Allele and genotype frequencies of 5 functional gene polymorphisms in RM patients and controls

Location	Allele frequency				Genotype frequency				
	Allele	Case (n=196)	Control (n=284)	P value	Genotype	Case (n=98)	Control (n=142)	P value	Odds ratio (95%CI)
VEGFA (−1154 G > A) rs1570360 G/A	G	156 (79.6 %)	235(82.7 %)	0.382	GG	62 (63.3 %)	100(70.4 %)	0.245	1.38(0.80–2.39)
	A	40 (20.4 %)	49 (17.3 %)		GA	32 (32.7 %)	35 (24.6 %)		
					AA	4 (4 %)	7 (5 %)		
KDR (Q472H) rs1870377 A/T	A	103 (52.6 %)	157 (55.3 %)	0.555	AA	34 (34.7 %)	39 (27.4 %)	**0.007**	2.07(1.11–3.83)
	T	93 (47.4 %)	127 (44.7 %)		AT	35 (35.7 %)	79 (55.6 %)		
					TT	29 (29.6 %)	24 (16.9 %)		
EG-VEGF (V67I) rs7514102 G/A	G	109 (55.6 %)	177 (62.3 %)	0.141	GG	41 (41.8 %)	72 (50.7 %)	0.176	1.43(0.85–2.40)
	A	87 (44.4 %)	107 (37.7 %)		GA	27(27.6 %)	33 (23.2 %)		
					AA	30(30.6 %)	37 (26.1 %)		
PROKR1 (I379V) rs34715748 A/G	A	186 (94.9 %)	256 (90.1 %)	0.040	AA	88 (89.8 %)	114 (80.3 %)	0.047	0.46(0.21–1.00)
	G	10 (5.1 %)	28 (9.9 %)		AG	10 (10.2 %)	28 (19.7 %)		
					GG	0 (0 %)	0 (0 %)		
PROKR2 (V331M) rs117106081 G/A	G	196 (100 %)	273(96.1 %)	**0.005**	GG	98 (100 %)	132 (93 %)	**0.007**	NA
	A	0 (0 %)	11 (3.9 %)		GA	0 (0 %)	9 (6.3 %)		
					AA	0 (0 %)	1 (0.7 %)		

Statistical significance P values were corrected for multiple testing by Bonferroni–Dunn method, and a P value of <0.01 is considered statistically significant. Significant P values were shown in boldface

1alpha (HIF-1α)-dependent mechanism [12, 24], and were also similarly hormonally regulated by estrogen, progesterone and human chorionic gonadotropin [25]. Both VEGFA/VEGFR and PROK1/PROKRs were expressed in the human placenta, but their expression patterns were temporally and spatially different in the feto-maternal interface [16, 26]. The EG-VEGF expression increased gradually in early pregnancy, reaching peaks at 8–10 weeks of gestation, but there was no significant change in the VEGFA level during the first trimester [16]. In human full-term gestation, VEGFA/VEGFR expressions were highest in the maternal side of the interface, and PROK1/PROKRs were highest in the fetal side of the placenta [26]. It was therefore suspected that the 2 systems play complementary roles during the first trimester; whereas in later gestation, the PROK1/PROKR system specifically mediates angiogenesis in the fetus, and the VEGFA/VEGFR system predominantly mediates maternal angiogenesis.

All 5 genetic variants investigated in the present study are biologically functional SNPs, and 4 of them are common polymorphisms in the general population. The common functional polymorphism of VEGFA (−1154G > A) in the promoter region have been reported to affect VEGF activity and expression [27], and was associated with an increased risk of RPL and recurrent implantation failure [28, 29]. KDR exhibits a strong tyrosine kinase activity towards pro-angiogenic signals, and the variant at residue 472H > Q was demonstrated to decrease VEGF binding efficiency and was believed to alter their downstream signaling pathways [30]. Several KDR polymorphisms are reported to be associated with RPL, but genetic susceptibility varies in different ethnicity [20, 31]. We speculated that these variations may result in changing the efficiency of KDR on VEGF binding and interfere with pregnancy establishment/maintenance. The biological interaction of EG-VEGF and PROKR1/2 is a ligand-receptor relationship. The effects of amino acid changes in EG-VEGF and its receptors are not quite clear, and their genetic polymorphisms are not reported except our preceding studies [21, 32]. In our previous study, tag SNPs of PROKR1 and PROKR2 were

Table 3 Summary of mutifactorical dimension reduction (MDR) analysis

	Best model	Testing results		10-fold cross-validation	
		Accuracy (%)	P-value[a]	Consistency	P-value[a]
1. VEGFA(−1154G/A)	2	60.0	**0.02**	10/10	0.74
2. KDR(Q472H)	2, 5	53.4	0.24	7/10	0.95
3. EG-VEGF(V67I)	1, 2, 3	48.5	0.61	8/10	0.91
4. PROKR1(I379V)	1, 2, 3, 4	49.1	0.60	10/10	0.74
5. PROKR2(V331M)	–	–	–	–	–

[a] Empirical P-values were calculated by permutation analysis, and significant P values were shown in boldface

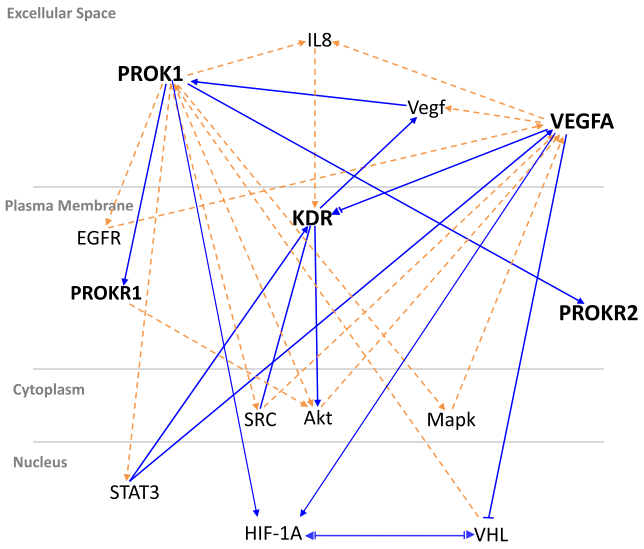

Fig. 1 VEGFA and prokineticin systems theme analysis. Genes/proteins are illustrated as nodes and molecular relationships as connecting lines between 2 nodes (direct relationships as *normal lines*; indirect relationships as *dashed lines*). Molecular relationships are supported by at least 1 literature reference, or by canonical information stored in the Ingenuity Pathways Knowledge Base (IPKB). *Black-bold* nodes represent genes of interest (VEGFA and EG-VEGF systems), while *black non-bold* nodes represent hubs that were added by the IPA algorithm to connect a set of genes of interest. Each gene/protein was categorized in the network according to its subcellular localization

associated with RPL, and EG-VEGF was a modifier gene [32]. We further demonstrated 2 specific variants of receptor genes, PROKR1 (I379V) and PROKR2 (V331M), may play as protective genotypes of human early pregnancy through altering trophoblast calcium influx and facilitate cell invasive ability [21]. We also found the gene expressions of V67I in RNA and protein levels are significantly lower compared with its wild-type (data not shown). Since all these 5 genetic variants in 2 major angiogenesis systems have functional effects on gene expression or/and cell behaviors, the biological impact of gene-gene interaction is difficult to investigate either in vitro or in vivo studies. Therefore, to evaluate the clinical data using in silico method is a good solution to answer the question of genetic interaction in complex disease.

RPL is a complex disease, and a woman carries different genetic polymorphisms will confer variable susceptibility to recurrent abortions. To characterize the genetic background of each individual may help to understand the etiology of RPL and to design diagnostic and treatment program. IPA is a powerful tool to explore gene-gene/protein interactions, and it provides comprehensive pathways and network analysis of complex biological data by integrating web-based information from a variety of experimental platforms. In the study, we demonstrated 8 molecules or genes (Akt, IL-8, EGFR, MAPK, SRC, VHL, HIF-1A and STAT3) that directly or indirectly linked the EG-VEGF/PROKRs and VEGFA/KDR systems. Among these genes or molecules, the AKT, EGFR and MAPK pathways were common canonical signaling pathways in both the EG-VEGF and VEGFA systems, and were involved in regulating cell growth, proliferation, apoptosis, migration and cell adhesion in a variety of pathophysiological conditions, including tumor angiogenesis, polycystic ovarian syndrome (PCOS), and preeclampsia [18, 33, 34]. IL-8 and SRC provided evidence of the role of EG-VEGF and VEGFA in the inflammatory and immune response in the decidua and placenta, and that EG-VEGF and VEGFA may be involved in embryo implantation, term or preterm labor and preeclampsia [17, 18, 35, 36]. SRC and STAT3 play a role as transcriptional factors or complexes that mediate gene transcription and activation, and were related to tumor growth, tumor angiogenesis, embryo implantation and antiphospholipid syndrome [17, 36–39]. HIF-1α activated transcription of VEGFA and EG-VEGF gene expression in a variety of developmental and physiological processes. VHL (von Hippel-Lindau) is a tumor suppressor gene, and is acquired for HIF-1alpha ubiquitination and degradation under normoxic conditions [37, 39, 40].

There are some limitations in this study. First, the sample size was relatively small, and thereby attenuated the power of the statistical significance. The MDR method was therefore introduced to identify gene–gene interactions that are associated with idiopathic RPL. The MDR method reduces high-dimensional genetic data into a single dimension, thus permitting gene-gene or gene-environmental interactions to be detected in relatively small sample sizes. Second, the IPA-identified hubs shared by the 2 angiogenesis systems were not validated in this study. Although these shared molecules were identified from evidence of complex biological data, the exact interactions between the 2 systems are not clear and warrant further study.

The individual functional relevance of the EG-VEGF and VEGFA systems in human early pregnancy is already known. The co-existent expressions of the 2 angiogenesis systems in reproductive tissues (placenta, endometrium, myometrium and embryo) further suggest their functional interactions. In the present study, although gene-gene interactions were not shown to be significant between the 2 major angiogenesis systems using statistical methods, their possible functional interactions were demonstrated by a literature search updated with the IPA database. Eight molecules or genes (Akt, IL-8, EGFR, MAPK, SRC, VHL, HIF-1A and STAT3) were shown to directly or indirectly link the EG-VEGF/PROKRs and VEGFA/KDR systems in the shortest paths. In conclusion, KDR (Q472H) and PROKR2 (V331M) gene polymorphisms are associated with idiopathic RPL; VEGFA and EG-VEGF gene systems may jointly contribute to idiopathic RPL through several shared canonical pathways. Our study supports the roles of the VEGFA/KDR and EG-VEGF/PROKR systems in human early pregnancy and may provide genetic information of RPL for future personalized medicine.

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