Association between vascular endothelial growth factor promoter polymorphisms and the risk of recurrent implantation failure

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Abstract. The objective of the present study was to investigate the association between recurrent implantation failure (RIF) and vascular endothelial growth factor (*VEGF*) gene polymorphisms that are associated with various female infertility disorders. A total of 116 women diagnosed with RIF and 218 control subjects were genotyped for the *VEGF* -2578C>A, -1154G>A, -634C>G and 936C>T polymorphisms using a polymerase chain reaction-restriction fragment length polymorphism assay. The *VEGF* -2578AA genotype was associated with an increased prevalence (≥4) of RIF [adjusted odds ratio (AOR)=2.77; 95% confidence interval (CI)=1.10-7.02; P=0.031], whereas the *VEGF* -634CG+GG genotype was associated with an increased incidence of total RIF (AOR=2.03; 95% CI=1.02-4.05; P=0.044) and ≥4 RIF (AOR=3.16; 95% CI=1.19-8.37; P=0.021). The results of the haplotype analysis indicated that -2578A/-1154A/-634G/936C (AOR=1.76; 95% CI=1.03-3.00; P=0.040 for total RIF and AOR=2.11; 95% CI=1.12-3.97; P=0.021 for ≥4 RIF) was associated with the occurrence of RIF. In addition, it was revealed that there was a significant difference in serum prolactin level associated with the *VEGF* -634C>G polymorphism (P=0.013). Therefore the findings of the present study indicate that the *VEGF* -2578AA genotype, -634G allele and -2578A/-1154A/-634G/936C haplotype may be genetic markers for susceptibility to RIF. However, further studies on *VEGF* promoter polymorphisms that include an independent randomized-controlled population are required to confirm these results.

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Introduction

Recurrent implantation failure (RIF) refers to when an implanted embryo repeatedly fails to result in the development of an intrauterine gestational sac following embryo transfer (ET), as determined by ultrasonography (1). RIF may simply be defined as two or more continuous implantation failures (2); however, researchers now prefer to define RIF as the failure to maintain a clinical pregnancy following three cycles of ET (3,4). Several causes of RIF have been reported, including embryo, uterine and immunological factors, as well as thrombophilic conditions, however, the genetic mechanisms underlying RIF remain unclear (1).

Embryo implantation is a multifactorial event that depends on the interaction of the blastocyst with the receptive endometrium and consists of molecular signaling by the embryo, followed by apposition and attachment to the endometrium (5). Following the formation of the fetal-maternal interface, the essential second step involves the invasion of the embryo endometrium (6). This invasion induces endometrial angiogenesis, which is promoted by numerous growth factors, including vascular endothelial growth factor (VEGF). VEGF increases vascular permeability and activates endothelial cell proliferation, migration, differentiation and capillary formation (6). The intrauterine concentrations of VEGF during the menstrual cycle were determined in women experiencing infertility and it was revealed that VEGF concentrations are cycle-dependent and increase during the late secretory and premenstrual phases (7). VEGF concentrations are also correlated with levels of insulin-like growth factor-binding protein 1, the decidualization marker of the endometrium (8). Sugino *et al* (9) examined the expression of VEGF and its receptors throughout the menstrual cycle and in early pregnancy. The

results revealed that the expression of VEGF and its receptor were higher in the mid-secretory phase compared with the proliferative phase during normal menstrual cycles. There was also a marked expression of VEGF in decidual cells in early pregnancy. The authors concluded that VEGF contributes to the successful implantation and maintenance of pregnancy by increasing vascular permeability or by forming the vascular network in the decidua. Kapiteijn *et al* (10) cultured human embryos in VEGF-conditioned media as an *in vitro* model and demonstrated that VEGF induced endometrial angiogenesis in the embryos. The results of these previous studies indicate that VEGF may be a key regulator in angiogenesis and decidualization of the endometrium, which are essential processes for the maintenance of a successful pregnancy.

VEGF is located on chromosome 6p21.3 and is comprised of eight exons (11). Several single nucleotide polymorphisms (SNPs) have been previously detected in *VEGF*, including -2578C>A, -1154G>A, -634C>G and 936C>T, which are associated with altered *VEGF* expression (12-16). There have been several previous reports that *VEGF* polymorphisms are associated with the development and prognosis of a variety of obstetrical and gynecological diseases (17-19). Additionally, there have been reports that the *VEGF* -1154G>A and -634C>G polymorphisms are associated with the occurrence of RIF (20-23), however, only a small number of studies have evaluated the association between other functional *VEGF* polymorphisms and the incidence rate of RIF (24,25).

The objective of the present study was to examine whether single *VEGF* SNPs or the functional *VEGF* polymorphism haplotype -2578C>A (rs699947), -1154G>A (rs1570360), -634C>G (rs2010963) and 936C>T (rs3025039) may affect the susceptibility to RIF in Korean females.

Materials and methods

Study population. Blood samples were obtained from 116 females with RIF [median age (range), 34 years (27-45 years)] and 218 healthy female controls [median age (range); 33 years (24-66 years)]. All study participants were recruited from the Department of Obstetrics and Gynecology of CHA Bundang Medical Center (Seongnam, South Korea) between March 2010 and December 2012.

In the present study, RIF was defined as the failure to achieve pregnancy following the completion of two fresh *in vitro* fertilization-ET cycles with >10 cleaved embryos and serum human chorionic gonadotrophin concentrations <5 U/ml 14 days after ET. All embryos were examined by an embryologist prior to transfer and judged to be of a good quality. The male and female partner in each couple experiencing RIF was evaluated. Subjects who were diagnosed with RIF due to anatomical, chromosomal, hormonal, infectious, autoimmune or thrombotic causes were excluded from the present study. Anatomical abnormalities were evaluated using several imaging modalities, including sonography, hysterosalpingogram, hysteroscopy, computerized tomography and magnetic resonance imaging. Karyotyping was conducted using standard protocols to assess chromosomal abnormalities (26,27). Hormonal causes of RIF, including hyperprolactinemia, luteal insufficiency and thyroid disease were excluded by measuring the concentrations of prolactin (PRL), thyroid-stimulating hormone (TSH), free thyroxine, follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E2) and progesterone in samples of peripheral blood. Lupus anticoagulant and anticardiolipin antibodies were examined according to the protocols of a previous study (28) to exclude lupus and antiphospholipid syndrome as autoimmune causes of RIF. Thrombotic causes of RIF were defined as thrombophilia and were evaluated by the detection of protein C and S deficiencies and by the presence of anti-α2 glycoprotein antibodies using methods described in a previous study (29).

The enrollment criteria for the control group included regular menstrual cycles, normal karyotype (46, XX), a history of at least one naturally conceived pregnancy and no history of pregnancy loss, including abortion. Data collection methods for each group were identical.

The study protocol was approved by the Institutional Review Board of CHA Bundang Medical Center on 23 February 2010 (reference no. CHAMC2009-12-120). All study participants provided written informed consent prior to participating in the present study. All the methods applied in the study were performed in accordance with the approved guidelines.

Hormone assays. Blood samples were collected by venipuncture on day 2 or 3 of the menstrual cycle for the measurement of FSH, LH, E2, TSH and PRL levels. Serum was prepared as previously described (30) and hormone levels were determined using either radioimmunoassays [E2 (cat. no., A21854), TSH (cat. no., IM3712) and PRL (cat. no., IM2121); Beckman Coulter, Inc., Brea, CA, USA], or enzyme immunoassays using IMMULITE® 1000 Systems (FSH and LH; Siemens AG, Munich, Germany) according to the manufacturer's protocol.

Genotype analysis. Genomic DNA was extracted from whole blood using the G-DEX IIc Genomic DNA Extraction kit (Intron Biotechnology Inc., Seongnam, Korea) and purified using the high-salt buffer method (31). DNA was diluted to 100 ng/μl with 1X TE (Tris-EDTA) buffer and subsequently 1 μl from each sample was used to amplify *VEGF* polymorphisms. Genotyping was performed by polymerase chain reaction (PCR) restriction fragment length polymorphism using the following primers: *VEGF* -2578C>A polymorphism forward, 5'-GGATGGGGCTGACTAGGTAAG-3' and reverse, 5'-AGC CCCCTTTTCCTCCAAC-3' to generate a 308-bp (C allele) or 326-bp (A allele) product; *VEGF* -1154G>A polymorphism forward, 5'-CGCGTGTCTCTGGACAGAGTTTCC-3' and reverse, 5'-CGGGGACAGGCGAGCTTCAG-3' to generate a 173-bp (A allele) or 141-bp (G allele) product; *VEGF* -634C>G polymorphism forward, 5'-CAGGTCACTCACTTTGCCCCG GTC-3' and reverse, 5'-GCTTGCCATTCCCCACTTGAA TCG-3' to generate a 204-bp (C allele) or 180-bp (G allele) product; *VEGF* 936C>T polymorphism forward, 5'-AAGGAA GAGGAGACTCTGCGCAGAGC-3' and reverse, 5'-TAAATG TATGTATGTGGGTGGGTGTGTCTACAGG-3' to generate a 208-bp (C allele) or 122-bp (T allele) fragment. The thermocycling conditions for each set of primers are presented in Table I and all PCR experiments were performed using an AccuPower® HotStart PCR PreMix (Bioneer Corporation, Daejeon, Korea). *VEGF* polymorphisms were identified by digesting the *VEGF* -2578C>A and -634G>C PCR products with the *Ava*II restriction endonuclease (New England

Table I. PCR conditions, primers and restrict enzyme used in the present study.

Gene	rs#	PCR condition			Primer sequence (5'-3')	R/E
VEGF -2578C>A	rs699947	94˚C	5 min	35 cycles	F: GGATGGGGCTGACTAGGTAAG	*Ava*II
		94˚C	30 sec		R: AGCCCCCTTTTCCTCCAAC	
		-62˚C	30 sec			
		-72˚C	30 sec			
		72˚C	7 min			
VEGF -1154G>A	rs1570360	94˚C	5 min	38 cycles	F: CGCGTGTCTCTGGACAGAGTTTCC	*Mnl*I
		94˚C	30 sec		R: CGGGGACAGGCGAGCTTCAG	
		-59˚C	40 sec			
		-72˚C	30 sec			
		72˚C	5 min			
VEGF -634C>G	rs2010963	94˚C	5 min	40 cycles	F: CAGGTCACTCACTTTGCCCCGGTC	*Ava*II
		94˚C	30 sec		R: GCTTGCCATTCCCCACTTGAATCG	
		-63˚C	35 sec			
		-72˚C	30 sec			
		72˚C	7 min			
VEGF 936C>T	rs3025039	94˚C	5 min	35 cycles	F: AAGGAAGAGGAGACTCTGCGCAGAGC	*Nla*III
		94˚C	30 sec		R: TAAATGTATGTATGTGGGTGGGTGTGTCTACAGG	
		-68˚C	1 min			
		-72˚C	30 sec			
		72˚C	7 min			

PCR, polymerase chain reaction; rs#, RefSNP(rs) number; R/E, restriction enzyme; VEGF, vascular endothelial growth factor; F, forward primer; R, reverse primer.

BioLabs, Inc., Ipswich, MA, USA) and the *VEGF* -1154G>A and 936C>T PCR products with *Mnl*I and *Nla*III restriction endonucleases (New England BioLabs, Inc., Ipswich, MA, USA). All restriction digests were performed at 37˚C for 16 h and detected using gel electrophoresis with 3% agarose gel and visualized with ethidium bromide on a Gel-Doc XR+ version system (Bio-Rad, Hercules, CA, USA).

Statistical analysis. Differences in the genotype and haplotype frequencies between RIF subjects and controls were compared using multivariate logistic regression. Allelic frequencies were calculated to identify deviations from Hardy-Weinberg equilibrium (HWE), using $P<0.05$ as the significance threshold as previously described (32,33). Adjusted odds ratios (AORs) and 95% confidence intervals (CI) were used to measure the strength of the association between different genotypes and RIF. Association analysis was performed among groups that were stratified by implantation failure number. Patients with RIF were defined as those with ≥2 implantation failures and patients were divided into the following groups: Total RIF, ≥3 implantation failures (≥3 RIF) and ≥4 implantation failures (≥4 RIF). As there was no significant difference between the ≥3 RIF group and the total RIF group, the ≥4 RIF group was compared with the total RIF group. $P<0.05$ was considered to indicate a statistically significant difference. Differences in hormone concentrations (E2, FSH, LH, PRL and TSH) in accordance with *VEGF* genotypes and alleles were evaluated using a one-way analysis of variance with a post-hoc Scheffé test for all pairwise comparisons and independent two-sample t-tests as appropriate. Data are presented as the mean ± standard deviation. Statistical analyses were performed using GraphPad Prism version 4.0 (GraphPad Software, Inc., La Jolla, CA, USA), StatsDirect version 2.4.4 (StatsDirect Ltd., Altrincham, UK) and PLINK version 1.07 (http://zzz.bwh.harvard.edu/plink/). Statistical power was calculated using the G*Power program version 3.1.7 (http://www.gpower.hhu.de/).

Transcription factor binding site prediction. The DNA sequence of the *VEGF* promoter was used to predict transcription factor binding sites. The P-Match (http://www.gene-regulation.com) (34,35) was used to predict the transcription factors that would bind to the region in VEGF promoter. P-Match is interconnected with the TRANSFAC® database (http://gene-regulation.com/).

Results

Baseline characteristics and the frequency of VEGF polymorphisms. The demographic characteristics of the study participants are presented in Table II. Patient age and RIF was matched with the relevant control groups and the following characteristics were examined: Age, BMI, gestational age and hormone levels including estradiol, FSH, LH, TSH and PRL. The results demonstrated that there were no significant differences in RIF between patients with RIF and controls. The genotypic distribution and haplotype frequencies of

Table II. Baseline characteristics of patients with RIF and control subjects.

Characteristic	Control subjects (n=218)	Patients with RIF (n=116)	P-value
Age (years)	33.34±5.88	34.22±3.35	0.127
Body mass index (kg/m^2)	21.77±3.41	21.05±2.77	0.081
Previous implantation failure (n)	NA	4.75±2.29	-
Live births (n)	1.80±0.74	NA	-
Gestational age (weeks)	39.34±1.66	None	-
Estradiol (pg/ml)	NA	36.06±961	-
FSH (mIU/ml)	NA	8.60±4.29	-
LH (mIU/ml)	NA	4.86±2.31	-
TSH (ng/ml)	NA	2.28±1.45	-
PRL (ng/ml)	NA	12.78±6.17	-

Data are presented as the mean ± standard deviation. RIF, recurrent implantation failure; NA, not applicable; FSH, follicle-stimulating hormone; LH, luteinizing hormone; TSH, thyroid-stimulating hormone; PRL, prolactin.

VEGF -2578C>A, -1154G>A, -634C>G and 936C>T for all study participants are detailed in Table III. The HWE was observed for all *VEGF* polymorphic sites analyzed in each group. The frequencies of the *VEGF* -2578CC, -1154GG, -634CC and 936CC genotypes corresponding to the reference genotypes of the four polymorphisms were 51.4, 72.0, 18.8 and 69.3% in the control group and 48.3, 67.2, 10.3 and 67.2% in the RIF group. Furthermore, the frequency of the *VEGF* -2578C/-1154G/-634C/936C haplotype was 42.0% in the control group and 31.9% in the RIF group. In the present study, patients with RIF exhibited higher frequencies of the *VEGF* variant genotypes and haplotypes, leading to an increased number of implantation failures, compared with controls.

Genetic susceptibility of single and multiple markers. The AORs for RIF prevalence according to the *VEGF* genotypes are provided in Table IV. The *VEGF* -2578AA genotype was associated with a significantly increased prevalence (≥4) of RIFs (AOR=2.77; 95% CI=1.10-7.02; P=0.031). The *VEGF* -634CG+GG genotype was associated with a significantly increased incidence of total RIF (AOR=2.03; 95% CI=1.02-4.05; P=0.044) and ≥4 RIFs (AOR=3.16; 95% CI=1.19-8.37; P=0.021). No statistically significant differences were observed between the control and RIF groups for any of the other genotypes.

The linkage disequilibrium of the *VEGF* polymorphisms at loci -2578(rs699947)/-1154(rs1570360)/-634 (rs2010963)/936(rs3025039) in the RIF and control groups are detailed in Fig. 1. There was a clear linkage disequilibrium between loci -1154 and -634 (D'=0.945) and -2578 and -634 (D'=0.885) in the control group (Fig. 1A). Polymorphisms -1154G>A and -634G>C had a clear linkage disequilibrium in the RIF group (D'=1.000; Fig. 1B). The selected haplotypes with the four *VEGF* polymorphisms were constructed to determine if any specific haplotypes were associated with RIF prevalence (Table IV). The C-G-G-C (AOR=1.59; 95% CI=1.06-2.39; P=0.026 for total RIF and AOR=2.00; 95% CI=1.23-3.26; P=0.006 for ≥4 RIF), C-G-G-T (AOR=2.29; 95% CI=1.10-4.79; P=0.027 for total RIF) and A-A-G-C (AOR=1.76; 95% CI=1.03-3.00; P=0.040 for total RIF and AOR=2.11; 95% CI=1.12-3.97; P=0.021 for ≥4 RIF) haplotype frequencies of the *VEGF* -2578C>A, -1154G>A, -634C>G and 936C>T variants, respectively, were significantly different between the RIF and the control group.

Differences in hormones according to VEGF polymorphisms in the RIF group. Possible associations between RIF and the serum level of PRL were examined. The results are presented in Fig. 2 according to *VEGF* gene polymorphisms. Patients with the *VEGF* -634GG genotype had significantly lower serum PRL levels compared with patients with the *VEGF* -634CC genotype (Fig. 2A). Additionally, patients with the *VEGF* -634G allele had significantly lower serum PRL levels compared with patients with the *VEGF* -634C allele (Fig. 2B). Levels of the other hormones investigated (E2, FSH, LH and TSH) were not significantly associated with the polymorphisms examined in the present study (Table V). Table VI summarizes the statistical power of genetic associations in the present case-control study. The analysis of *VEGF* -634C>G polymorphism was statistically significant when compared with the additive model (94.49%), dominant model (99.91%) and recessive model (94.79%) for AORs with RIF risk in total RIFs. In ≥4 RIFs, the statistical power of *VEGF* -634C>G polymorphism was strongly significant when compared with the additive model (99.25%), dominant model (100%) and recessive model (99.85%). In addition, the *VEGF* -2578C>A polymorphism was demonstrated to possess a greater statistical power than that of the recessive model (99.99%) in AORs of ≥4 RIFs.

Discussion

The present study evaluated the association between four functional *VEGF* SNPs (-2578C>A, -1154G>A, -634C>G and 936C>T) and the prevalence of RIF in Korean females. The results indicated that the *VEGF* -2578AA genotype, -634G allele and -2578C/-1154G/-634G/936C, -2578C/-1154G/-634G/936T and -2578A/-1154A/-634G/936C haplotypes may be genetic markers for susceptibility to RIF.

Table III. Genotype and haplotype frequencies of *VEGF* polymorphisms.

A, Genotype frequencies

	Without RIF	With RIF	
Genotype	Controls, n=218 (%)	Total RIFs, n=116 (%)	≥4 RIFs, n=72 (%)
VEGF -2578CC	112 (51.4)	56 (48.3)	33 (45.8)
VEGF -2578CA	95 (43.6)	49 (42.2)	30 (41.7)
VEGF -2578AA	11 (5.0)	11 (9.5)	9 (12.5)
HWE-P	0.105	0.953	0.596
VEGF -1154GG	157 (72.0)	78 (67.2)	46 (63.9)
VEGF -1154GA	53 (24.3)	33 (28.4)	22 (30.6)
VEGF -1154AA	8 (3.7)	5 (4.3)	4 (5.6)
HWE-P	0.197	0.533	0.532
VEGF -634CC	41 (18.8)	12 (10.3)	5 (6.9)
VEGF -634CG	112 (51.4)	56 (48.3)	33 (45.8)
VEGF -634GG	65 (29.8)	48 (41.4)	34 (47.2)
HWE-P	0.554	0.461	0.424
VEGF 936CC	151 (69.3)	78 (67.2)	45 (62.5)
VEGF 936CT	62 (28.4)	37 (31.9)	27 (37.5)
VEGF 936TT	5 (2.3)	1 (0.9)	0 (0.0)
HWE-P	0.642	0.130	0.050

B, Haplotype frequencies

	Without RIF	With RIF	
Haplotype	Controls (2n=436, %)	Total RIFs (2n=232, %)	≥4 RIFs (2n=144, %)
VEGF -2578C/-1154G/-634C/936C	183 (42.0)	74 (31.9)	40 (27.8)
VEGF -2578C/-1154G/-634C/936T	6 (1.4)	1 (0.4)	0 (0.0)
VEGF -2578C/-1154G/-634G/936C	107 (24.5)	68 (29.3)	46 (31.9)
VEGF -2578C/-1154G/-634G/936T	17 (3.9)	16 (6.9)	9 (6.3)
VEGF -2578C/-1154A/-634C/936C	1 (0.2)	0 (0.0)	0 (0.0)
VEGF -2578C/-1154A/-634C/936T	0 (0.0)	0 (0.0)	0 (0.0)
VEGF -2578C/-1154A/-634G/936C	3 (0.7)	1 (0.4)	0 (0.0)
VEGF -2578C/-1154A/-634G/936T	2 (0.5)	1 (0.4)	1 (0.7)
VEGF -2578A/-1154G/-634C/936C	4 (0.9)	5 (2.2)	3 (2.1)
VEGF -2578A/-1154G/-634G/936C	23 (5.3)	14 (6.0)	8 (5.6)
VEGF -2578A/-1154G/-634G/936T	27 (6.2)	11 (4.7)	8 (5.6)
VEGF -2578A/-1154A/-634G/936C	43 (9.9)	31 (13.4)	20 (13.9)
VEGF -2578A/-1154A/-634G/936T	20 (4.6)	10 (4.3)	9 (6.3)

RIF, recurrent implantation failure; HWE, Hardy-Weinberg equilibrium; VEGF, vascular endothelial growth factor.

The -2578C>A and -1154G>A variants are located in the *VEGF* promoter region (26). The *VEGF* -2578CC and -1154GG genotypes appear to confer increased VEGF secretion compared with the presence of a minor allele (36,37). However, the functional effect of *VEGF* -634C>G is contested. Watson *et al* (15) and Hansen *et al* (38) proposed that the *VEGF* -634C allele was associated with the decreased production of *VEGF*, whereas Wongpiyabovorn *et al* (16) and Awata *et al* (39) reported that the *VEGF* -634G allele was associated with decreased VEGF production. These conflicting results are potentially due to the effect of haplotype combinations. Therefore, based on the *VEGF* haplotypes containing other *VEGF* SNPs, the effect of *VEGF* -634C>G should be examined further. It has previously been reported that there is an association between the haplotypes resulting from polymorphisms in the promoter region (-2578/-1154/-634) and *VEGF* expression (36). Lambrechts *et al* (36) reported that the -2578A/-1154A/-634G and -2578A/-1154G/-634G *VEGF*

Table IV. AORs for RIF prevalence according to *VEGF* genotype and haplotype.

A, *VEGF* genotypes

			Total RIFs			≥4 RIFs		
Genotype	Model	Reference type	AOR (95% CI)	P	Adjusted P[a]	AOR (95% CI)	P	Adjusted P[a]
VEGF -2578C>A	Additive	-2578CC	1.21 (0.84-1.75)	0.303	0.606	1.39 (0.91-2.12)	0.131	0.262
	Dominant	-2578CC	1.12 (0.71-1.75)	0.637	0.764	1.23 (0.72-2.10)	0.454	0.454
	Recessive	-2578CC	2.02 (0.85-4.83)	0.113	0.226	2.77 (1.10-7.02)	0.031	0.047
VEGF -1154G>A	Additive	-1154GG	1.17 (0.78-1.75)	0.461	0.615	1.34 (0.84-2.12)	0.215	0.287
	Dominant	-1154GG	1.22 (0.75-1.99)	0.433	0.764	1.42 (0.81-2.50)	0.225	0.427
	Recessive	-1154GG	1.15 (0.37-3.62)	0.811	0.811	1.52 (0.44-5.25)	0.504	0.504
VEGF -634C>G	Additive	-634CC	1.56 (1.10-2.19)	0.012	0.048	1.95 (1.28-2.98)	0.002	0.008
	Dominant	-634CC	2.03 (1.02-4.05)	0.044	0.176	3.16 (1.19-8.37)	0.021	0.084
	Recessive	-634CC	1.65 (1.03-2.65)	0.037	0.148	2.10 (1.22-3.64)	0.008	0.024
VEGF 936C>T	Additive	936CC	1.00 (0.64-1.56)	0.995	0.995	1.15 (0.69-1.93)	0.587	0.587
	Dominant	936CC	1.08 (0.66-1.75)	0.764	0.764	1.33 (0.76-2.32)	0.32	0.427
	Recessive	936CC	0.32 (0.04-2.86)	0.308	0.411	NA	NA	NA

B, *VEGF* haplotypes

		Total RIFs			≥4 RIFs		
Haplotype	Reference type	AOR (95% CI)	P	Adjusted P[a]	AOR (95% CI)	P	Adjusted P[a]
VEGF -2578C/ -1154G/-634C/936T	-2578C/-1154G/ -634C/936C	0.38 (0.04-3.26)	0.378	0.504	NA	NA	
VEGF -2578C/ -1154G/-634G/936C	-2578C/-1154G/ -634C/936C	1.59 (1.06-2.39)	0.026	0.107	2.00 (1.23-3.26)	0.006	0.042
VEGF -2578C/ -1154G/-634G/936T	-2578C/-1154G/ -634C/936C	2.29 (1.10-4.79)	0.027	0.107	2.38 (0.99-5.75)	0.053	0.124
VEGF -2578A/ -1154G/-634C/936C	-2578C/-1154G/ -634C/936C	3.09 (0.81-1.82)	0.100	0.200	3.34 (0.72-15.55)	0.124	0.174
VEGF -2578A/ -1154G/-634G/936C	-2578C/-1154G/ -634C/936C	1.52 (0.74-3.12)	0.253	0.405	1.60 (0.67-3.84)	0.292	0.174
VEGF -2578A/ -1154G/-634G/936T	-2578C/-1154G/ -634C/936C	1.02 (0.48-2.16)	0.961	0.961	1.37 (0.58-3.24)	0.475	0.341
VEGF -2578A/ -1154A/-634G/936C	-2578C/-1154G/ -634C/936C	1.76 (1.03-3.00)	0.040	0.107	2.11 (1.12-3.97)	0.021	0.475
VEGF -2578A/ -1154A/-634G/936T	-2578C/-1154G/ -634C/936C	1.20 (0.53-2.68)	0.666	0.761	2.01 (0.85-4.74)	0.113	0.074

[a]False discovery rate-adjusted P-value for multiple hypotheses testing using the Benjamini-Hochberg method. AOR, adjusted odds ratio; CI, confidence intervals; RIF, recurrent implantation failure; NA, not applicable; VEGF, vascular endothelial growth factor. AORs and P-values were adjusted by age and body mass index.

haplotypes were significantly correlated with decreased *VEGF* mRNA expression and plasma concentrations.

Among these polymorphisms, the mechanism of *VEGF* -2578C>A is well known (36,40,41). This SNP is in complete linkage with the deletion/insertion of an 18-bp fragment in the -2549 region and a construct containing the 18-bp deletion (linkage with the C allele) causing a 1.95-fold increase in transactivation (42). As evidence of the *VEGF* -1154G>A and -634C>G function is limited, the transcription factor binding sites containing these SNPs were predicted using P-Match. *VEGF*-1154G>A is contained within a predicted binding site for myeloid zinc finger-1 (MZF1) in which the MZF1 binding site is substituted for the Pax2 or Sp1 binding site by -1154A (prediction data attained using P-Match; gene-regulation.com). *VEGF* -634C>G is likewise identified within the predicted MZF1 or Pax2 binding site (prediction data attained

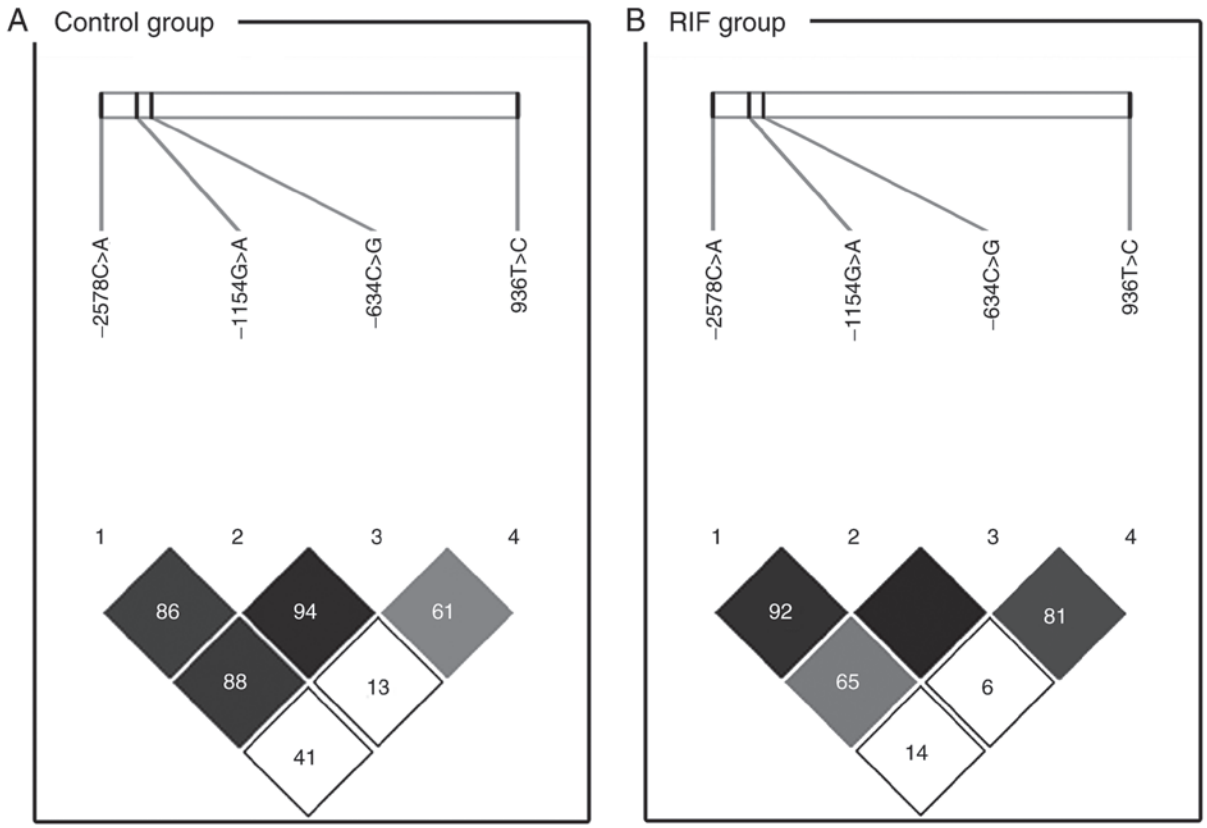

Figure 1. LD patterns of *VEGF* single nucleotide polymorphisms. The values in the squares denote LD between single markers. (A) Control subjects exhibited strong LD between loci *VEGF* -1154G>A (rs1570360) and -634C>G (rs2010963; D'=0.945), *VEGF* -2578C>A (rs699947) and -1154G>A (rs1570360; D'=0.863) and *VEGF* -2578C>A (rs699947) and -634C>G (rs2010963; D'=0.885). (B) Patients with recurrent implantation failure exhibited strong LD between loci *VEGF* -2578C>A (rs699947) and -1154G>A (rs1570360; D'=0.923) and between *VEGF* -1154G>A and -634C>G (D'=1.000). Dark squares indicate high r^2 values and light squares indicate low r^2 values. LD, linkage disequilibrium; VEGF, vascular endothelial growth factor.

using P-Match; gene-regulation.com), however, a change in the predicted transcription factor binding sites is not produced by the substitution.

In addition to VEGF, serum levels of the classical decidualization marker PRL should also be considered in association with decidualization of the endometrium. The present study revealed an association between the *VEGF* -634C>G polymorphism and plasma PRL levels. A previous study reported that *VEGF* rs3025039C>T was correlated with plasma PRL levels in polycystic ovary syndrome (43). A number of previous studies have also revealed that secreted PRL affects tissue vascularization at the lactation stage and present a role of PRL as a pro-angiogenic factor (44-48). Based on this, the current authors hypothesized that *VEGF* polymorphisms may affect the plasma PRL levels, however this hypothesis was not clearly confirmed or rejected through the experiments of the present study. Therefore, further studies are necessary to determine the potential association between *VEGF* polymorphisms and PRL levels.

Furthermore, previous studies have demonstrated that differences in PRL levels affect the formation of the placenta and decidualization of the endometrium following fertilization (44,49-52). PRL also regulates inflammatory cytokine (TNF-α, IL-1α, IL-1β, TGF-β and IL-8) levels and immune system homeostasis in early pregnancy (53-55). Therefore, normal PRL levels are critical for maintaining a successful pregnancy. In addition, PRL regulates gonadal function and serves roles in steroidogenesis, formation of the corpus luteum and modulation of the effects of gonadotropins (55). PRL stimulates the process of ovulation, implantation and placental development (49-52,56). In addition, PRL stimulates the growth, development and metabolism of the fetus, serves key roles in the formation of the corpus luteum, decreases levels of sex steroids during the menstrual cycle and stimulates the production of milk during the postpartum period (57).

The present study had several limitations. Firstly, the serum VEGF levels in the participants were not measured. Although the association between *VEGF* polymorphisms and serum VEGF levels has been elucidated in other conditions (36,42), the data associated with RIF is limited. Secondly, it was not possible to explain the exact role of VEGF in the pathogenesis of RIF development. Accordingly, in future research an association between the *VEGF* polymorphisms and *VEGF* expression in the tissues in which implantation events occur, as opposed to serum concentrations, should be elucidated. VEGF expression occurs within biochemical pathways and is not the only risk factor for disorders associated with implantation and pregnancy maintenance. Therefore, the interactions between VEGF and other factors expressed during implantation are also potential risk factors. To overcome these constraints, further studies on the functional role of *VEGF* polymorphisms in the pathogenesis of RIF are required.

Table V. Differences in various reproduction-related endocrine parameters according to *VEGF* polymorphism in patients with RIF.

	Estradiol (pg/ml)		FSH (mIU/ml)		LH (mIU/ml)		PRL (ng/ml)		TSH (ng/ml)	
Genotype	Mean ± SD (n)	CV, %	Mean ± SD (n)	CV, %	Mean ± SD (n)	CV, %	Mean ± SD (n)	CV, %	Mean ± SD (n)	CV, %
VEGF -2578CC	30.18±9.09 (11)	30.1	7.92±3.96 (9)	50	5.16±1.58 (8)	30.6	12.81±5.11 (9)	39.9	1.76±0.63 (9)	35.8
VEGF -2578CA	34.39±24.95 (44)	72.6	9.16±5.38 (40)	58.7	4.49±2.18 (39)	48.6	12.49±6.65 (40)	53.2	2.25±1.22 (39)	54.2
VEGF -2578AA	39.27±25.79 (43)	65.7	8.21±3.07 (43)	37.4	5.15±2.53 (41)	49.1	13.03±6.03 (44)	46.3	2.42±1.74 (43)	71.9
P-value	0.449		0.535		0.41		0.926		0.47	
C allele	32.99±21.01 (66)	63.7	8.78±4.96 (58)	56.5	4.68±2.02 (55)	43.2	12.59±6.13 (58)	48.7	2.10±1.09 (57)	51.9
A allele	37.62±25.42 (130)	67.6	8.51±3.95 (126)	46.4	4.94±2.42 (121)	49	12.86±6.19 (128)	48.1	2.37±1.59 (125)	67.1
P-value	0.204		0.7		0.497		0.785		0.25	
VEGF -1154GG	28.14±9.95 (5)	35.4	8.69±5.38 (5)	61.9	4.61±1.47 (4)	31.9	14.12±4.74 (5)	33.6	1.89±0.71 (5)	37.6
VEGF -1154GA	32.36±13.26 (28)	41	8.45±4.61 (26)	54.6	4.73±2.39 (26)	50.5	12.46±6.16 (28)	49.4	2.29±1.03 (27)	45
VEGF -1154AA	38.26±28.04 (65)	73.3	8.65±4.14 (61)	47.9	4.93±2.34 (58)	47.5	12.81±6.35 (60)	49.6	2.31±1.66 (59)	71.9
P-value	0.424		0.978		0.915		0.858		0.83	
G allele	31.25±12.38 (38)	39.6	8.51±4.67 (36)	54.9	4.70±2.18 (34)	46.4	12.89±5.76 (38)	44.7	2.19±0.96 (37)	43.8
A allele	37.21±26.01 (158)	69.9	8.62±4.20 (148)	48.7	4.90±2.33 (142)	47.6	12.75±6.27 (148)	49.2	2.31±1.55 (145)	67.1
P-value	0.171		0.898		0.664		0.896		0.649	
VEGF -634CC	44.80±36.38 (11)	81.2	9.35±2.95 (10)	31.6	5.60±2.98 (9)	53.2	17.68±9.13 (10)	51.6	2.37±1.78 (9)	75.1
VEGF -634CG	36.72±19.73 (46)	53.7	8.14±3.55 (48)	43.6	4.87±2.23 (45)	45.8	12.89±5.42 (46)	42	2.24±1.62 (46)	72.3
VEGF -634GG	32.97±24.74 (41)	75	9.01±5.46 (34)	60.6	4.64±2.25 (34)	48.5	11.30±5.55 (37)	49.1	2.32±1.15 (36)	49.6
P-value	0.345		0.565		0.546		0.013		0.953	
C allele	39.33±25.91 (68)	65.9	8.50±3.39 (68)	39.9	5.08±2.43 (63)	47.8	14.34±6.95 (66)	48.5	2.27±1.64 (64)	72.2
G allele	34.32±22.95 (128)	66.9	8.65±4.74 (116)	54.8	4.73±2.22 (113)	46.9	11.91±5.51 (120)	46.3	2.29±1.34 (118)	58.5
P-value	0.166		0.814		0.34		0.01		0.956	
VEGF 936CC	37.27±27.93 (67)	74.9	8.16±3.75 (61)	46	5.00±2.41 (58)	48.2	12.99±6.01 (63)	46.3	2.41±1.61 (62)	66.8
VEGF 936CT	33.76±12.61 (30)	37.4	9.46±5.15 (31)	54.4	4.59±2.11 (30)	46	12.32±6.57 (30)	53.3	2.02±1.01 (29)	50
VEGF 936TT	24.00 (1)		-		-		-		-	
P-value	0.712		0.169		0.437		0.627		0.238	
C allele	36.63±25.72 (164)	70.2	8.42±4.08 (153)	48.5	4.91±2.34 (146)	47.7	12.86±6.09 (156)	47.4	2.33±1.52 (153)	65.2
T allele	33.15±12.43 (32)	37.5	9.46±5.15 (31)	54.4	4.59±2.11 (30)	46	12.32±6.57 (30)	53.3	2.02±1.01 (29)	50
P-value	0.456		0.217		0.486		0.661		0.286	

RIF, recurrent implantation failure; FSH, follicle-stimulating hormone; LH, luteinizing hormone; PRL, prolactin; TSH, thyroid-stimulating hormone; VEGF, vascular endothelial growth factor; CV, coefficient of variation.

Table VI. Statistical powers of genetic associations in the present case-control study.

			Total RIFs		≥4 RIFs	
Genotype	Model	Reference type	AOR (95% CI)	Statistical power (%)	AOR (95% CI)	Statistical power (%)
VEGF -2578C>A	Additive	-2578CC	1.21 (0.84-1.75)	27.94	1.39 (0.91-2.12)	58.78
	Dominant	-2578CC	1.12 (0.71-1.75)	14.7	1.23 (0.72-2.10)	28.02
	Recessive	-2578CC	2.02 (0.85-4.83)	99.98	2.77 (1.10-7.02)	99.99
VEGF -1154G>A	Additive	-1154GG	1.17 (0.78-1.75)	24.42	1.34 (0.84-2.12)	49.69
	Dominant	-1154GG	1.22 (0.75-1.99)	36.6	1.42 (0.81-2.50)	64.89
	Recessive	-1154GG	1.15 (0.37-3.62)	21.23	1.52 (0.44-5.25)	80.35
VEGF -634C>G	Additive	-634CC	1.56 (1.10-2.19)	94.49	1.95 (1.28-2.98)	99.25
	Dominant	-634CC	2.03 (1.02-4.05)	99.91	3.16 (1.19-8.37)	100.00
	Recessive	-634CC	1.65 (1.03-2.65)	94.79	2.10 (1.22-3.64)	99.85
VEGF 936C>T	Additive	936CC	1.00 (0.64-1.56)	5.0	1.15 (0.69-1.93)	16.07
	Dominant	936CC	1.08 (0.66-1.75)	8.38	1.33 (0.76-2.32)	47.53
	Recessive	936CC	0.32 (0.04-2.86)	100.0	NA	

AORs and P-values were adjusted by age and body mass index. AOR, adjusted odds ratio; CI, confidence interval; RIF, recurrent implantation failure; NA, not applicable; VEGF, vascular endothelial growth factor.

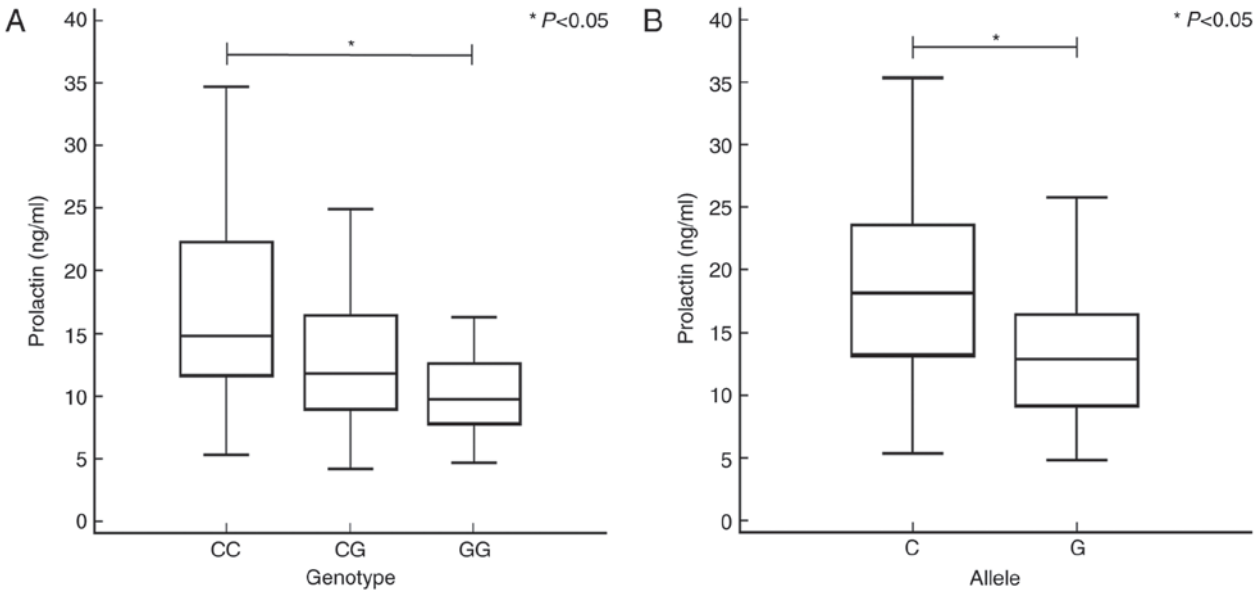

Figure 2. Association between differences in PRL levels and *VEGF* -634C>G in patients with recurrent implantation failure. Data was analyzed using one-way analysis of variance with a post-hoc Scheffé test for all pairwise comparisons or Student's t-test for each *VEGF* -634C>G genotype and allele, respectively. (A) PRL levels in the serum differed significantly (P<0.05) between patients with the *VEGF* -634CC [median (range): 14.81 (5.30-34.72)] and -GG [9.77 (4.73-17.40)] genotypes. (B) Patients with the *VEGF* -634G allele had significantly lower PRL levels compared with patients with the -634C allele. *P<0.05. PRL, prolactin; VEGF, vascular endothelial growth factor.

In conclusion, the -2578C>A, -1154G>A and -634C>G polymorphisms in the *VEGF* promoter region were associated with the occurrence of RIF. The results revealed an association between the *VEGF* -634C>G polymorphism and serum PRL levels. However, further studies of the *VEGF* promoter polymorphisms involving an independent randomized-controlled population are required to confirm these results. Additionally, the results of the present study warrant additional studies to elucidate the functional role of *VEGF* promoter polymorphisms in the RIF etiologies. Therefore, the present study indicates that the *VEGF* -2578AA genotype, -634G allele and -2578A/-1154A/-634G/936C haplotype may be utilized as biomarkers for patients with RIF. However, further studies are required to confirm this.

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