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ARTICLE

Genetic variants of vascular endothelial growth factor are associated with recurrent implantation failure in Korean women

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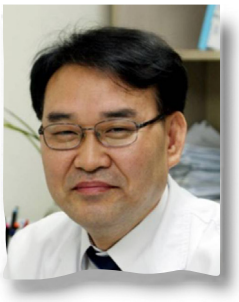

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Abstract Vascular endothelial growth factor (VEGF) is involved in embryonic development, decidual vascularization and placenta angiogenesis. This study was performed to determine whether there is an association between genetic polymorphisms in the *VEGF* gene and the development of recurrent implantation failure (RIF) in Korean women. A total of 119 women diagnosed with RIF and 236 control subjects were genotyped for *VEGF* polymorphic sites including rs833061 (-460T>C), rs25648 (-7C>T) and rs3025020 (-583C>T) using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assays and real-time PCR. The *VEGF* rs833061 C allele and rs25648 T allele were significantly associated with increased RIF risk (odds ratio [OR] = 1.813 [1.161–2.831], $P = 0.009$, OR = 2.213 [1.254–3.903], $P = 0.005$). The rs833061/rs25648 TC/CT, TC/CT+TT, and rs833061/rs3025020 TC+CC/TT genotypes were more frequent in the RIF group compared with the control group (OR = 2.130 [1.092–4.156], $P = 0.025$, OR = 2.130 [1.092–4.156], OR = 4.261 [1.163–15.620], $P = 0.028$, respectively). The results of this study suggests that *VEGF* polymorphisms are associated with RIF development. Therefore, we postulate that *VEGF* polymorphisms might be useful markers to predict RIF development. Further studies are warranted to elucidate the role of *VEGF* variants and RIF development.

KEYWORDS: female infertility, genetic association study, recurrent implantation failure, single nucleotide polymorphism, VEGF

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Introduction

Implantation is defined as when the human embryo attaches to the epithelium of the uterus and penetrates through the epithelial lining to embed into the endometrium. In clinical practice, implantation is generally considered successful when the intrauterine gestational sac is identified by ultrasonography.

Recurrent implantation failure (RIF) is defined as repeated failure to achieve a clinical pregnancy following the transfer of adequate quality of embryos (Coughlan et al., 2014). Although there is no consensus definition for RIF among researchers, Polanski et al. proposed defining RIF as the absence of attachment of the embryo to the womb lining after two consecutive cycles of IVF, intracytoplasmic sperm injection (ICSI) or frozen embryo transfer cycles, where the cumulative number of transferred embryos was no less than four for day-2 embryos and no less than two for day-5 embryos (blastocysts), using good quality and developmental stage embryos (Polanski et al., 2014). As with recurrent pregnancy loss, there are several suggested causes associated with RIF, such as gamete/embryo factors, uterine factors, immunological factors and thrombophilic conditions (Choi et al., 2014; Coughlan et al., 2014).

Of those factors, appropriate endometrium is considered to be crucial for successful implantation. After the successful implantation, the fetus is supplied with oxygen and nutrients through adequate placental development. These two processes, implantation and placentation, are required for healthy pregnancy in early stages. Angiogenesis is responsible for these essential processes.

Vascular endothelial growth factor (VEGF) is a key regulator of angiogenesis and vasculogenesis. Single nucleotide polymorphism (SNP), a DNA sequence variation occurring commonly within a population, affects protein expression and function. There are several reports that *VEGF* gene polymorphisms have an association with various obstetrics and gynaecologic disease development and prognosis (Cheng et al., 2013; Lee et al., 2010; Perini et al., 2014). In addition, there are reports that *VEGF* rs2010963 (+405G/C) genotype and *VEGF* rs1570360 (-1154A/A) genotype is associated with RIF (Boudjenah et al., 2012; Goodman et al., 2008).

Other *VEGF* SNP, including rs833061 (-460T>C), rs25648 (-7C>T) and rs3025020 (-583C>T), have been reported to affect VEGF expression and activity (Al-Habboubi et al., 2011; Almawi et al., 2013). However, there are a limited number of studies that evaluate the association between *VEGF* polymorphism and RIF development in Korean subjects.

The objective of this study was to elucidate the association between *VEGF* polymorphisms (rs833061 T > C and rs25648 C > T, and rs3025020 C > T) and RIF in Korean subjects.

Materials and methods

Study participants

This study was a prospective case-control study. Blood samples were collected from 119 subjects with RIF (mean age ± SD, 34.22 ± 3.35 years), and 236 control participants (mean age ± SD, 33.36 ± 5.81). The study population consisted of participants recruited from the Department of Obstetrics and Gynecology of CHA Bundang Medical Center, CHA University (Seongnam-si, Korea) between March 2010 and December 2012. The Institutional Review Board of CHA Bundang Medical Center reviewed and approved the study on 23 February 2010 (reference no. PBC09–120). Informed consent was obtained from all participants.

RIF was defined as failure to achieve pregnancy after two completed fresh IVF–embryo transfer (IVF–ET) cycles with more than 10 cleaved embryos. Serum human chorionic gonadotrophin (HCG) concentrations were less than 5 mIU/ml 14 days after embryo transfer. All transferred embryos were examined by the embryologist before transfer and judged to be of good quality. Both the male and female partner of couples experiencing recurrent implantation failure were evaluated. The following exclusion criteria are commonly adopted to diagnose RIF. Subjects who were diagnosed with RIF due to anatomic, chromosomal, hormonal, infectious, autoimmune or thrombotic causes were excluded from the study group. Anatomical abnormalities were evaluated using several imaging methods, including sonography, hysterosalpingogram, hysteroscopy, computerized tomography and magnetic resonance imaging. Karyotyping was conducted using standard protocols. By measuring the concentrations of prolactin, thyroid-stimulating hormone, free T4, follicle-stimulating hormone, luteinizing hormone and progesterone in peripheral blood, hormonal causes were excluded, including hyperprolactinaemia, luteal insufficiency and thyroid disease. Lupus anticoagulant and anticardiolipin antibodies were examined for autoimmune causes such as lupus and antiphospholipid syndrome. Thrombotic causes were defined as thrombophilia, and were evaluated by protein C and protein S deficiencies and by the presence of anti-α2 glycoprotein antibody. Semen analysis, karyotyping and hormonal assays, including oestradiol, testosterone, FSH and LH, were performed for male partners. Among the initial 152 patients who were enrolled for the study, 33 subjects who had Müllerian anomaly, hypothyroidism, chromosomal abnormality or antiphospholipid syndrome were excluded from the patient group, leaving 119 patients for the study.

Enrolment criteria for the control group included regular menstrual cycles, normal karyotype (46XX), a history of at least one naturally conceived pregnancy and no history of pregnancy loss including abortion history. Data were collected identically for all groups.

Genotyping

Peripheral blood samples were collected for genotyping. Genomic DNA was extracted from anticoagulated peripheral blood using a G-DEX for blood Genomic DNA Extraction Kit (iNtRON Biotechnology, Seongnam, Korea). Nucleotide changes were examined using PCR-restriction fragment length polymorphism (PCR-RFLP) analyses (rs3025020) or real-time PCR (rs833061, rs25648). *VEGF* rs833061T>C, rs25648 C > T and rs3025020C>T were selected using the human genome SNP database (dbSNP: www.ncbi.nlm.nih.gov/snp). The PCR primers used in this study are shown in **Supplementary Table S1**. Restriction enzyme digestion for PCR-RFLP was performed using the following enzyme and conditions: *Msp*I (*VEGF* rs3025020; New England BioLabs, Ipswich, MA) at 37°C for 16 h.

Three independent investigators confirmed the genotype results determined by RFLP on identical samples with matching conditions. Genotypes were further confirmed by sequencing 20% of the samples by random selection. Real-time PCR was performed using the allelic discrimination method. TaqMan SNP genotyping assays (Applied Biosystems, Foster City, CA) were used. TaqMan probes were available directly from Applied Biosystems, and reactions were performed in 10 μl on a StepOne Real-time PCR System as recommended by the manufacturer (Applied Biosystems).

Statistical analyses

Differences in genotype and allele combination frequencies between RIF subjects and controls were compared using multivariate logistic regression and Fisher's exact tests, respectively. Allele frequencies were calculated to identify deviations from Hardy–Weinberg equilibrium (HWE) using $P = 0.05$ as a threshold. Since the inferences of this study are derived as a result of multiple testing, the Benjamini and Hochberg strategy has been adopted, which effectively reduces the potential impact of spurious significant results (Benjamini and Hochberg, 1995). Odds ratios (OR) and 95% confidence intervals (CI) were used to measure the strength of association between genotypes and RIF. Two-tailed *P*-values less than 0.05 were considered statistically significant.

Gene–gene interactions among SNP loci were analysed using the log-linear model-based multifactor dimensionality reduction (LM-MDR) and MDR software version 2.0 (available at www.epistasis.org) (Hahn et al., 2003; Lee et al., 2007; Ritchie et al., 2001). The allele combination frequencies for the selected models by MDR analysis were estimated using the HAPSTAT program version 3.0 (www.bios.unc.edu/~lin/hapstat). Statistical analyses were performed using GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA) and StatsDirect software version 2.4.4 (StatsDirect Ltd., Altrincham, UK). The statistical power was calculated using G*POWER 3.0 (Institut für Psychologie, Christian-Albrechts-Universität Kiel, Kiel, Germany).

Results

The demographic characteristics of study participants are presented in **Table 1**. The genotypic distribution and allele frequencies of rs833061, rs25648 and rs3025020 were determined. **Table 2** represents the distribution of the rs833061, rs25648 and rs3025020 genotypes and allelic frequencies for all groups. All *VEGF* polymorphic sites that were analysed showed polymorphisms, which occurred in HWE in all groups. The minor allele frequencies of the three polymorphisms (rs833061 T > C, rs25648 C > T, rs3025020 C > T) were 23.1%, 6.4% and 35.2% in control group participants and 32.4%, 12.2% and 34.5% in the RIF group, respectively. The rs833061 TC+CC genotype was associated with an increased prevalence of RIF (OR = 1.813, 95% CI = 1.161–2.831, $P = 0.009$). The frequency of the rs25648 CT genotype was significantly higher in the RIF group compared with control (OR = 2.213, 95% CI = 1.254–3.903, $P = 0.005$). For the rs3025020 genotype, there was no statistically significant difference between the control and RIF groups.

Next, combination analyses were performed for these *VEGF* gene polymorphisms (**Table 3**). The combination analyses revealed that the rs833061 TC/rs25648 CT and rs833061 TC+CC/rs3025020 TT genotypes were more frequently observed in RIF patients compared with control subjects (OR = 2.130, 95% CI = 1.092–4.156, $P = 0.025$, OR = 4.261, 95% CI = 1.163–15.620, $P = 0.028$).

The rs833061 C allele was associated with RIF. According to the combination analyses, this association was maintained when the rs833061 genotype was combined with rs25648 or rs3025020. Therefore, both individual and combined analyses suggested that the rs833061 TC genotype is associated with RIF development. MDR-based allele combination analyses of the three *VEGF* polymorphisms were conducted to examine whether there are synergistic effects on RIF risk by polymorphic site interactions (**Table 4**). Three allele combination analyses indicated that rs833061/rs25648/rs3025020 C-C-T and C-T-T genotypes exerted synergistic effects on increased RIF risk (OR = 1.991, 95% CI = 1.056–3.753, $P = 0.031$ and OR = 17.880, 95% CI = 0.953–335.300, $P = 0.013$, respectively). Among the models of two loci, the rs833061/rs3025020 C-T genotype and rs833061/rs25648 C-T genotype were associated with increased RIF prevalence (OR = 2.741, 95% CI = 1.460–5.145, $P = 0.001$ and OR = 1.984, 95% CI = 1.106–3.560, $P = 0.020$, respectively).

Furthermore, power analyses were conducted to detect associations in this case-control study at the 5% significance level. The power varies for an OR by the proportion of exposure in the control group. The sample size of 236 controls and 119 subjects in the study group could have statistical power to detect an OR of 2.0 when the proportions of

Table 1 Clinical characteristics of idiopathic recurrent implantation failure (RIF) patients and control subjects.

Characteristic	*Control* (n = *236*)	*RIF* (n = *119*)
Age (years, mean ± SD) [a]	33.36 ± 5.81	34.22 ± 3.35
BMI (kg/m^2, mean ± SD) [a]	21.72 ± 3.41	20.96 ± 2.52
Previous pregnancy losses (*n*, mean ± SD)	None	NA
Previous implantation failure (*n*, mean ± SD)	None	4.75 ± 2.29
Live birth (*n*, mean ± SD)	1.71 ± 0.71	None
Mean gestational age (week, mean ± SD)	39.28 ± 1.67	None

BMI = body mass index; NA = not applicable.
[a]No statistically significant difference (Student *t*-test).

Table 2 Genotype frequencies of *VEGF* (rs833061T>C, rs25648C>T and rs3025020C>T) polymorphisms in recurrent implantation failure patients and control subjects.

Genotype	*Control (n = 236)*	*RIF (n = 119)*	*OR (95% CI)*	P[a]	P[b]	*Statistical power (%)*
VEGF rs833061T>C	no. (%)	no. (%)				
TT	136 (57.6)	51 (42.9)	1.000 (reference)	–	–	–
TC	91 (38.6)	59 (49.6)	1.729 (1.092–2.737)	0.019	0.029	66.6
CC	9 (3.8)	9 (7.6)	2.667 (1.002–7.095)	0.043	NS	48.6
TC+CC	–	–	1.813 (1.161–2.831)	0.009	0.014	74.7
HWE *P*-value	0.189	0.148	–	–	–	–
VEGF rs25648C>T						
CC	206 (87.3)	90 (75.6)	1.000 (reference)	–	–	–
CT	30 (12.7)	29 (24.4)	2.213 (1.254–3.903)	0.005	0.016	75.6
TT	–	–	NA	NA	NA	–
CT+TT	–	–	2.213 (1.254–3.903)	0.005	0.014	–
HWE *P*-value	0.297	0.13	–	–	–	–
VEGF rs3025020C>T						
CC	94 (39.8)	50 (42.0)	1.000 (reference)	–	–	–
CT	118 (50.0)	56 (47.1)	0.892 (0.559–1.425)	NS	NS	7.8
TT	24 (10.2)	13 (10.9)	1.018 (0.478–2.172)	NS	NS	4.9
CT+TT	–	–	0.914 (0.584–1.430)	NS	NS	6.8
HWE *P*-value	0.138	0.648	–	–	–	–

CI = confidence interval; HWE = Hardy–Weinberg equilibrium; NA = not applicable; NS = not significant; OR = odds ratio; RIF = recurrent implantation failure.
[a]Chi-square test.
[b]False discovery rate-adjusted *P*-value for multiple hypotheses testing using the Benjamini–Hochberg method.

exposure in the control were 20% (power = 76.1%), 30% (power = 75.1%) and 40% (power = 82.4%). Additionally, this sample size has reasonable power to detect an OR of 0.5 for the proportions of 20% (power = 61.6%), 30% (power = 75.1%) and 40% (power = 82.4%) in the control.

Discussion

This study evaluated the possible association between three *VEGF* SNP (rs3025020, rs833061 and rs25648) and RIF development in Korean women. The SNP evaluated in the study were selected because they were reported to be correlated with VEGF protein expression and few studies have evaluated their association with RIF. The data compared genotype frequencies of these polymorphisms and demonstrated that the rs833061 C allele and rs25648 T allele were associated with RIF development.

Successful implantation is a complex process between mother and embryo. There are several factors affecting that complicated process, including uterine factors, immunologic factors, thrombophilic factors and embryonic factors. In this study, the subjects who were diagnosed with uterine anomaly and underlying medical disease were excluded. In addition, an embryologist evaluated embryo quality for IVF. Therefore, in this study, we may assume that *VEGF* polymorphism is confined to endometrial factors for implantation failure.

The intrauterine concentration of VEGF during the menstrual cycle was determined in humans (Licht et al., 2003). The cycle-dependent nature of VEGF was observed with increasing concentrations during the late secretory and premenstrual phases. In addition, VEGF concentration was correlated with the decidualization marker of endometrium, IGFBP-1. Sugino et al. also examined the expression of VEGF and its receptors throughout the menstrual cycle and in early pregnancy (Sugino et al., 2002). VEGF and its receptor expression increased mid secretary phase compared with proliferative phase during normal menstrual cycle. Decidual cells strongly expressed VEGF in early pregnancy. The authors concluded that VEGF contributes to successful implantation and maintenance of pregnancy by increasing vascular permeability or forming vascular network in the deciduas. Kapiteijin et al. cultured human embryos in VEGF conditioned media (Kapiteijn et al., 2006). The study showed that VEGF induced human embryo to stimulate endometrial angiogenesis in an in-vitro model. Combining these results, VEGF is a key regulator in angiogenesis and decidualization of the endometrium, which are essential processes for successful pregnancy.

The SNP rs833061 is located in the promoter region of *VEGF*. Stevens et al. suggested that rs833061 genotype alter promoter activity and responsiveness in their haplotype analysis (Stevens et al., 2003). Almawi et al. reported that there was a progressive decline in serum VEGF concentration as the subjects had more rs833061 C allele (Almawi et al., 2013). In addition, the authors demonstrated that serum VEGF concentration was significantly reduced in subjects diagnosed with recurrent pregnancy loss than in control subjects. This observation is consistent with our result.

The rs25648 is located in the 5′ untranslated region of the gene. The TT genotype of the rs25648 was associated with a higher concentration of serum VEGF in the southern Italy population (Ruggiero et al., 2011). It was reported that CT and TT genotypes of rs25648 resulted in higher levels of *VEGF* mRNA expression in colorectal cancer (Yamamori et al., 2004). These

Table 3 Genotype combination analyses of *VEGF* (rs833061T>C, rs25648C>T, rs3025020C>T) polymorphisms in recurrent implantation failure.

Combined genotype	*Control (n = 236)*	*RIF (n = 119)*	*OR (95% CI)*	P-*value*[a]	P-*value*[c]	*Statistical power (%)*
VEGF rs833061T>C/rs25648C>T						
TT/CC	133 (56.4)	57 (47.9)	1.000 (reference)	–	–	–
TT/CT	3 (1.3)	0 (0.0)	0.332 (0.017–6.530)	NS[b]	NS	19.4
TT/TT	0 (0.0)	0 (0.0)	NA	NA	NA	–
TT/CT+TT	–	–	0.332 (0.017–6.530)	NS[b]	NS	19.4
TC/CC	68 (28.8)	32 (26.9)	1.098 (0.651–1.851)	NS	NS	6.5
TC/CT	23 (9.7)	21 (17.6)	2.130 (1.092–4.156)	0.025	NS	60.4
TC/TT	0 (0.0)	0 (0.0)	NA	NA	NA	–
TC/CT+TT	–	–	2.130 (1.092–4.156)	0.025	NS	–
CC/CC	5 (2.1)	6 (5.0)	2.800 (0.821–9.551)	NS	NS	30.6
CC/CT	4 (1.7)	3 (2.5)	1.750 (0.379–8.075)	NS[b]	NS	6.8
CC/TT	0 (0.0)	0 (0.0)	NA	NA	NA	
CC/CT+TT	–	–	1.750 (0.379–8.075)	NS[b]	NS	6.8
VEGF rs833061T>C/rs3025020C>T						
TT/CC	49 (20.8)	23 (19.3)	1.000 (reference)	–	–	–
TC/CC	40 (16.9)	24 (20.2)	1.278 (0.630–2.596)	NS	NS	12.7
CC/CC	5 (2.1)	3 (2.5)	1.278 (0.281–5.816)	NS[b]	NS	4.2
TC+CC/CC	–	–	1.278 (0.643–2.543)	NS	NS	13.6
TT/CT	67 (28.4)	23 (19.3)	0.731 (0.369–1.452)	NS	NS	23.7
TC/CT	47 (19.9)	28 (23.5)	1.269 (0.642–2.509)	NS	NS	13.4
CC/CT	4 (1.7)	5 (4.2)	2.663 (0.653–10.860)	NS[b]	NS	21.7
TC+CC/CT	–	–	1.379 (0.712–2.671)	NS	NS	22.1
TT/TT	20 (8.5)	5 (4.2)	0.533 (0.178–1.597)	NS	NS	30.2
TC/TT	4 (1.7)	7 (5.9)	3.728 (0.991–14.03)	NS[b]	NS	45.8
CC/TT	–	1 (0.8)	6.319 (0.248–161.200)	NS[b]	NS	89.1
TC+CC/TT	–	–	4.261 (1.163–15.620)	0.028[b]	NS	57.8
VEGF rs25648C>T/rs3025020C>T						
CC/CC	77 (32.6)	38 (31.9)	1.000 (reference)	–	–	–
CT/CC	17 (7.2)	12 (10.1)	1.430 (0.621–3.297)	NS	NS	11.9
TT/CC	0 (0.0)	0 (0.0)	NA	NA	NA	–
CT+TT/CC	–	–	1.430 (0.621–3.297)	NS	NS	11.9
CC/CT	105 (44.5)	45 (37.8)	0.868 (0.515–1.464)	NS	NS	9.8
CC/CT	13 (5.5)	11 (9.2)	1.715 (0.703–4.185)	NS	NS	19.2
TT/CT	0 (0.0)	0 (0.0)	NA	NA	NA	–
CT+TT/CT	–	–	1.715 (0.703–4.185)	NS	NS	–
CC/TT	24 (10.2)	12 (10.1)	1.013 (0.458–2.243)	NS	NS	4.9
CT/TT	0 (0.0)	1 (0.8)	6.039 (0.240–151.800)	NS[b]	NS	<1
TT/TT	0 (0.0)	0 (0.0)	NA	NA	NA	–
CT+TT/TT	–	–	6.039 (0.240–151.800)	NS[b]	NS	–

CI = confidence interval; NA = not applicable; NS = not significant; OR = odds ratio; RIF = recurrent implantation failure.
[a]Chi-square test.
[b]Fisher's exact test.
[c]False discovery rate-adjusted *P*-value for multiple hypotheses testing using the Benjamini–Hochberg method.

results suggested that rs25648 T genotypes increased serum VEGF concentration by increasing *VEGF* mRNA expression. According to these results, it could be expected that the subjects with rs25648 C genotype were more likely to experience RIF. However, the patients with rs25648 CT+TT genotypes showed an increased risk of RIF development in this study (OR = 2.213, 95% CI = 1.254–3.903, $P = 0.005$). These results were not consistent with the previous observations. Similar results were observed in other studies. In 2011, Al-Habboubi et al. evaluated the distribution of *VEGF* polymorphism and the effect of those SNP on VEGF expression in serum in Arab populations (Al-Habboubi et al., 2011). The authors demonstrated that rs2010963 genotypes did not affect serum VEGF concentration. However, Boudjenah et al. reported that *VEGF* rs2010963 (+405G/C) genotype was higher in women with implantation failure after IVF than in control (Boudjenah et al., 2012). Goodman et al. showed that *VEGF* rs1570360 (-1154A/A) genotype was more frequently observed in women experiencing recurrent implantation failure than in fertile women (Goodman et al., 2008). However, there was no difference in serum VEGF concentration among rs1570360 GG, GA and AA genotypes (Al-Habboubi et al., 2011).

Table 4 Haplotype frequencies of *VEGF* (rs833061T>C, rs25648C>T, rs3025020C>T) polymorphisms in recurrent implantation failure.

Haplotype	*Control (2n = 472)*	*RIF (2n = 238)*	*OR (95% CI)*	P-*value*[a]	P-*value*[c]	*Statistical power (%)*
VEGF rs833061T>C/rs25648C>T/ rs3025020C>T						
T-C-C	219 (46.4)	110 (46.2)	1.000 (reference)	–	–	–
T-T-C	0 (0.0)	0 (0.0)	NA	NA	NA	–
C-C-C	60 (12.7)	25 (10.5)	0.830 (0.493–1.395)	NS	NS	12.7
C-T-C	27 (5.7)	20 (8.4)	1.475 (0.792–2.747)	NS	NS	21.2
T-C-T	140 (29.7)	57 (23.9)	0.811 (0.552–1.190)	NS	NS	22.4
T-T-T	3 (0.6)	0 (0.0)	0.284 (0.015–5.546)	NS[b]	NS	20.9
C-C-T	22 (4.7)	22 (9.2)	1.991 (1.056–3.753)	0.031	NS	53.9
C-T-T	0 (0.0)	4 (1.7)	17.880 (0.953–335.300)	0.013[b]	NS	49.0
VEGF rs833061T>C/rs3025020C>T						
T-C	216 (45.8)	112 (47.1)	1.000 (reference)	–	–	–
C-C	90 (19.1)	44 (18.5)	0.943 (0.615–1.445)	NS	NS	6.1
T-T	147 (31.1)	55 (23.1)	0.722 (0.491–1.060)	NS	NS	45.8
C-T	19 (4.0)	27 (11.3)	2.741 (1.460–5.145)	0.001	0.004	86.5
VEGF rs25648C>T/rs3025020C>T						
C-C	276 (58.5)	136 (57.1)	1.000 (reference)	–	–	–
T-C	30 (6.4)	20 (8.4)	1.353 (0.741–2.470)	NS	NS	15.2
C-T	166 (35.2)	78 (32.8)	0.954 (0.680–1.338)	NS	NS	6.0
T-T	0 (0.0)	4 (1.7)	18.230 (0.974–341.300)	0.013[b]	0.039	50.9
VEGF rs833061T>C/rs25648C>T						
T-C	359 (76.1)	167 (70.2)	1.000 (reference)	–	–	–
T-T	4 (0.8)	0 (0.0)	0.239 (0.013–4.458)	NS[b]	NS	28.3
C-C	83 (17.6)	47 (19.7)	1.217 (0.814–1.820)	NS	NS	15.5
C-T	26 (5.5)	24 (10.1)	1.984 (1.106–3.560)	0.020	NS	59.6

CI = confidence interval; NA = not applicable; NS = not significant; OR = odds ratio; RIF = recurrent implantation failure.
[a]Chi-square test.
[b]Fisher's exact test.
[c]False discovery rate-adjusted *P*-value for multiple hypotheses testing using the Benjamini–Hochberg method.

There are several possible explanations for these discrepancies among *VEGF* SNP, serum VEGF concentrations and RIF. The *VEGF* SNP show different effects on serum VEGF concentrations according to the population (Ruggiero et al., 2011). Three isolated populations in Italy were examined to find specific polymorphisms in *VEGF* gene associated with a variation in protein concentration. In the study, the authors found that none of the SNP influencing serum VEGF concentrations in one population was associated with the others. In addition, there was a discrepancy between rs833061 genotypes and serum VEGF concentration according to the researchers even in the similar Bahrain population (Al-Habboubi et al., 2011; Almawi et al., 2013). This study did not examine the VEGF protein concentrations in serum according to the *VEGF* SNP. There are not enough data about the relationship between *VEGF* genotypes and serum VEGF protein expression. Secondly, there are ethnic differences in MAF of *VEGF* SNP (Xu et al., 2012). Recently, endocrine gland-derived vascular endothelial growth factor has been identified as a specific placental angiogenic factor involved in physiological and pathological processes such as recurrent pregnancy loss (LeCouter et al., 2001; Su et al., 2010). The effect of *VEGF* gene polymorphism on endometrium should be considered and evaluated to answer the question of how *VEGF* SNP show various effects without influencing serum VEGF concentrations and RIF development. Although there are few reports demonstrating how *VEGF* SNP affect VEGF expression locally in endometrium, we suggest that *VEGF* SNP may locally affect angiogenesis and decidualization in endometrium during implantation and pregnancy maintenance in early pregnancy.

There are several limitations in the study. Firstly, the serum VEGF concentrations in our population were not examined. There are few studies evaluating the contribution of each *VEGF* polymorphism to serum VEGF expression in Korea (Kim et al., 2009). In that study, the authors showed that *VEGF* 936 C/T polymorphism was associated with plasma concentrations of VEGF in the patients with type 2 diabetes. Secondly, functional studies for *VEGF* SNP were not performed to elucidate the RIF-related pathogenesis. Although several studies have reported an association between *VEGF* polymorphisms and RIF, few have evaluated the pathogenesis by which *VEGF* polymorphisms affect implantation and early pregnancy. This study could not propose a detailed pathogenesis by which *VEGF* polymorphism affects successful implantation. Thirdly, VEGF expression depending on *VEGF* SNP genotypes in local tissue such as decidua was not examined. The VEGF expression in decidua is more important than in serum. Lastly, the sample size of RIF group is not big enough to sufficiently provide enough statistical power in demonstrating associations between *VEGF* SNP and RIF.

To the best of our knowledge, this is the first study to investigate the association between rs833061, rs3025020 and rs25648 and the prevalence of RIF in a Korean population. In this study, we demonstrated an association between the *VEGF*

polymorphisms rs833061 and rs25648 and RIF development. The data highlight the possibility of *VEGF* polymorphisms as genetic markers to predict RIF occurrence. However, future studies of *VEGF* polymorphisms with larger sample sizes will be required to confirm these results. Additionally, our results warrant additional functional studies to elucidate the functional role of *VEGF* polymorphisms in the pathogenesis of RIF.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.rbmo.2015.10.010.

References

Al-Habboubi, H.H., Sater, M.S., Almawi, A.W., Al-Khateeb, G.M., Almawi, W.Y., 2011. Contribution of VEGF polymorphisms to variation in VEGF serum levels in a healthy population. Eur. Cytokine Netw. 22, 154–158.

Almawi, W.Y., Saldanha, F.L., Mahmood, N.A., Al-Zaman, I., Sater, M.S., Mustafa, F.E., 2013. Relationship between VEGFA polymorphisms and serum VEGF protein levels and recurrent spontaneous miscarriage. Hum. Reprod. 28, 2628–2635.

Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. 57, 289–300.

Boudjenah, R., Molina-Gomes, D., Wainer, R., de Mazancourt, P., Selva, J., Vialard, F., 2012. The vascular endothelial growth factor (VEGF) +405 G/C polymorphism and its relationship with recurrent implantation failure in women in an IVF programme with ICSI. J. Assist. Reprod. Genet. 29, 1415–1420.

Cheng, D., Hao, Y., Zhou, W., Ma, Y., 2013. Vascular endothelial growth factor +936C/T, -634G/C, -2578C/A, and -1154G/A polymorphisms with risk of preeclampsia: a meta-analysis. PLoS ONE 8, e78173.

Choi, T.Y., Lee, H.M., Park, W.K., Jeong, S.Y., Moon, H.S., 2014. Spontaneous abortion and recurrent miscarriage: a comparison of cytogenetic diangosis in 250 cases. Obstet. Gynecol. Sci. 57, 518–525.

Coughlan, C., Ledger, W., Wang, Q., Liu, F., Demirol, A., Gurgan, T., Cutting, R., Ong, K., Sallam, H., Li, T.C., 2014. Recurrent implantation failure: definition and management. Reprod. Biomed. Online 28, 14–38.

Goodman, C., Jeyendran, R.S., Coulam, C.B., 2008. Vascular endothelial growth factor gene polymorphism and implantation failure. Reprod. Biomed. Online 16, 720–723.

Hahn, L.W., Ritchie, M.D., Moore, J.H., 2003. Multifactor dimensionality reduction software for detecting gene-gene and gene-environment interactions. Bioinformatics 19, 376–382.

Kapiteijn, K., Koolwijk, P., van der Weiden, R.M., van Nieuw Amerongen, G., Plaisier, M., van Hinsbergh, V.W., Helmerhorst, F.M., 2006. Human embryo-conditioned medium stimulates in vitro endometrial angiogenesis. Fertil. Steril. 85 (Suppl. 1), 1232–1239.

Kim, H.W., Ko, G.J., Kang, Y.S., Lee, M.H., Song, H.K., Kim, H.K., Cha, D.R., 2009. Role of the *VEGF* 936 C/T polymorphism in diabetic microvascular complications in type 2 diabetic patients. Nephrology 14, 681–688.

LeCouter, J., Kowalski, J., Foster, J., Hass, P., Zhang, Z., Dillard-Telm, L., Frantz, G., Rangell, L., DeGuzman, L., Keller, G.A., Peale, F., Gurney, A., Hillan, K.J., Ferrara, N., 2001. Identification of an angiogenic mitogen selective for endocrine gland endothelium. Nature 412, 877–884.

Lee, H.H., Hong, S.H., Shin, S.J., Ko, J.J., Oh, D., Kim, N.K., 2010. Association study of vascular endothelial growth factor polymorphisms with the risk of recurrent spontaneous abortion. Fertil. Steril. 93, 1244–1247.

Lee, S.Y., Chung, Y., Elston, R.C., Kim, Y., Park, T., 2007. Log-linear model-based multifactor dimensionality reduction method to detect gene gene interactions. Bioinformatics 23, 2589–2595.

Licht, P., Russu, V., Lehmeyer, S., Wissentheit, T., Siebzehnrubl, E., Wildt, L., 2003. Cycle dependency of intrauterine vascular endothelial growth factor levels is correlated with decidualization and corpus luteum function. Fertil. Steril. 80, 1228–1233.

Perini, J.A., Cardoso, J.V., Berardo, P.T., Vianna-Jorge, R., Nasciutti, L.E., Bellodi-Privato, M., Machado, D.E., Abrao, M.S., 2014. Role of vascular endothelial growth factor polymorphisms (-2578C>A, -460 T>C, -1154G>A, +405G>C and +936C>T) in endometriosis: a case-control study with Brazilians. BMC Womens Health 14, 117.

Polanski, L.T., Baumgarten, M.N., Quenby, S., Brosens, J., Campbell, B.K., Raine-Fenning, N.J., 2014. What exactly do we mean by "recurrent implantation failure"? A systematic review and opinion. Reprod. Biomed. Online 28, 409–423.

Ritchie, M.D., Hahn, L.W., Roodi, N., Bailey, L.R., Dupont, W.D., Parl, F.F., Moore, J.H., 2001. Multifactor-dimensionality reduction reveals high-order interactions among estrogen-metabolism genes in sporadic breast cancer. Am. J. Hum. Genet. 69, 138–147.

Ruggiero, D., Dalmasso, C., Nutile, T., Sorice, R., Dionisi, L., Aversano, M., Broet, P., Leutenegger, A.L., Bourgain, C., Ciullo, M., 2011. Genetics of VEGF serum variation in human isolated populations of cilento: importance of VEGF polymorphisms. PLoS ONE 6, e16982.

Stevens, A., Soden, J., Brenchley, P.E., Ralph, S., Ray, D.W., 2003. Haplotype analysis of the polymorphic human vascular endothelial growth factor gene promoter. Cancer Res. 63, 812–816.

Su, M.T., Lin, S.H., Lee, I.W., Chen, Y.C., Hsu, C.C., Pan, H.A., Kuo, P.L., 2010. Polymorphisms of endocrine gland-derived vascular endothelial growth factor gene and its receptor genes are associated with recurrent pregnancy loss. Hum. Reprod. 25, 2923–2930.

Sugino, N., Kashida, S., Karube-Harada, A., Takiguchi, S., Kato, H., 2002. Expression of vascular endothelial growth factor (VEGF) and its receptors in human endometrium throughout the menstrual cycle and in early pregnancy. Reproduction 123, 379–387.

Xu, S., Wu, W., Sun, H., Lu, J., Yuan, B., Xia, Y., De Moor, B., Marchal, K., Wang, X., Xu, P., Cheng, W., 2012. Association of the vascular endothelial growth factor gene polymorphisms (-460C/T, +405G/C and +936T/C) with endometriosis: a meta-analysis. Ann. Hum. Genet. 76, 464–471.

Yamamori, M., Sakaeda, T., Nakamura, T., Okamura, N., Tamura, T., Aoyama, N., Kamigaki, T., Ohno, M., Shirakawa, T., Gotoh, A., Kuroda, Y., Matsuo, M., Kasuga, M., Okumura, K., 2004. Association of VEGF genotype with mRNA level in colorectal adenocarcinomas. Biochem. Biophys. Res. Commun. 325, 144–150.

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