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GENETICS

The −2549 insertion/deletion polymorphism in the promoter region of the *VEGFA* gene in couples with idiopathic recurrent spontaneous abortion

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Abstract
Purpose The vascular endothelial growth factor A (*VEGFA*) is crucial for normal vasculogenesis and angiogenesis during pregnancy, and alterations in the *VEGFA* gene expression were detected in women with idiopathic recurrent spontaneous abortion (IRSA) and spontaneously aborted conceptuses. Our aim was to evaluate whether there is an association between the functional −2549 insertion/deletion (I/D) polymorphism in the promoter region of the *VEGFA* gene and IRSA in reproductive couples.
Methods We performed a case-control study involving 149 women and their 140 partners with three or more IRSA and 149 control women and men. Allele-specific polymerase chain reaction was used for genotyping.
Results We found no association of the −2549 I/D polymorphism with IRSA in women. However, men with the DD genotype have a 1.75-fold increased risk of IRSA compared with men carrying the ID and II genotypes (95 % confidence interval (CI)=1.05–2.93, P=0.032). In addition, the D allele in men contributes to a 1.42-fold increased risk of IRSA (95 % CI= 1.02–1.97, P=0.036) compared to men carrying the I allele.
Conclusions Our results indicate that the −2549 I/D polymorphism in the *VEGFA* gene in men might be associated with IRSA. Additional genetic association studies including both partners, as well as expression studies, are needed to elucidate the role of this polymorphism in IRSA.

Capsule The −2549 insertion/deletion polymorphism in the promoter region of the *VEGFA* gene in men might be associated with idiopathic recurrent spontaneous abortion.

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Keywords Genetic polymorphism · Pregnancy · Recurrent spontaneous abortion · Vascular endothelial growth factor A

Introduction

Recurrent spontaneous abortion (RSA) is a pregnancy complication that includes at least three consecutive spontaneous abortions [1]. Despite numerous researches, only antiphospholipid syndrome, structural uterine anomalies, and parental chromosomal anomalies have been confirmed as causes of RSA and can be identified in 40 % of couples [1]. Genetic variability has been proposed as a predisposing factor for idiopathic RSA (IRSA), and over a hundred candidate genes were tested [2]. A large number of these genes are involved in the regulation of angiogenesis at the feto-placental unit.

Establishment of proper and sufficient blood flow in the placenta is crucial for successful pregnancy outcome and depends on appropriate vasculogenesis and angiogenesis, which are mediated by diverse angiogenic factors, including vascular endothelial growth factor A (*VEGFA*) [3]. Vascular endothelial growth factor A is a major angiogenic factor secreted by both maternal and fetal/trophoblastic cells, promoting endothelial cell proliferation and survival, vascular permeability, and hematopoesis [4, 5]. It has various functions in human reproduction, including the regulation of fetal and placental angiogenesis, as well as gametogenesis and (pre)decidualization [6]. The levels of *VEGFA* messenger RNA (mRNA) and protein in the endometrium and placenta differ in normal and complicated pregnancies, including

IRSA, which might lead to abnormalities in angiogenesis during implantation, placentation, and early pregnancy [3, 6]. The altered gene expression in placenta suggests a potential contribution of both parental genomes.

The *VEGFA* gene is highly polymorphic, and more than 25 different polymorphisms have been described [7]. Some of these polymorphisms are functional and organized into haplotypes. Genetic association with IRSA was tested for 20 polymorphisms, among which, the most common are −1154 G/A, +936 C/T, −2578 C/A, and −634 G/C single-nucleotide polymorphisms (SNPs). However, results of individual studies are opposite. Meta-analyses indicate that +936 C/T, +583 T/C, and −634 G/C SNPs in women might be predisposing factors for IRSA, whereas the results for the −1154 G/A SNP are inconsistent [8–10].

Although SNPs have been widely investigated in IRSA and other reproductive disorders, there is an increasing interest in the research of insertion/deletion (I/D) polymorphisms, considering their contribution to genetic and phenotypic divergence and diversity [11, 12]. It is estimated that there are 1.6–2.5 million I/D polymorphisms in the human genome, making them an important source of genetic markers [12]. In the *VEGFA* gene, a functional I/D polymorphism is located at position −2549 in the promoter region [13]. Deletion of an 18 base pair (bp) long sequence (D allele) leads to a 1.95-fold increased transcriptional activity compared to the allele containing the insertion (I allele) [14].

Considering the important roles of *VEGFA* during pregnancy and alterations in the *VEGFA* gene expression in IRSA women and spontaneously aborted conceptuses, we aimed to evaluate whether there is an association between the functional −2549 I/D polymorphism in the promoter region of the *VEGFA* gene and IRSA in Slovenian reproductive couples.

Subjects and methods

Subjects

We performed a case-control study involving IRSA couples and controls from the Slovenian population. The group of IRSA couples consisted of 149 women and their 140 male partners with three or more unexplained consecutive spontaneous abortions before the 22nd week of gestation. Exclusion criteria were chromosomal anomalies in either partner, endocrine or metabolic disorders, antiphospholipid syndrome, autoimmune disease or other systemic diseases, previous venous or arterial thrombosis, or structural uterine anomalies detected by ultrasonography and/or hysteroscopy. A total of 98 (65.8 %) of couples had no live births (primary IRSA), whereas 51 (34.2 %) had at least one live born child (secondary IRSA). Ninety-two percent of couples had the spontaneous abortions (SAs) in the first trimester and 8 % in the second trimester. Couples were described in more detail previously [15]. The control group consisted of 149 unrelated, healthy women and men with at least two live births and no history of SA or any other pregnancy complication. All women and men were recruited through the Institute of Medical Genetics, Department of Obstetrics and Gynaecology, UMC Ljubljana, Slovenia, and written informed consent was obtained from all individual participants included in the study. The study was approved by Slovenian and Croatian National Ethics' Committees.

Molecular genetic methods

Genomic DNA was isolated from peripheral blood leukocytes by standard procedures using a commercially available kit (Qiagen_FlexiGene kit; QIAGEN GmbH, Hilden, Germany). Genotyping was performed by allele-specific polymerase chain reaction (PCR) as described previously [16]. The primers were 5′CCTGGAGCGTTTTGGTTAAA3′ and 5′ATATAGGAAGCAGCTTGGAA3′. All PCRs were carried out in thermal cyclers (Mastercycle Personal, Eppendorf, Hamburg, Germany, and 2720 Thermal Cycler, Applied Biosystems, Carlsbad, CA, USA). The PCR products were visualized under ultraviolet light after electrophoresis on 2 % agarose gels, stained with GelRed™ (Olerup SSP®, Saltsjöbaden, Sweden). Sizes of PCR products were 234 bp for the I allele and 216 bp for the D allele (Fig. 1).

Statistical analysis

Statistical analyses were carried out using Statistica for Windows, version 10 (StatSoft Inc., Tulsa, OK, USA) and MedCalc for Windows, version 14.12.0 (MedCalc Software,

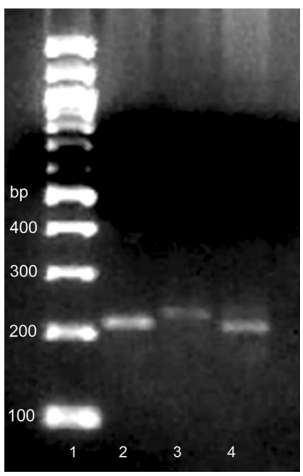

Fig. 1 Electrophoresis on 2 % agarose gel showing the PCR amplification products for *VEGFA* −2549 I/D polymorphism. *Lane 1*, 100-bp DNA ladder. *Lane 2*, homozygote for deletion allele (DD) (216 bp). *Lane 3*, homozygote for insertion allele (II) (234 bp). *Lane 4*, heterozygote (ID) (234 and 216 bp)

Table 1 Genotype and allele frequencies of the *VEGFA* −2549 I/D polymorphism in IRSA and control women

	Genotype/*N* (%)				Allele/*N* (%)		
Women	DD	ID	II	X^2; *P*	D	I	X^2; *P*
IRSA	54 (36.2)	63 (42.3)	32 (21.5)	0.96; 0.617	171 (57.4)	127 (42.6)	0.68; 0.410
Control	46 (30.9)	68 (45.6)	35 (23.5)		160 (53.7)	138 (46.3)	
Primary IRSA	35 (34.7)	41 (41.8)	22 (22.5)	0.16; 0.922	111 (56.6)	85 (43.4)	0.06; 0.811
Secondary IRSA	19 (37.3)	22 (43.1)	10 (19.6)		60 (58.8)	42 (41.2)	

IRSA idiopathic recurrent spontaneous abortion, X^2 chi-squared

Mariakerke, Belgium). Statistical power was calculated using DSS Researcher's Toolkit (www.dssresearch.com/toolkit/spcalc/power_p2.asp). Deviations from Hardy-Weinberg equilibrium (HWE) were calculated using the Simple Hardy-Weinberg Calculator–Court Lab (Washington State University College of Veterinary Medicine, Pullman, WA, USA). Pearson's chi-squared (X^2) test was used for the evaluation of differences in genotype and allele frequencies between study groups. Odds ratios (ORs) and 95 % confidence interval (CI) were calculated to test the associations of the −2549 I/D polymorphism with the risk of IRSA under dominant, recessive, and co-dominant genetic models. *P* values <0.05 were considered statistically significant.

Results

The power of the present study was 100 % to detect a 2-fold increase in the frequency of the I allele. There were no deviations from HWE in any of the study groups (data not shown).

Genotype and allele frequencies of the −2549 I/D polymorphism in the promoter region of the *VEGFA* gene in IRSA couples and controls, as well as couples with primary and secondary IRSAs, are shown in Tables 1 and 2. Statistically significant higher frequency of the D allele was found in IRSA men compared to controls (X^2=4.06, *P*=0.044), whereas the distribution of genotype frequencies were similar between these two groups. The differences in genotype and allele frequencies were not statistically significant between IRSA and control women, women with primary and secondary IRSAs, and men with primary and secondary IRSAs. The most frequent genotype combination in IRSA couples regardless of maternal and paternal origin was DD+ID (32.1 %).

The association of the −2549 I/D polymorphism with IRSA under dominant, recessive, and co-dominant genetic models is shown in Tables 3 and 4. We found no association of the polymorphism with IRSA in women under any model. However, men with the DD genotype have a 1.75-fold increased risk of IRSA compared with men carrying the ID and II genotypes (recessive model) (95 % CI=1.05–2.93, *P*=0.032). In addition, the D allele in men contributes to a 1.42-fold increased risk of IRSA (95 % CI=1.02–1.97, *P*=0.036) compared to the I allele. Finally, there was no association between the −2549 I/D polymorphism in men and primary or secondary IRSA under any genetic model.

Discussion

In the present case-control study, we tested the genetic association between the −2549 I/D polymorphism in the promoter region of the *VEGFA* gene and IRSA in Slovenian reproductive couples. Our results indicate that the DD genotype in men is associated with a 1.75-fold increased risk of IRSA compared to men carrying the ID and II genotypes, making it a potential predisposing factor for IRSA.

The −2549 I/D polymorphism has been extensively studied in many diseases. The DD genotype was found to be associated with breast cancer [17], diabetic retinopathy [18], diabetic nephropathy [19], and Kawasaki disease [20], whereas the ID genotype was associated with systemic sclerosis [21]. However, the association of the −2549 I/D polymorphism

Table 2 Genotype and allele frequencies of the *VEGFA* −2549 I/D polymorphism in IRSA and control men

	Genotype/*N* (%)				Allele/*N* (%)		
Men	DD	ID	II	X^2; *P*	D	I	X^2; *P*
IRSA	49 (35.0)	58 (41.4)	33 (23.6)	4.75; 0.093	156 (55.7)	124 (44.3)	4.06; 0.044
Control	35 (23.5)	70 (47.0)	44 (29.5)		140 (47.0)	158 (53.0)	
Primary IRSA	35 (38.5)	38 (41.7)	18 (19.8)	2.48; 0.289	108 (59.3)	74 (40.7)	2.37; 0.124
Secondary IRSA	14 (28.6)	20 (40.8)	15 (30.6)		48 (49.0)	50 (51.0)	

IRSA idiopathic recurrent spontaneous abortion, X^2 chi square

Table 3 Association of the *VEGFA* −2549 I/D polymorphism with IRSA in women

VEGFA I/D genetic model		W_{IRSA} vs. W_C		$W_{Primary\ IRSA}$ vs. $W_{Secondary\ IRSA}$	
		OR (95 % CI)	*P*	OR (95 % CI)	P
Dominant	DD+ID vs. II	1.12 (0.65–1.93)	0.677	0.84 (0.36–1.95)	0.689
Recessive	DD vs. ID+II	1.27 (0.79–2.06)	0.327	0.93 (0.46–1.89)	0.853
Co-dominant	DD vs. II	1.28 (0.69–2.39)	0.429	0.84 (0.33–2.13)	0.709
Alleles	DD vs. ID	1.27 (0.75–2.13)	0.374	0.99 (0.46–2.12)	0.976
	II vs. ID	0.99 (0.55–1.78)	0.965	1.18 (0.47–2.93)	0.721
	D vs. I	1.16 (0.84–1.60)	0.365	0.91 (0.56–1.48)	0.717

95 % CI 95 % confidence interval, *OR* odds ratio, W_C control women, W_{IRSA} women with idiopathic recurrent spontaneous abortion, $W_{Primary\ IRSA}$ women with primary idiopathic recurrent spontaneous abortion, $W_{Secondary\ IRSA}$ women with secondary idiopathic recurrent spontaneous abortion

with IRSA was previously tested in only one study, which included women of the South Indian population [22]. Similar to our study, no statistically significant differences were found in the distribution of genotype frequencies between IRSA and control women. In addition, the association with IRSA under different genetic models was not determined.

Although other polymorphisms of the *VEGFA* gene were previously tested in many studies, our study is the first to include male partners of IRSA women. Regardless of the fact that most genetic association studies include only women, recent evidence points to the importance of the male genome in reproductive success [23] and particularly in the pathogenesis of IRSA [2, 24, 25]. This contribution most likely manifests through the transmission of risk alleles through the feto-placental unit to the embryo [23]. Interestingly, certain paternal SNPs in the VEGF gene family increase the risk for pregnancy complications such as preeclampsia and small for gestational age infants [26]. In addition, a number of different paternal SNPs were found to be in strong association with preeclampsia, indicating an important role of the male genome in reproductive disorders [23]. Although the samples of spontaneously aborted conceptuses were not available to us for this study, we can deliberate about the contribution of the DD genotype in men to IRSA. Considering that both higher and lower VEGF protein levels along with lower mRNA levels were detected in the chorionic villi of spontaneously aborted conceptuses compared with conceptuses obtained by induced abortion [27–29], it is possible that the inheritance of the risk D allele might lead to altered *VEGFA* gene expression in the embryo. Consequently, this may lead to abnormal angiogenesis and spontaneous abortion.

Furthermore, a combined effect of both partners cannot be excluded. Our analysis showed that the combination of DD and ID genotypes is the most common combination in IRSA couples, and therefore, the transmission of the risk D allele in homozygous or heterozygous form might contribute to spontaneous abortion. Although we also found a higher frequency of the DD genotype in IRSA women compared to control women, the difference did not reach statistical significance and was not associated with IRSA under any genetic model. Nevertheless, additional research is needed in different populations and on a larger number of participants, especially because altered VEGF mRNA and protein levels were detected in IRSA women compared to control women, which might be under genetic control. Women with IRSA have lower VEGF mRNA and protein levels in the endometrium during the implantation window, leading to abnormal vascular function [30–33]. On the contrary, these women have higher serum VEGF protein levels, the significance of which is not known [30, 31].

In addition to being a functional polymorphism, the −2549 I/D polymorphism is in perfect linkage disequilibrium with the −2578 C/A SNP [13]. The I allele is linked with the

Table 4 Association of the *VEGFA* −2549 I/D polymorphism with IRSA in men

VEGFA I/D genetic model		M_{IRSA} vs. M_C		$M_{Primary\ IRSA}$ vs. $M_{Secondary\ IRSA}$	
		OR (95 % CI)	*P*	OR (95 % CI)	*P*
Dominant	DD+ID vs. II	1.36 (0.80–2.30)	0.253	1.79 (0.81–3.99)	0.152
Recessive	DD vs. ID+II	1.75 (1.05–2.93)	0.032	1.56 (0.74–3.31)	0.243
Co-dominant	DD vs. II	1.87 (1.00–3.49)	0.051	2.08 (0.83–5.25)	0.119
Alleles	DD vs. ID	1.69 (0.97–2.95)	0.064	1.31 (0.58–3.00)	0.513
	II vs. ID	0.74 (0.43–1.24)	0.253	0.56 (0.25–1.24)	0.152
	D vs. I	1.42 (1.02–1.97)	0.036	1.52 (0.93–2.49)	0.097

95 % CI 95 % confidence interval, *OR* odds ratio, M_C control men, M_{IRSA} men with idiopathic recurrent spontaneous abortion, $M_{Primary\ IRSA}$ men with primary idiopathic recurrent spontaneous abortion, $M_{Secondary\ IRSA}$ men with secondary idiopathic recurrent spontaneous abortion

−2578 A allele, whereas the D allele is linked with the −2578 C allele. In the study by Eller et al. [34], there was a statistically significant higher frequency of the −2578 CC genotype in IRSA women compared to control women, which suggests that these women also have an increased frequency of the −2549 DD genotype.

Our study has several strengths. The selection criteria for couples were strict and based on exclusion of known causes of RSA, including structural uterine anomalies, antiphospholipid syndrome, and chromosomal anomalies in both partners. Statistical power was sufficient, and the association between the −2549 I/D polymorphism was conducted in Slovenian population for the first time. Our study also included both reproductive partners. On the other hand, one of the limitations of this study is that genotyping was not performed on spontaneously aborted conceptuses. Despite sufficient power analysis, another limitation might be the sample size, which might not allow us to detect minor effects. Therefore, before any clinical guidance might be offered, additional genetic association studies including both partners, as well as expression studies, are needed to elucidate the role of *VEGFA* −2549 I/D polymorphism in IRSA.

In conclusion, our results indicate that the −2549 I/D polymorphism in the promoter region of the *VEGFA* gene in men might be associated with IRSA.

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Ethical approval All procedures involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Compliance with ethical standards

The authors declare that they have no conflicts of interest.

Written informed consent was obtained from all individual participants included in the study. The study was approved by Slovenian and Croatian National Ethics' Committees and was performed in accordance with the ethical standards as described in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

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