Original Article

Vascular Endothelial Growth Factor Genetic Variant Is Associated with in-Stent Restenosis after Percutaneous Coronary Intervention

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Abstract

Background: *In-stent restenosis (ISR) is an inevitable complication of percutaneous coronary intervention, with genetic factors thought to play a role in its pathogenesis. The vascular endothelial growth factor (VEGF) gene can have an inhibitory effect on ISR development. Accordingly, in the present study, we investigated the role of −2549 VEGF (insertion/deletion [I/D]) variants in ISR formation.*

Methods: *Patients with ISR (ISR$^+$) (n=53) and patients without ISR (ISR-) (n=67) were enrolled in this case-control study based on follow-up angiography 1 year after percutaneous coronary intervention between 2019 and 2020. The clinical characteristics of the patients were evaluated, and the frequencies of the alleles and genotypes of −2549 VEGF (I/D) variants were determined using polymerase chain reaction. The χ^2 test was performed for the calculation of genotypes and alleles. A P value of less than 0.05 was considered the level of significance*

Results: *This study recruited 120 individuals at a mean age of 61.43±8.91 years in the ISR+ group and 62.09±7.94 years in the ISR- group. Women and men, respectively, comprised 26.4% and 73.6% of the ISR+ group and 43.3% and 56.7% of the ISR- group. A significant association was observed between the VEGF −2549 genotype frequency and ISR. The frequency of the insertion/insertion (I/I) allele was significantly higher in the ISR$^+$ group than in the ISR- group, while the frequency of the D/D allele was higher in the latter group.*

Conclusion: *Regarding ISR development, the I/I allele may be a risk allele and the D/D allele a protective allele.*

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Keywords: *Coronary restenosis; Drug-eluting stent; Gene variant; Vascular endothelial growth factor (VEGF)*

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Introduction

Vascular stenosis is the most common manifestation of cardiovascular diseases, the leading cause of death in the world.[1, 2] Stent implantation inside artery walls is the most popular percutaneous coronary intervention in vascular stenosis treatment.[3] Stents are categorized into 2 types: the bare-metal stent (BMS) and the drug-eluting stent (DES).[4] However, in-stent restenosis (ISR) is the most significant challenge of therapeutic stents.[4] The placement of a stent inside an artery wall causes vascular injury and ISR occurrence.[5, 6] Age, sex, diabetes mellitus, genetics, and stent type are all influential factors in ISR development.[4] Blood vessel narrowing, an inflammatory response to vascular injury, leads to the migration and proliferation of vascular smooth muscle cells (VSMC) in ISR.[7] Neointima is scar tissue formation as a result of VSMC proliferation inside the artery wall, probably due to stent placement.[8]

Although DES use has greatly lowered ISR incidence by reducing VSMC proliferation by 70%, ISR is an inevitable limitation of percutaneous coronary intervention.[9] Mounting evidence strongly suggests the role of genetics in ISR formation.[4, 10] One of the advances in DES use is the delivery of antiproliferative agents, such as genes, at the site of vascular injury and stenosis.[11, 12, 1] Therefore, the identification of effective genes and variants can be helpful in ISR prevention and treatment. Endothelial cells are one of the main cells lining the inner surface of blood vessels and play a significant role in regulating intravascular blood flow by controlling inflammation, proliferating VSMC, and inhibiting hyperplasia.[13] The vascular endothelial growth factor (VEGF) gene is involved in the proliferation and differentiation of endothelial cells.[14] The chief function of *VEGF*, which has different isoforms, is its contribution to angiogenesis.[14, 15] VEGF has also been reported to cause endothelial cell permeability to low-density lipoprotein as one of the major causes of atherosclerosis.[9] In addition, oxidized-low-density lipoprotein increases *VEGF* expression in macrophages, one of the contributing factors to atherosclerosis.[16]

DES implantation by inhibiting VSMC proliferation can reduce neointima formation and ISR.[17] Endothelial cell proliferation can lessen ISR, while it seems that the *VEGF* gene by initiating endothelium formation can suppress the neointimal formation and ISR occurrence.[11, 17] Therefore, *VEGF* use in DES implantation is an intriguing technique for ISR management. Nonetheless, given the role of this gene in endothelial cell regrowth and neointima reduction in the arterial wall, it is used as a target gene in DES implantation.[18, 11] Previous research has shown an association between the *VEGF* gene and ISR and cardiovascular diseases.[19, 20-23] Several single-nucleotide polymorphisms have been reported in the *VEGF* gene promoter.[24] The insertion/deletion (I/D) −2549 polymorphism is located at the promoter site, where the D allele can lead to an increase, and allele I can lead to a decrease in *VEGF* expression.[25] The role of variations in the regulatory region of the *VEGF* gene, especially in the promoter region, has been reported in a variety of diseases such as cancer, diabetes, and rheumatoid arthritis.[26, 27] The variants can cause different *VEGF* expression levels.[28, 29]

Therefore, in the current study, we intended to evaluate the role of *VEGF* gene (I/D) −2549 variants in ISR in patients undergoing angioplasty with a DES. Determining the role of genetic variants in ISR occurrence can help design future DESs with a view to regulating genes that are influential in ISR formation.

Methods

The present case-control study recruited 120 subjects, consisting of patients with ISR (ISR^+) (n=53) and patients without ISR (ISR-) (n=67), from the Cardiology Unit of Dr. Shariati Hospital between 2019 and 2020. ISR was defined as stenosis exceeding 50% of the vessel lumen diameter at the stented place or its edges (5 mm segments adjacent to the stent) after percutaneous coronary intervention. ISR patterns were determined based on the classification by Mehran et al.[5, 30-31] The study population was composed of individuals who underwent repeat coronary angiography 1 year after angioplasty with a DES. Individuals who had ISR within less than 1 year after stenting, coronary angioplasty with a BMS, and age less than 18 years old were excluded from the study. Both case and control groups were homogenous regarding age and sex.

Participants who had significant restenosis and those without restenosis at the stent place in repeat angiography were considered the case and control groups, respectively, based on the criteria introduced by Mehran et al.[32] A questionnaire to collect detailed medical history and demographic information was completed for the entire study population.

Hypertension was defined as a blood pressure of 140/90 mmHg or greater and a history of hypertension medication use. Diabetes mellitus was defined as a fasting plasma glucose level of 126 mg/dL or greater, a 2-hour post-load glucose level of 200 mg/dL or greater, or oral hypoglycemic agent use based on the American Diabetes Association (ADA) guidelines.[33, 34] Metabolic syndrome was diagnosed based on the National Cholesterol Education Program Adult Treatment Panel (NCEP ATP) III (2004) definition in accordance with the ADA updated definition of impaired fasting glucose and was composed of the following components: elevated fasting plasma glucose (≥6.1 mmol/L [110 mg/dL], modified in 2004 to ≥5.6 mmol/L [100 mg/dL]), blood pressure (≥130/ ≥85 mmHg), triglycerides (≥1.7 mmol/L [150 mg/dL]), high-density lipoprotein-cholesterol (<1.03 mmol/L [40 mg/dL] in men and <1.29 mmol/L [50

mg/dL] in women), and obesity (waist circumference >102 cm in men and >88 cm in women).[35-39]

Data such as age; sex; body mass index (BMI); waist circumference; family history of hypercholesterolemia; risk factors for myocardial infarction, including hyperlipidemia, dyslipidemia, and smoking; and medications were gathered.

The study protocol was approved by the Ethics Committee of Tehran University of Medical Sciences, and written informed consent was obtained from all the participants.

Total DNA was extracted from blood samples via the phenol-chloroform method. The −2549 variant region in the *VEGF* gene was amplified by polymerase chain reaction (PCR) using the following primer sequences: forward 5'-GCTGAGG ATGGGGCTGACTAGGTA-3' and reverse: 5'-GTTTCTGACCTGGCTATTTCCAGG-3'.

The PCR test was performed with the first denaturation step of 6 minutes at 95 ˚C, followed by 35 cycles of denaturation at 95 ˚C for 30 seconds, annealing at 63.5 ˚C for 30 seconds, extension at 72 ˚C for 30 seconds, and final extension at 72 °C for 5 minutes. The products were separated and detected on 3% agarose gel electrophoresis.

A band of 229 base pairs (bp) was considered to represent an 18 bp insertion (I allele) and a band of 211 bp an 18 bp deletion (D allele). The I/D allele resulted in 229 bp and 211 bp bands.

The χ^2 test, the odds ratio (OR), and the 95% confidence interval (CI) were employed for the calculation of the genotypes, allele frequencies, and clinical characteristics of the patients. The hardy–Weinberg equilibrium was also evaluated in the case and control groups. The unpaired test-test was used for the comparison of clinical characteristics between the 2 groups. A P value of less than 0.05 was considered the level of significance. The SPSS software, version 16, was applied for the statistical analyses.

Results

The ISR$^+$ and ISR- groups were not statistically significantly different vis-à-vis the mean age (61.43±8.91 y vs 62.09±7.94 y; P=0.676), BMI (26.42±3.85 vs 27.43±3.69; P=0.244), and waist circumference (93.55±12.43 vs 90.44±12.74; P=0.382).

Clinical characteristics, such as sex, family history of hypercholesterolemia, diabetes mellitus, hypertension, hyperlipidemia, metabolic syndrome, dyslipidemia, smoking, and drugs (statins and acetylsalicylic acid [ASA]), showed no statistically significant differences between the ISR$^+$ and ISR- groups. Nevertheless, the number of patients who received clopidogrel was significantly different between the 2 groups (P=0.006) (Table 1).

The genotype analysis showed no significant deviation from the hardy–Weinberg equilibrium in the control (P=0.506) and case (P=0.182) groups. The genotyping is shown in Figure 1, and the genotype and allele distributions are presented in Table 2. The genotype frequency was significantly different between the ISR+ and ISR- groups (P=0.029). The genotype frequency was also evaluated in dominant, recessive, and co-dominant genetic models. The dominant (II+ID vs deletion [DD]; OR, 2.22; 95% CI, 0.91 to 5.56) and recessive (II vs ID+DD; OR, 2.98; 95% CI, 1.12 to 8.11) models both showed a significant difference between the case and control groups. The co-dominant model was statistically significantly different in the II vs DD model between the ISR$^+$ and ISR- groups (OR, 3.9; 95% CI, 1.24 to 12.47), while the difference between the 2 groups did not constitute statistical significance in the II vs ID model (OR, 2.43; 95% CI, 0.83 to 7.19). The allelic distribution was also statistically significantly different between the 2 study groups (OR, 2.13; 95% CI, 1.21 to 3.78).

Table 1. Clinical characteristics of the patients with ISR compared with the patients without ISR*

	ISR+	ISR-	P
Sex			0.056
Female	14 (26.4)	29 (43.3)	
Male	39 (73.6)	38 (56.7)	
FH	5 (9.4)	3 (4.5)	0.280
DM	21 (39.6)	26 (38.8)	0.927
HTN	29 (54.7)	40 (59.7)	0.583
HPL	16 (30.2)	19 (28.4)	0.827
Metabolic syndrome	10 (18.9)	17 (25.4)	0.397
Dyslipidemia	5 (9.4)	11 (16.4)	0.264
Smoking	9 (45.0)	15 (42.9)	0.877
Drugs			
Statins	23 (44.2)	37 (56.1)	0.202
ASA	32 (60.4)	42 (63.6)	0.716
Clopidogrel	12 (22.6)	31 (47.0)	0.006**

*The χ^2 test was used for the genotypes and allele evaluation. Data are presented as n (%).
**P<0.05 has been considered significant
ISR, In-stent restenosis; FH, Family history of hypercholesterolemia; DM, Diabetes mellitus; HTN, Hypertension; HPL, Hyperlipidemia; ASA, Acetylsalicylic acid

Table 2. Genotypes and allele frequencies of the VEGF −2549 variants in the cases and controls*

	Case (ISR+) (N=50)	Control (ISR-) (N=63)	P	OR (95% CI)
Genotypes				
II	18 (36.0)	10 (15.9)	0.029**	
ID	20 (40.0)	27 (42.9)		
DD	12 (24.0)	26 (41.3)		
Dominant				
II+ID vs DD	38 (76.0)	37 (58.7)	0.054**	2.22 (0.91-5.56)
	12 (24.0)	26 (41.3)		
Recessive				
II vs ID+DD	18 (36.0)	10 (15.9)	0.014**	2.98 (1.12-8.11)
	32 (64.0)	53 (84.1)		
Co-dominant				
II vs DD	18 (60.0)	10 (27.8)	0.008**	3.9 (1.24-12.47)
	12 (40.0)	26 (72.2)		
IIvs.ID	18 (47.4)	10 (27.0)	18 (47.4)	10 (27.0)
	20 (52.6)	27 (73.0)		
Allele			0.005**	2.13 (1.21-3.78)
II	56 (56.0)	47 (37.3)		
DD	44 (44.0)	79 (62.7)		

*The χ^2 test was used for the genotypes and allele evaluation. Data are presented as n (%).
**P<0.05 has been considered significant
ISR, In-stent restenosis; II, Insertion/insertion; ID, Insertion-deletion; DD, Deletion

Table 3. Comparison of genotype frequencies according to clinical symptoms in the groups with and without in-stent restenosis*

Groups	Genotypes			
	DD	ID	II	P
ISR+				0.173
Male	11	15	11	
Female	1	5	7	
ISR-				0.597
Male	16	13	5	
Female	10	14	5	
ISR+ DM	2	7	9	0.175
ISR- DM	5	12	7	0.013**
ISR+ HTN	7	10	10	0.888
ISR- HTN	13	19	7	0.264
ISR+ HPL	4	7	5	0.887
ISR- HPL	8	10	1	0.281
ISR+ Metabolic syndrome	5	2	3	0.086
ISR- Metabolic syndrome	9	6	2	0.515
ISR+ Dyslipidemia	0	1	4	0.087
ISR- Dyslipidemia	2	1	4	0.006**
ISR+ Smoking	5	3	1	0.044**
ISR- Smoking	6	7	2	0.554

*The χ^2 test was used for the evaluation
**P<0.05 has been considered significant
ISR, In-stent restenosis; DM, Diabetes mellitus; HTN, Hypertension; HPL, Hyperlipidemia

Table 4. Correlations between clinical characteristics and vascular endothelial growth factor (VEGF) genotypes*

Pearson Correlation	Sex	BMI	Waist Circumference	DM	HTN	HPL	Metabolic Syndrome	Dyslipidemia
r	0.09	-0.36	-0.51	-0.29	-0.05	0.08	0.18	-0.26
P	0.249	0.001**	0.000**	0.002**	0.580	0.389	0.046**	0.005**

*The Pearson correlation test was conducted.
**P<0.05 has been considered significant
BMI, Body mass index; DM, Diabetes mellitus; HTN, Hypertension; HPL, Hyperlipidemia

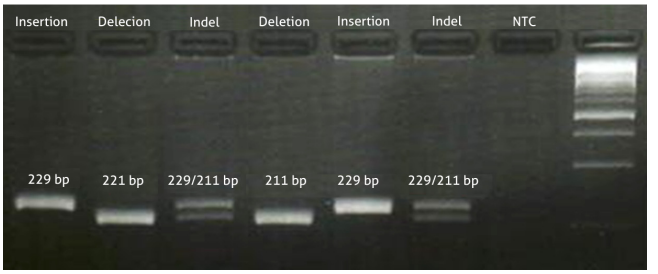

Figure 1. The image depicts the −2549 VEGF genotyping. The genotypes were visualized on 3% gel electrophoresis. The 229 bp represents an 18 bp insertion (I allele), the 211 bp band indicates the deletion allele (D allele), and the insertion/deletion (I/D) allele has 229 bp and 211 bp bands.
NTC, Non-template control

Table 3 demonstrates the genotype frequencies examined in the patients according to underlying clinical characteristics. The comparison of genotypes based on sex, hypertension, hyperlipidemia, and metabolic syndrome was not significantly different in each group separately. The genotype frequency was significant in individuals with diabetes mellitus in the ISR- group (P=0.013). In the ISR^+ group, the distribution of genotypes among individuals with smoking habits was also statistically significant compared with patients without smoking habits (P=0.044). The comparison for the distribution of genotypes in patients with dyslipidemia and without dyslipidemia was also significant in the ISR- group (P=0.006). The results concerning genotype distribution in the different groups using the Pearson correlation are presented in Table 4. There were significant correlations between BMI, waist circumference, diabetes mellitus, metabolic syndrome, dyslipidemia and *VEGF* genotypes. However, sex, hypertension, and hyperlipidemia showed no correlations with genotypes.

Discussion

VEGF, a glycoprotein produced by endothelial cells in the walls of blood vessels, is influential in ISR development.[40,41] The transfer of the *VEGF* gene using plasmids at the neointima site shows that the expression of this gene can increase endothelial cell proliferation and inhibit ISR development.[40] The transcription regulatory region is located at −930 bp of the transcription start site and is effective on *VEGF* expression.[27] It appears that a variation in the regulatory region of the *VEGF* gene increases its expression, while the addition of 18 bp in the promoter region leads to a decrease in its expression.[25]

In our study, we found a significant association between −2549 variants in the *VEGF* gene and ISR. The dominant, recessive, and co-dominant genetic models of the *VEGF* gene variant showed a significant correlation with ISR. Furthermore, allelic distribution was also significantly different between the ISR^+ and ISR^- groups. It has been reported that the insertion/insertion (I/I) allele reduces the expression of the *VEGF* gene compared with the D/D allele.[25] The distribution of the I/I allele was significantly higher in the ISR^+ (56.0%) group than in the ISR- group (47.0%). The *VEGF* (I/D) variant may have a suppressing effect on ISR development and neointima formation.[18,11] In the present study, the higher frequency of the I/I allele in the case group may have resulted in the lower expression of the *VEGF* gene. On the other hand, the higher frequency of the D/D allele in the control group (ISR-), reported to cause a 1.95-fold increase in *VEGF* expression, might have conferred a protective effect against ISR development.[25] In contrast, in a previous investigation, the frequency of the I/I allele was higher in patients with myocardial infarction than in the control group.[42] The results of that study also showed that the I/I allele was associated with increased *VEGF* expression and atherosclerosis.[42] In another study, no association was observed between the I/D variant and *VEGF* and messenger RNA expression in the placenta of women with preeclampsia.[43] Previous investigations have also reported an association between *VEGF* rs699947 and rs2010963 polymorphisms and ISR formation.[19,21] According to prior studies, coronary artery disease was associated with rs2305948 (G>A) and rs1870377 (A>T),[20] and rs6999447 in *VEGF* were linked to restenosis in coronary artery disease.[22]

The *VEGF* gene product is involved in many cancers.[44] The role of the *VEGF* −2549 polymorphism in uterine leiomyoma was investigated in the Iranian population in a previous investigation, whose results, similar to ours, demonstrated that the frequency of the I/I genotype was higher than that of the D/D genotype in the patient group than in the control group.[44] That study also proposed a higher risk of uterine leiomyoma in women carrying the I/I genotype compared with the D/D genotype.[44] The *VEGF* gene is involved in angiogenesis and tumor genesis in cancer.[45] The frequency of the D/D genotype was higher in patients with breast cancer than in the control group, and the level of *VEGF* was higher in patients than in the control group in a prior study.[46] Another investigation reported that the frequency of genotype I/I was higher in the case group (patients with anterior cruciate ligament injuries) than in the control group.[46] The *VEGF* gene can have a protective role against muscle injuries because it seems that the function of the *VEGF* gene is reduced in individuals with the I/I genotype compared with the D/D genotype.[46]

A prior investigation reported that the −2549 polymorphism had no association with peripheral arterial disease in patients with diabetic nephropathy; additionally, although serum *VEGF* levels in individuals with the I/I genotype were lower than those of I/D and D/D genotypes, the difference was not statistically significant.[41] Diabetes is one of the influential factors in neointima formation and ISR development.[47] The role of the *VEGF* gene in the microvascular complications of diabetes, such as diabetic retinopathy, was indicated in a previous investigation, reporting that the frequency of

the D/D genotype was higher in patients with diabetic retinopathy than in the control group, and the serum level of *VEGF* was higher in subjects with the D/D genotype, although the difference was not statistically significant.[48] The D allele also increases the risk of diabetic retinopathy.[49] We observed a significant correlation between BMI, waist circumference, metabolic syndrome, and dyslipidemia with *VEGF* genotypes. We also found a significant correlation between diabetes mellitus and the −2549 genotypes.

Clopidogrel, an antiplatelet and antithrombotic drug, is deemed influential in neointima formation.[50-52] Even though the role of clopidogrel in ISR is controversial, our study showed that the number of patients who received clopidogrel in the ISR+ group was significantly lower than that in the ISR- group, which may represent the preventive effect of clopidogrel against ISR formation. It has been reported that the use of VEGF/PTX NP-coated stents compared with other DES forms can cause re-endothelialization and reduce ISR.[11] Transfected endothelial cells with *VEGF* showed suppressed neointimal and ISR as a result of re-endothelialization in a prior study.[53] Parental umbilical cord blood-derived mesenchymal stem cell (UCB-MSC)-coated stents with hepatocyte growth factor and *VEGF* reduced vascular stenosis and induced re-endothelialization according to another investigation.[54]

Conclusion

The results of the present study showed an association between the −2549 variants of the *VEGF* gene and ISR development in patients who underwent coronary angioplasty with a DES. The evaluation of *VEGF* expression and serum levels can better elucidate the role of *VEGF* in ISR. We recommend that the −2549 polymorphism be investigated in a larger sample size.

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