Vascular endothelial growth factor (VEGF)-related polymorphisms rs10738760 and rs6921438 are not risk factors for proliferative diabetic retinopathy (PDR) in patients with type 2 diabetes mellitus (T2DM)

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ABSTRACT

Vascular endothelial growth factor (VEGF) is an important regulator of angiogenesis and has been investigated as a candidate gene in a number of conditions, including diabetes and its microvascular complications (e.g., retinopathy and nephropathy). Several *VEGF*-related polymorphisms have been shown to contribute to nearly half of the variability in circulating VEGF levels in healthy individuals. Our aim was to assess the association between *VEGF*-related rs10738760 and rs6921438 polymorphisms and proliferative diabetic retinopathy (PDR) in Slovenian patients with type 2 diabetes mellitus (T2DM). We also investigated the effect of these polymorphisms on VEGF receptor 2 (VEGFR-2) expression in fibrovascular membranes (FVMs) from patients with PDR. This case-control study enrolled 505 unrelated patients with T2DM: 143 diabetic patients with PDR as a study group, and 362 patients with T2DM of >10 years duration and with no clinical signs of PDR as a control group. Patient clinical and laboratory data were obtained from their medical records. rs10738760 and rs6921438 polymorphisms were genotyped using TaqMan SNP Genotyping assay. VEGFR-2 expression was assessed by immunohistochemistry in 20 FVMs from patients with PDR, and numerical areal density of VEGFR-2-positive cells was calculated. The occurrence of PDR was 1.7 times higher in diabetic patients carrying GA genotype of rs6921438 compared to patients with GG genotype, with a borderline statistical significance (OR = 1.7, 95% CI = 1.00 – 2.86, $p = 0.05$). In addition, A allele of rs6921438 was associated with increased VEGFR-2 expression in FVMs from PDR patients. However, we observed no association between AA genotype of rs6921438 nor between rs10738760 variants and PDR, indicating that the two polymorphisms are not genetic risk factors for PDR.

KEY WORDS: Vascular endothelial growth factor; VEGF; SNP; single nucleotide polymorphism; proliferative diabetic retinopathy; PDR; T2DM; diabetes; VEGFR-2

DOI: http://dx.doi.org/10.17305/bjbms.2018.3519 *Bosn J Basic Med Sci. 2019;19(1):94-100. © 2018 ABMSFBIH*

INTRODUCTION

Diabetic retinopathy (DR) is a common microvascular complication of diabetes and is the leading cause of blindness in adults aged 20–74 years in developed countries [1-5]. It occurs in more than 60% of patients with type 2 diabetes mellitus (T2DM) and in almost all patients with type 1 diabetes mellitus (T1DM) within the first decade of diabetes onset [6-8]. DR may progress through several stages, including early non-proliferative diabetic retinopathy (NPDR), moderate NPDR, severe NPDR and finally advanced, proliferative, DR (PDR) [9,10]. The main characteristics of NPDR are microaneurysms, hemorrhage, cotton wool spots and hard exudates (lipid deposits). The distinctive features of PDR include increased vascular permeability, tissue ischemia and neovascularization that leads to fibrovascular changes, vitreoretinal traction and retinal detachment, eventually resulting in blindness [3,5,11].

It is accepted that both genetic and environmental factors influence the severity and progression of DR [12,13]. As an important regulator of angiogenesis, vascular endothelial growth factor (*VEGF*) has been investigated as a candidate gene in a large number of conditions including cardiovascular,

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Submitted: 22 April 2018/Accepted: 19 July 2018

inflammatory diseases, diabetes, DR, diabetic nephropathy (DN) and cancer [14]. Several studies have shown that VEGF is involved in the progression of DR, leading to the proliferative stage of the disease [15]. Under physiological conditions VEGF is expressed at low levels, whereas it is markedly upregulated in the vitreous and aqueous humours of the eyes from patients with PDR [16-19]. VEGF is a potent angiogenic and vascular permeability factor (VPF); in addition to its involvement in neovascularization process in PDR, VEGF has been implicated in the development of early DR, as well as in breakdown of the blood-retina barrier in diabetic macular edema [14,20]. Hypoxia is the major stimulus for VEGF production in retinal areas with reduced perfusion. The mechanisms involve increased *VEGF* transcription, mRNA stability, mRNA translation as well as increased production of the chaperone oxygen-regulated protein 150 (ORP150). The increase in *VEGF* gene expression is predominantly mediated by hypoxia-inducible factor-1 (HIF-1) [12].

The human *VEGF* gene is located on chromosome 6p21.3 and consists of 8 exons separated by 7 introns [21,22]. Single nucleotide polymorphisms (SNPs) of or related to the *VEGF* gene have been associated with VEGF expression/serum levels [23-27]. SNPs located in the *VEGF* gene, such as rs833061 and rs699947 (both located in the promoter), rs2010963 (in the 5′ untranslated region [5′ UTR]) and rs3025039 (in the 3′ UTR) have been best studied in the context of diabetes microvascular complications [26]. On the other hand, only a few studies investigated the association of diabetes and diabetic complications with SNPs related to but located outside the *VEGF* gene. A genome-wide association (GWA) study showed that the polymorphism rs10738760 (located on chromosome 9p24.2, between the *VLDLR* and *KCNV2* genes) and rs6921438 (on 6p21.1 chromosome 171 kb downstream of the *VEGFA* and close to the *MRPL14* gene) together with rs4416670 (also located on 6p21.1) and rs6993770 (on 8q23.1 within the *ZFPM2* gene) explain nearly half of the variability in circulating VEGF levels [27].

In otherwise healthy individuals, the rs6921438 polymorphism was shown to interact with hypertension resulting in decreased VEGF levels [28] as well as to contribute to decreased high-density lipoprotein-cholesterol (HDL-C) and increased low-density lipoprotein cholesterol (LDL-C) levels [29], which may have a negative effect on the cardiovascular system. In addition, an association of the rs10738760 polymorphism with increased risk of metabolic syndrome, higher VEGF and lower HDL levels was demonstrated in patients with metabolic syndrome [30]. Bonnefond et al. [31] investigated the effect of rs6921438 and rs10738760 on the risk of T2DM, nephropathy and retinopathy in Danish and/or French populations. Overall they reported no significant association between the two *VEGF*-related SNPs and the risk of T2DM, DN or DR [31]. Similarly, Terzić et al. [32] showed that rs6921438 and rs10738760 were not associated with DR in Slovenian patients with T2DM.

In this study, we aimed to assess the association between the rs6921438 and rs10738760 polymorphisms and PDR in Slovenian patients with T2DM. Moreover, we investigated the effect of these SNPs on VEGF receptor 2 (VEGFR-2) expression in fibrovascular membranes (FVMs) from patients with PDR.

MATERIALS AND METHODS

Patients

This case-control study enrolled 505 unrelated Slovenian (white) patients with T2DM who had a defined ophthalmologic diagnosis. The participants were recruited from the University Eye Clinics Ljubljana and from the internal medicine clinics (cardiology, diabetic, and endocrinology clinics) of the University Clinical Center in Ljubljana and Maribor. The patients were classified as having type 2 diabetes according to the 2003 American Diabetes Association criteria for the diagnosis and classification of T2DM [33]. For a dilated fundus examination, the pupils were dilated with 2.5% tropicamide and phenylephrine and a senior ophthalmologist (M.G.P.) performed the examination using a slit lamp biomicroscope with non-contact lens. The result was electronically documented by a fundus camera with a 50-degree angle (Topcon-TRC 40-IX, Tokyo, Japan). DR staging was determined according to the Early Treatment Diabetic Retinopathy Study (ETDRS) diabetic retinopathy severity scale [34].

The study group consisted of 143/505 patients with T2DM and PDR (evidence of new blood vessel formation at the optic disc or elsewhere in the retina and/or fibrous proliferation with or without vitreous hemorrhage). The control group consisted of 362/505 patients with T2DM of more than 10 years duration who had NPDR or had no clinical signs of DR. Demographic, clinical and laboratory parameters such as age, gender, diabetes duration, insulin therapy, systolic blood pressure, diastolic blood pressure, body mass index (BMI), history of hypertension, cigarette smoking, total cholesterol, LDL-C, HDL-C, triglycerides and glycated hemoglobin (HbA1c), were retrieved from the patients' medical records.

To avoid the confounding effect of impaired kidney function, patients with overt nephropathy were excluded from the study. The study was approved by the National Medical Ethics Committee. After informed consent was obtained, a detailed interview with patients was performed.

Genotyping

DNA was isolated from peripheral blood using the QIAamp DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany). The

rs10738760 and rs6921438 polymorphisms were genotyped using the TaqMan SNP Genotyping assay (Applied Biosystems, Foster City, CA, USA), according to the manufacturer protocol. Genotyping was done for cases and multiple controls (homozygous sample or one allele, heterozygous sample and homozygous sample for another allele and negative control).

Immunohistochemistry

FVMs were obtained from 20 patients with T2DM and PDR during pars plana vitrectomy, as described previously [17]. Formalin-fixed paraffin-embedded (FFPE) tissue sections of FVMs were used for hematoxylin-eosin (HE) staining [18]. Consecutive 5-µm tissue sections were cut from each paraffin block and then mounted and dried on glass slides. Tissues were deparaffinized and dehydrated in graded alcohol solutions. Detection of VEGFR-2-positive cells was performed with the NovoLink Max Polymer Detection System (Leica Biosystems Newcastle Ltd, United Kingdom) following the manufacturer's instructions. The slides were incubated with anti-VEGFR-2 monoclonal antibodies (diluted 1:100, ThermoFisher, USA) overnight at 4°C. Placental tissue was used as a positive and tonsils as a negative control. The cells were defined as VEGFR-2-positive/negative. The area with VEGFR-2-positive cells was manually marked and numerical areal density of VEGFR-2-positive cells was calculated (the number of positive cells per mm^2) [17].

Statistical analysis

Allelic discrimination analysis was conducted using StepOne Software version 2.2 (Applied Biosystems, Foster City, CA, USA). Statistics was performed using IBM SPSS Statistics for Windows, Version 21.0 (IBM Corp., Armonk, NY). Categorical variables were expressed as the number and percentage of patients. Continuous variables were expressed as mean ± standard deviation (SD) and compared by unpaired Student's *t*-test. Discrete variables were compared using Chi-square test. All variables showing significant differences in the univariate analysis ($p < 0.05$) were analyzed by logistic regression analysis. A value of $p < 0.05$ was considered statistically significant. The deviation from Hardy –Weinberg equilibrium (HWE) was assessed by Fisher's exact test (http://ihg.gsf.de/) [35].

RESULTS

Clinical and laboratory characteristics of study group (patients with T2DM and PDR) and control group (patients with T2DM without PDR) are presented in Table 1. The two groups of T2DM patients were well matched for age, systolic and diastolic blood pressure, HbA1c, total cholesterol and triglyceride levels. A statistically significant difference between T2DM patients with and without PDR was observed in diabetes duration ($p < 0.001$), insulin therapy ($p < 0.001$), LDL-C ($p = 0.001$), HDL-C levels ($p = 0.001$), and BMI ($p < 0.001$). Diabetic patients with PDR had a longer duration of type 2 diabetes, higher LDL-C, lower HDL-C levels, and higher prevalence of insulin therapy compared to controls. BMI was significantly higher in controls. Moreover, hypertension and cigarette smoking were more frequent in the group of diabetics with PDR compared to control group.

Table 2 shows the genotype and allele frequencies of rs10738760 and rs6921438 polymorphisms. The two SNPs conformed to HWE in both groups (diabetic patients with and without PDR); i.e., no deviations from HWE were observed ($p > 0.05$).

The allele frequencies of rs10738760 and rs6921438 in our cohort were consistent with the 1000 Genomes Project data

TABLE 1. Clinical and laboratory characteristics of study group (patients with T2DM and PDR) and control group (patients with T2DM without PDR)

Characteristics	Patients with T2DM and PDR	Patients with T2DM without PDR	*p*
Number	143	362	
Age (years)	63.4±9.5	64.7±10.2	0.2
Male sex (%)	70 (49.0)	161 (44.6)	0.4
Duration of diabetes (years)	18.8±6.2	11.8±2.6	<0.001
Patients on insulin therapy (%)	104 (72.9)	174 (48.1)	<0.001
Systolic blood pressure (mmHg)	143.9±23.1	143.1±18.4	0.7
Diastolic blood pressure (mmHg)	83.8±10.2	83.5±10.1	0.8
BMI (kg/m^2)	28.4±4.6	30.1±5.3	<0.001
History of hypertension (%)	114 (80.0)	269 (74.4)	0.2
Cigarette smokers (%)	13 (9.4)	21 (5.8)	0.2
Total cholesterol (mmol/l)	5.1±1.2	4.9±1.1	0.09
LDL cholesterol (mmol/l)	3.1±1.0	2.7±0.9	0.001
HDL cholesterol (mmol/l)	1.1±0.3	1.2±0.3	0.001
Triglycerides (mmol/l)	2.3±1.3	2.4±1.8	0.3
HbA1c (%)*	8.0±1.4	7.8±1.4	0.3

The values are expressed as mean±standard deviation or frequencies (percentages). *The average value of HbA1c. T2DM: Type 2 diabetes mellitus; PDR: Proliferative diabetic retinopathy; BMI: Body mass index; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; HbA1c: Glycated hemoglobin

of the European population. We observed no significant difference in the genotype or allele frequencies of rs10738760 and rs6921438 polymorphisms between diabetics with PDR and diabetic patients without PDR (Table 2).

We then performed a logistic regression analysis using a co-dominant model of inheritance to evaluate whether rs10738760 and rs6921438 polymorphisms were independently associated with PDR after adjusting for duration of diabetes, insulin therapy, BMI, LDL-C and HDL-C (Table 3). The logistic regression analysis showed a tendency for increased risk of PDR in patients with the GA genotype of rs6921438, with a borderline statistical significance (OR = 1.70, 95% CI = 1.00 – 2.86, $p = 0.05$) compared to patients with the GG genotype. Namely, the GA genotype of rs6921438 was found to modify the susceptibility to PDR, since the occurrence of PDR was 1.70 times higher in diabetic patients with GA genotype compared to the reference group (diabetics with GG genotype). However, the AA genotype of rs6921438 did not achieve a statistically significant association with PDR. Additionally, no association between rs10738760 variants and PDR was observed.

In FVMs from diabetic patients with PDR a significantly higher numerical areal density of VEGFR-2-positive cells (Figure 1) was found in patients with the A allele of rs6921438 (AA+AG genotypes) compared to the homozygotes for wild type G allele (105 ± 31/mm² vs. 65 ± 20/mm², respectively; $p < 0.001$). On the contrary, there was no significant difference in numerical areal density of VEGFR-2-positive cells between rs10738760 genotypes (patients with AA+AG genotypes vs. patients with GG genotype: 75 ± 25/mm² vs. 67 ± 28/mm²; $p < 0.001$).

DISCUSSION

Previously, it was suggested that rs10738760 and rs6921438 polymorphisms affect the levels of VEGF in serum, i.e., in healthy individuals, these SNPs contributed to nearly half of the variability in circulating VEGF levels [27]. As a potent angiogenic and vascular permeability factor, VEGF has been implicated in the development of diabetic microvascular complications. In the present study, we investigated the association between rs10738760 and rs6921438 polymorphisms and PDR in Slovenian patients with T2DM. The logistic regression analysis showed a tendency of rs6921438 GA genotype for an increased risk of PDR ($p = 0.05$). In addition, the A allele of rs6921438 was found to affect the levels of VEGFR-2 in FVMs

TABLE 2. Distribution of rs10738760 and rs6921438 genotypes and alleles in study group (patients with T2DM and PDR) and control group (patients with T2DM without PDR)

Genotypes/alleles	Patients with T2DM and PDR (n=143)	Patients with T2DM without PDR (n=362)	*p*
rs10738760			
GG	30 (21.0)	89 (24.6)	0.5
GA	81 (56.6)	186 (51.4)	
AA	32 (22.4)	87 (24.0)	
G allele (%)	141 (49.3)	364 (50.3)	0.8
A allele (%)	145 (50.7)	360 (49.7)	
PHWE†	0.1	0.6	
rs6921438			
GG	40 (28.0)	90 (24.9)	0.4
GA	63 (44.0)	183 (50.5)	
AA	40 (28.0)	89 (24.6)	
G allele (%)	143 (50.0)	363 (50.1)	1.0
A allele (%)	143 (50.0)	361 (49.9)	
PHWE†	0.2	0.8	

T2DM: Type 2 diabetes mellitus; PDR: Proliferative diabetic retinopathy; PHWE: Probability of adherence to Hardy–Weinberg equilibrium. †The deviation from Hardy-Weinberg equilibrium (HWE) was assessed by the exact test (http://ihg.gsf.de/)

TABLE 3. Association between rs10738760 and rs6921438 polymorphisms and the risk for PDR

Inheritance model	Genotype	Patients with T2DM and PDR (n=143)	Patients with T2DM without PDR (n=362)	Adjusted OR, 95% CI/*p*†
rs10738760 Co-dominant	GG	30 (21.0)	89 (24.6)	Reference
	GA	81 (56.6)	186 (51.4)	0.89 (0.50–1.63)/0.7
	AA	32 (22.4)	87 (24.0)	1.08 (0.52–2.23)/0.8
rs6921438 Co-dominant	GG	40 (28.0)	90 (24.9)	Reference
	GA	63 (44.0)	183 (50.5)	1.70 (1.00–2.86)/0.05
	AA	40 (28.0)	89 (24.6)	1.12 (0.64–1.98)/0.7

†*p* values were adjusted for diabetes duration, insulin therapy, BMI, LDL-C and HDL-C. Genotypes are expressed as frequencies (percentages).
T2DM: Type 2 diabetes mellitus; PDR: Proliferative diabetic retinopathy; OR: Odds ratio; CI: Confidence interval; BMI: Body mass index; HDL-C: High-density lipoprotein-cholesterol; LDL-C: Low-density lipoprotein cholesterol

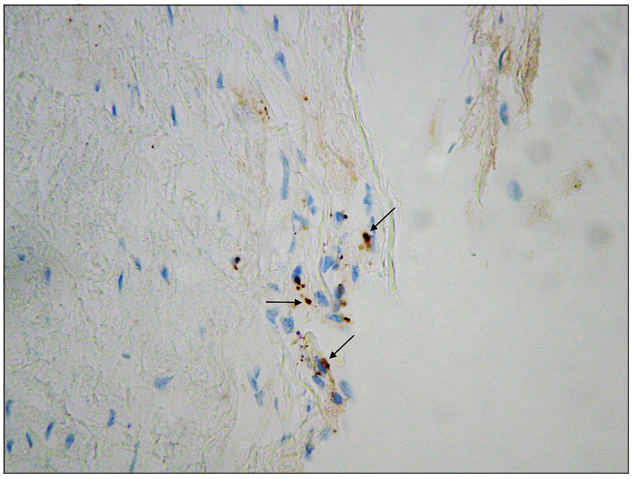

FIGURE 1. VEGF receptor 2-positive cells in fibrovascular membranes from patients with PDR (anti-VEGF receptor 2 monoclonal antibody, ×63 magnification). PDR: Proliferative diabetic retinopathy; VEGF: Vascular endothelial growth factor.

of diabetic patients with PDR. On the contrary, we did not observe an association between rs10738760 variants and PDR in our group of patients with T2DM.

Bonnefond et al. [31] investigated the contribution of rs6921438 and rs10738760 polymorphisms to T2DM and microvascular complications (DR and DN) in several case-control studies including French and Danish populations. Although in the French population the G allele of rs6921438 (increases circulating VEGF levels in the general population) was associated with increased T2DM risk, this was not confirmed in the Danish population. Also, rs10738760 SNP was not associated with T2DM in neither of those populations. Similarly, they did not observe significant effects of rs6921438 or rs10738760 on diabetic microvascular complications in patients with T2DM [31]. In line with these and our findings are also the results of another study on Slovenian patients with T2DM [32], which reported no significant association between DR and rs6921438 and rs10738760 polymorphisms. Overall, the results from our and the previous studies indicate a more complex and perhaps indirect relationship between VEGF and T2DM and its microvascular complications, as suggested by Bonnefond et al. [31]. In addition to genetic factors including polymorphisms in the *VEGF* and other related genes [36-39], environmental factors such as diabetes duration, poor glycemic control, and hypertension also affect the development of PDR.

VEGF is considered as a possible molecular target/biomarker for PDR since the heritability of circulating VEGF levels is very high (60–80% heritability) [27] and markedly higher VEGF levels have been observed in diabetic patients with PDR [12,37]. In addition, inhibition of VEGF-mediated retinal neovascularization and retinal vascular permeability using anti-VEGF agents or inhibitory VEGF isoforms has a potential as a therapeutic strategy in diabetic retinopathy [40-43].

Finally, our results suggested that the expression of VEGFR-2 is affected by the presence of *VEGF*-related polymorphisms, since the numerical areal density of VEGFR-2-positive cells was significantly higher in FVMs from PDR patients carrying the A allele of rs6921438 (AA+AG genotypes) compared to PDR patients with the GG genotype (wild type).

There are several limitations to our study. First, we enrolled a relatively small number of patients and a larger sample size may be necessary to demonstrate significant association. Second, we only assessed the association between two *VEGF*-related polymorphisms and PDR. Therefore, we cannot exclude the effect of other *VEGF* or *VEGF*-related polymorphisms on the development of PDR. Third, we did not evaluate the levels of VEGF in the vitreous fluid or serum of diabetic patients with PDR, and it would be informative to see if rs6921438 and/or rs10738760 polymorphism affect VEGF expression in PDR. In the general population, the minor A allele of rs6921438 was associated with decreased and the G allele with increased levels of serum VEGF [27].

Overall, our results suggest that *VEGF*-related rs10738760 and rs6921438 polymorphisms do not contribute to genetic susceptibility to PDR, despite the effect of rs6921438 on VEGFR-2 expression. Due to the aforementioned limitations, further studies with a larger sample size should be carried out to clarify the role of *VEGF*-related SNPs in PDR.

ACKNOWLEDGMENTS

The authors thank Mrs. Polona Sajovic and Mrs. Petra Nussdorfer for the preparation of histological slides, and Mrs. Visam Bajt, BA for revising the English.

DECLARATION OF INTERESTS

The authors declare no conflict of interests.

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