Vascular endothelial growth factor (VEGF)-related single nucleotide polymorphisms rs10738760 and rs6921438 are not associated with diabetic retinopathy (DR) in Slovenian patients with type 2 diabetes mellitus (T2DM)

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ABSTRACT

Diabetic retinopathy (DR) is a complication of diabetes characterized by vascular permeability, increased tissue ischemia, and angiogenesis. One of the most important proteins involved in angiogenesis is vascular endothelial growth factor (VEGF, also known as VEGFA). A previous study demonstrated that two single nucleotide polymorphisms (SNPs), rs6921438 and rs10738760, account for nearly half the variation in circulating VEGF levels. The aim of our study was to assess the association between rs6921438 and rs10738760 and DR in Slovenian patients with type 2 diabetes mellitus (T2DM). This case-control study enrolled 1037 unrelated Slovenian individuals (Caucasians) with T2DM. DR group included 415 T2DM patients with DR, while control group included 622 T2DM patients with no clinical signs of DR. The clinical and laboratory data were obtained from the medical records of the patients. The genotyping of rs6921438 and rs10738760 SNPs was carried out with real-time PCR assays. Significant differences were observed between patients with DR and controls in the duration of diabetes ($p < 0.001$), insulin therapy ($p < 0.001$), glycated hemoglobin ($p = 0.001$), body mass index ($p = 0.002$), total cholesterol ($p = 0.002$), and low-density lipoprotein cholesterol ($p < 0.001$). However, we did not observe significant differences in the genotype and allele distribution of the two SNPs, between DR and control group ($p < 0.05$). Logistic regression analysis showed that rs6921438 and rs10738760 were not independent genetic risk factors for DR in the co-dominant model adjusted for the above-mentioned clinical and laboratory data. In conclusion, VEGF-related SNPs rs10738760 and rs6921438 are not associated with DR in our group of Slovenian patients (Caucasians) with T2DM.

KEY WORDS: Vascular endothelial growth factor; VEGF; VEGFA; diabetic retinopathy; DR; type 2 diabetes mellitus; T2DM; single nucleotide polymorphism; SNP

INTRODUCTION

Diabetic retinopathy (DR) is a microvascular complication of diabetes characterized by progressive damage to the eyes. DR is the leading cause of new cases of blindness in adults [1,2,3], and the development of DR is influenced by both genetic and environmental factors [4-8]. The clinical characteristics of DR include vascular permeability, increased tissue ischemia, and angiogenesis [9,10]. Vascular endothelial growth factor A (VEGFA, also known as VEGF) is a

chemokine involved in the process of angiogenesis, acting as a key regulator of vascular permeability [11,12]. Higher serum VEGF levels have been linked to DR [10,13,14].

The heritability of circulating VEGF levels is high [15] and several common single nucleotide polymorphisms (SNPs) were shown to be significantly related to serum VEGF levels [16,17]. A genome-wide association (GWA), community-based study [17], enrolling 3,527 individuals of European descent, analyzed the association between SNPs and circulating VEGF levels. A total of 140 SNPs reached genome-wide significance and four of these SNPs were independently associated with VEGF levels: rs6921438, rs4416670, rs6993770, and rs10738760 [17]. rs6921438 SNP is located on the short arm of chromosome 6 at 6p21.1, 171 kb downstream of the *VEGFA* gene, and close to the mitochondrial ribosomal protein L14

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gene (*MRPL14*) [16,17]. rs10738760 SNP is located on chromosome 9p24.2, close to the very low-density lipoprotein receptor (*VLDLR*) and potassium voltage-gated channel subfamily V, member 2 (*KCNV2*) genes [16,17]. In the GWA study, rs6921438 explained 41.2% and rs10738760 5% of the variability in circulating VEGF levels [17].

To date, only one study assessed the effect of the VEGF-related SNPs, rs6921438 and rs10738760, on the risk of type 2 diabetes mellitus (T2DM) and associated microvascular complications [14]. They found that the G allele of rs6921438 was associated with increased glycated haemoglobin (HbA1c) levels and increased risk for T2DM in a French population [14].

Considering the potential contribution of VEGF-related SNPs to the risk of T2DM as well as the lack of relevant studies, here, we assessed the association between rs6921438 and rs10738760 and DR in Slovenian patients with T2DM.

MATERIALS AND METHODS

Patients

This case-control study enrolled 1037 unrelated Caucasians with T2DM and defined ocular status. The patients were not evaluated with regard to the history of glycemic control. T2DM was diagnosed according to the current American Diabetes Association criteria [18]. Dilated fundus examination was performed by a senior ophthalmologist (M.P.) after pupil dilation with 2.5% tropicamide and phenylephrine, 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). The clinical and laboratory data were obtained from the medical records of the patients. DR staging was performed according to the Early Treatment Diabetic Retinopathy Study (ETDRS) diabetic retinopathy severity scale [19]. The study group (DR) included 415/1037 patients with T2DM and DR, while the control group involved 622 individuals with T2DM who had no clinical signs of DR. To avoid the confounding effect of impaired kidney function, we did not enroll patients with overt nephropathy (i.e., end-stage renal failure). The study was approved by the National Medical Ethics Committee (number 118/12/2011). After obtaining informed consent from the patients, a detailed interview was undertaken.

Genotyping

Genomic DNA was extracted from 200 μL of the whole blood using a FlexiGene DNA kit, according to the recommended protocol (Qiagen, Germany). We selected VEGF-related rs6921438 and rs10738760 SNPs for analysis. rs6921438 is located on the short arm of chromosome 6 at 6p21.1, 171 kb downstream of the *VEGFA*, and close to the *C6orf223* gene,

which encodes an uncharacterized protein. rs10738760 is located on the short arm of chromosome 9 (i.e., 9p24.2), between the *VLDLR* and *KCNV2* genes [14]. Two SNPs were genotyped using predesigned TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA, USA), C 1971047 1 and C 27464534 20. We used the StepOne Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) for SNP analysis, according to the manufacturer's instructions. The real-time PCR reactions were set up in a final volume of 5 μL, containing 2.5 μL of 2 × TaqMan Genotyping Master Mix (Applied Biosystems, Foster City, CA, USA), 0.12 μL of 40 × SNP Genotyping Assay (Applied Biosystems, Foster City, CA, USA), 1.88 μL of nuclease-free water, and 25 ng of genomic DNA. The PCR was carried out under the following conditions: enzyme activation for 10 minutes at 95°C, followed by 55 cycles of amplification at 95°C for 15 seconds and at 60°C for 1 minute.

Statistical analysis

Statistical analysis was conducted using SPSS Statistics for Windows, Version 20.0. (IBM Corp., Armonk, NY). Two researchers (D.P. and I.C.), blinded to the DR status of the patients, performed SNP genotyping. We used a Chi-square test to compare discrete variables. Continuous variables were compared by the unpaired Student's *t*-test. In addition, all variables that showed significant differences in the univariate analysis (i.e., Chi-square test and unpaired Student's *t*-test) were included in a logistic regression analysis. A value of $p < 0.05$ was considered statistically significant. The deviation from Hardy–Weinberg equilibrium (HWE) was assessed by the Fisher's exact test (http://ihg.gsf.de/).

RESULTS

This study enrolled 1037 individuals with T2DM of at least 10 years' duration. The control group included 622 patients with T2DM but with no evidence of DR, while 415 patients with T2DM and DR were included in DR group. The clinical and laboratory data, obtained from the medical records of the patients, are shown in Table 1. There were no significant differences in the age, sex, systolic and diastolic blood pressure, history of hypertension, smoking status, high-density lipoprotein (HDL) cholesterol, and triglycerides between the groups (Table 1). On the contrary, a statistically significant difference was observed between the groups in the following parameters: duration of diabetes ($p < 0.001$), insulin therapy ($p < 0.001$), HbA1c ($p = 0.001$), body mass index [BMI] ($p = 0.002$), total cholesterol ($p = 0.002$), and low-density lipoprotein (LDL) cholesterol [$p < 0.001$](Table 1). The duration of diabetes was more than 5 years longer in DR compared to control group

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

Characteristics	DR group (415)	Control group (622)	p
Age (years)	65.41±8.7	64.95±9.2	0.4
Male sex (%)	211 (50.8)	318 (51.1)	0.5
Duration of diabetes (years)	18.98±8.2	13.28±6.8	<0.001
Patients on insulin therapy (%)	312 (75.1)	293 (47.1)	<0.001
Systolic blood pressure (mmHg)	148.99±21.9	148.82±19.5	0.9
Diastolic blood pressure (mmHg)	84.17±10.7	84.44±10.8	0.7
BMI (kg/m²)	29.34±4.9	30.19±4.5	0.002
History of hypertension (%)	334 (80.5)	498 (80.1)	0.5
Cigarette smokers (%)	36 (8.6)	51 (8.2)	0.5
Total cholesterol (mmol/l)	4.97±1.2	4.74±1.1	0.002
LDL cholesterol (mmol/l)	2.91±0.9	2.67±0.9	<0.001
HDL cholesterol (mmol/l)	1.19±0.3	1.23±0.4	0.07
Triglycerides (mmol/l)	2.09±0.6	2.12±0.7	0.8
HbA1c (%)*	8.01±1.4	7.72±1.3	0.001

Data are expressed as means ± standard deviations or frequencies (percentages). *The average value of HbA1c. T2DM: Type 2 diabetes mellitus; DR: Diabetic retinopathy; BMI: Body mass index; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; HbA1c: Glycated hemoglobin

TABLE 2. Distribution of rs10738760 and rs6921438 genotypes and alleles in T2DM patients with DR and those without DR (controls)

Genotypes/Alleles	DR group (415)	Control group (622)	p
rs10738760			
GG	96 (23.1%)	137 (22.0%)	0.22
GA	228 (54.9%)	319 (51.3%)	
AA	91 (21.9%)	166 (26.7%)	
G allele (%)	420 (50.6%)	593 (47.7%)	0.21
A allele (%)	410 (49.4%)	651 (52.3%)	
PHWE†	0.04	0.5	
rs6921438			
GG	103 (24.8%)	179 (28.8%)	0.25
GA	208 (50.1%)	309 (49.7%)	
AA	104 (25.1%)	134 (21.5%)	
G allele (%)	414 (49.9%)	667 (53.6%)	0.10
A allele (%)	416 (50.1%)	577 (46.4%)	
PHWE†	0.9	0.9	

†The polymorphisms were tested for adherence to Hardy–Weinberg equilibrium using Pearson's goodness-of-fit test (1 df). T2DM: Type 2 diabetes mellitus; DR: Diabetic retinopathy; PHWE: Probability of adherence to Hardy–Weinberg equilibrium

[$p < 0.001$] (Table 1). A significantly higher proportion of the patients with DR required insulin therapy compared to control group (Table 1). The patients with DR had higher HbA1c, total cholesterol, and LDL cholesterol levels, while BMI was significantly lower in this group compared to the controls (Table 1).

As shown in Table 2, the frequencies of the GG, GA and AA genotypes of rs10738760 were 23.1%, 54.9%, and 21.9% in DR group, and 22.0%, 51.3%, and 26.7% in control group, respectively. The average frequencies of rs10738760 alleles were 50.6% for G allele and 49.4% for A allele in DR group, and 47.7% for G allele and 52.3% for A allele in control group (Table 2).

The distributions of the GG, GA and AA genotypes of rs6921438 were 24.8%, 50.1%, and 25.1% in DR group and 28.8%, 49.7%, and 21.5% in control group, respectively (Table 2). The average frequencies of rs6921438 alleles in DR group were 49.9% for G allele and 50.1% for A allele, and in control group 53.6% for G allele and 46.4% for A allele (Table 2).

The genotype distribution of rs6921438 conformed to HWE in both groups [p = 0.9 for DR and p = 0.9 for control group] (Table 2). The genotype distribution of rs10738760 conformed to HWE in control group (p = 0.5), but not in DR group [p = 0.04] (Table 2).

We used a logistic regression analysis to evaluate whether the two SNPs were independently associated with DR, after adjusting for duration of diabetes, insulin therapy, BMI, HbA1c, total cholesterol, and LDL cholesterol (Table 3). We found no significant association between rs10738760 or rs6921438 and DR [co-dominant model] (Table 3).

DISCUSSION

Previously, a GWA study on 3,527 individuals of European descent reported four SNPs that were independently associated with VEGF levels: rs6921438, rs4416670, rs6993770, and rs10738760 [17]. In this cross-sectional study, we analyzed the association between rs6921438 and rs10738760 SNPs and DR in a group of Slovenian patients with T2DM. Our results showed no association between the two SNPs and DR in the patients with T2DM. These results are in agreement with the French case-control study [14] on 1336 T2DM patients with DR and 1231 T2DM controls, where they also reported no significant association between rs6921438 or rs10738760 and DR. In addition, no significant association between the two SNPs and macular oedema was found in their study [14].

A previous study on a subset of Slovenian patients with T2DM reported the effect of another *VEGF* polymorphism, rs2010963, on the serum and vitreous levels of VEGF; however, this SNP did not contribute to the genetic susceptibility to proliferative diabetic retinopathy (PDR) [16].

Four proteins are proposed to have important roles in the pathogenesis of diabetes, DR, PDR, or diabetic macular oedema (DMO), including: VEGF, the receptor for advanced glycation end products (RAGE), endothelial nitric oxide synthase (eNOS), and aldose reductase (AR) [13,16,20,21]. These proteins are encoded by four well-studied genes, and in the context of DR, the *VEGFA* has been the most commonly investigated gene [22]. The best-studied VEGF-related polymorphisms as potential risk factors of DR include rs833061, rs699947, rs2010963, and rs3025039. Many, but not all studies, confirmed the association between candidate SNPs and DR [13, 16, 20-22]. These studies included different populations

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

Inheritance model	Genotype	DR group (415)	Control group (622)	Adjusted OR, 95% CI/p-value†
rs10738760	GG	96 (23.1%)	137 (22.0%)	Reference
Codominant	GA	228 (54.9%)	319 (51.3%)	0.98 (0.65-1.48)/0.9
	AA	91 (21.9%)	166 (26.7%)	0.69 (0.43-1.11)/0.1
rs6921438	GG	103 (24.8%)	179 (28.8%)	Reference
Codominant	GA	208 (50.1%)	309 (49.7%)	1.06 (0.72-1.56)/0.8
	AA	104 (25.1%)	134 (21.5%)	1.51 (0.96-2.37)/0.1

†p values were calculated using logistic regression analysis and were adjusted for diabetes duration, insulin therapy, BMI, HbA1c, total cholesterol, and LDL cholesterol. DR: Diabetic retinopathy; OR: Odds ratio; CI: Confidence interval; BMI: Body mass index; HbA1c: Glycated hemoglobin; LDL: Low-density lipoprotein

or races (i.e., Caucasians, Asians, or both)[22]. Similar to our study, another study including Caucasians (i.e., French patients) assessed the effect of rs6921438 and rs10738760 on DR in T2DM patients, and showed no association between these SNPs and the risk of DR [14].

In our study, rs10738760 SNP did not conformed to HWE in DR group, meaning that the allele and genotype frequencies for this SNP are not constant from generation to generation [23]. This could be the result of evolutionary forces, such as mate choice, mutation, selection, genetic drift, gene flow, and meiotic drive. The deviation from HWE present only in DR and not in the control group, indicates a possible association of the SNP locus with DR [24].

The strengths of our prospective study are the community-based sample of Caucasians with T2DM and exact assessment of DR. A limitation of the study may be the small number of participants. Nevertheless, the study was appropriately designed to detect the differences in the distribution of genotypes in this cohort of T2DM patients with and without DR.

Further studies should clarify the contribution of VEGF-related SNPs to the development of DR in patients with T2DM.

CONCLUSION

In summary, we showed that VEGF-related SNPs rs10738760 and rs6921438 are not associated with DR in our group of Slovenian patients (Caucasians) with T2DM.

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DECLARATION OF INTERESTS

The authors declare no conflict of interests.

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