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POLYMORPHISM OF THE MANGANESE SUPEROXIDE DISMUTASE GENE BUT NOT OF VASCULAR ENDOTHELIAL GROWTH FACTOR GENE IS A RISK FACTOR FOR DIABETIC RETINOPATHY

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ABSTRACT

Background: In diabetic retinopathy, the vascular endothelium is damaged due to oxidative stress and inflammation, and vitreous VEGF concentration becomes elevated. We studied the association of diabetic retinopathy with single nucleotide polymorphisms (SNPs) on two genes: VEGF, an important mediator of neovascularisation, and MnSOD, a major antioxidant enzyme.

Methods: The study population was 755 individuals consisting of 131 diabetic (type 1 or type 2) patients with diabetic retinopathy (DR group), 98 diabetic controls without retinopathy (DC group) and 526 non-diabetic controls. VEGF SNPs rs699947, rs2010963, rs2146232, rs3025033, rs3025039 and Ala16Val polymorphism of the MnSOD gene were genotyped.

Results: The frequencies of allele and genotype of the single genotyped VEGF SNPs or haplotypes reconstructed of these single SNPs did not differ between DR and DC groups. We found a higher frequency of the AlaAla genotype ($p=0.03$) and Ala16 allele ($p=0.04$) of the MnSOD gene in the DR group when compared to DC group.

Conclusions: In conclusion, the studied VEGF SNPs were not associated with the risk of diabetic retinopathy and therefore it is unlikely that the VEGF gene is a major locus determining the risk of diabetic retinopathy. A statistically significant association of MnSOD Ala16Val polymorphism with diabetic retinopathy was found.

INTRODUCTION

Diabetic retinopathy is a microvascular complication of diabetes in which vessel endothelium is damaged due to oxidative stress, leukocyte adhesion, altered coagulation and inflammation (1). Vascular alterations due to hypoxia and biochemical changes in the eye in diabetic retinopathy lead to accumulation of blood and fluid in the retinal tissue. These angiogenic stimuli and the subsequent increased production of vascular endothelial growth factor (VEGF) may lead to retinal neovascularisation. These fragile neovascular vessels can leak blood into the vitreous cavity compromising vision. Fibrous tissue growth may also lead to retinal detachment and blindness if left untreated (2).

Diabetic retinopathy is associated with diabetes duration and poor glycemic control, but several studies have indicated that variations in the onset and severity of diabetic retinopathy are not totally explained by known risk factors (3). Furthermore, familial aggregation of diabetic retinopathy has been found (4). Associations between diabetic retinopathy and several candidate genes have been reported and VEGF, among others, has been suggested to be one of the most promising genetic loci involved in diabetic retinopathy (3).

Several clinical studies have reported increased VEGF levels in aqueous and vitreous fluids in patients with diabetic retinopathy (5). VEGF is a multifunctional cytokine which promotes angiogenesis and vascular permeability in highly vascular tissues such as kidneys and retina. It induces endothelial cell proliferation, promotes endothelial cell migration and functions both as a vasodilator and as an antiapoptotic factor (6). The VEGF gene located on chromosome 6 is highly polymorphic containing eight exons.

The retinal damage caused by hyperglycaemia has been linked to increased oxidative stress. The overproduction of reactive oxygen species, especially superoxide, by the mitochondrial electron-transport chain is prevented by overexpression of the enzyme manganese superoxide dismutase (MnSOD), the mitochondrial form of superoxide dismutase. MnSOD catalyses the breakdown of superoxide into hydrogen peroxide and thus protects the endothelial cells from oxidative stress (7). The most common polymorphism of MnSOD (a missense polymorphism changing alanine to valine at position 9 from the first amino acid of the mature protein or the 16th amino acid from the beginning of the signal sequence, Ala16Val), evokes functional consequences and this mutation has been identified as a risk factor for diabetic nephropathy (8).

The purpose of this study is to investigate the role of VEGF and MnSOD polymorphisms in diabetic retinopathy. The identification of the genes associated with diabetic retinopathy would form a basis for understanding the biochemical mechanisms of the disease and provide new tools for identification of patients at risk.

MATERIALS AND METHODS

Study subjects

This study is a case-control study. Recruited subjects were diabetic patients (type 1 or type 2) attending fundus imaging for screening of diabetic retinopathy in Oulu City Health Centres or patients with diabetic retinopathy from the Department of Ophthalmology, Oulu University Hospital. The diagnosis and grading of diabetic retinopathy was conducted by

an expert ophthalmologist from 60 degree fundus photographs through dilated pupils and by clinical examination. In order to avoid diabetics being misclassified as free of diabetic retinopathy because of a brief diabetes duration, we included only patients with a diabetes duration of at least 15 years. All subjects were interviewed about their health status (e.g. type of diabetes, duration of diabetes, medications). We performed basic blood tests (e.g. fasting blood glucose level, glycated haemoglobin, creatinine, CRP) as well as determinations of plasma lipids and lipid metabolism.

A total of 313 patients were recruited and 229 diabetics completed the questionnaire and provided blood samples. They were divided into two study groups: The diabetic retinopathy (DR) group consisted of 131 diabetics with diabetic retinopathy and the diabetic control (DC) group of 98 diabetic controls without retinopathy or with very mild background retinopathy (only a few microaneurysms). The control group (C) consisted of the control cohort of 526 inhabitants of the city of Oulu (Opera Control Cohort). These samples have been collected earlier and the group has been characterised (9).

The vitreous samples were collected from 39 diabetic patients with proliferative diabetic retinopathy who underwent vitreous surgery in Oulu University Hospital, Oulu, Finland. None of these patients had had anti-VEGF treatment and all gave their informed consent. Vitreous collection was performed at the beginning of classic three-port pars plana vitrectomy before starting intravitreal infusion with balanced salt solution. Undiluted vitreous samples (200-500 μ l) were aspirated into a tuberculin syringe attached into a vitreous cutter and immediately refrigerated after collection. Vitreous was aliquoted, transferred into sterile tubes and frozen at -70 °C.

Participation in the study was voluntary and all subjects had the right to revoke their permission without affecting their future treatment. Patient data were collected from the medical records of the Oulu University Hospital and Oulu City Health Centres. This study was approved by the Ethical Committee of the Oulu University Hospital and all the study subjects provided an informed consent.

Methods

Genomic DNA was isolated from leukocytes using the salting-out technique. The genotyping for VEGF SNPs was done using TaqMan® SNP Genotyping Assays and Applied Biosystems 7000 Real-Time PCR System (Applied Biosystems, Foster City, CA). The current study focuses on five identified polymorphisms of the VEGF gene: rs699947 (-2578A>C, numbering from the beginning of translation, located at the promoter), rs2010963 (-634C>G, located at 5' UTR), rs2146323 (A>C, intron 2), rs3025033 (A>G, intron 7) and rs3025039 (+936C>T, located at 3' UTR). A further three SNPs rs699947, rs2010963 and rs3025039 were selected based on previous positive findings that these SNPs can affect VEGF expression (10,11) and rs2146323 and rs3025033 were selected (with selection criteria of minor allele frequency over 10 % and r^2 threshold of 0.8) from HapMap to cover all the haploblocks of the gene. The MnSOD polymorphism was selected based on its previous association with diabetic nephropathy (8). MnSOD genotyping was conducted as previously described (12).

Plasma and vitreous VEGF concentrations were assessed using a QuantiGlo Chemiluminescent human VEGF immunoassay (cat. no QVE00B, R&D Systems, Minneapolis, MN, USA). The assay was performed according to the manufacturer's

instructions. The detection limit of the assay is 6.4 pg/ml and the assay is linear up to 20 000 pg/ml. According to the manufacturer, the intra-assay coefficient of variation was 4.0 % with an inter-assay coefficient of variation 7.9 %. The laboratory analyses (glucose control, lipids etc.) were performed as routine samples in the Oulu University Hospital.

Statistical analysis

Statistical analysis was done using the SPSS program version 14.0 (SPSS Inc., Chicago, IL, USA). The differences in the distribution of genotypes and alleles between the study groups were analysed by Fisher's exact test for 2 × 2 contingency table. The differences between the VEGF levels were analysed by Student's t-test. Haplotypes were constructed and haplotype-based association analysis was performed with the THESIAS ("Testing Haplotype Effects In Association Studies") program based on the maximum likelihood model and linked to the SEM algorithm (13). With THESIAS it is possible to estimate haplotype frequencies and their effects on the phenotype of interest simultaneously with covariates. Covariates (sex, diabetes type and glycated haemoglobin) were included in the analysis when indicated.

The power of the study was estimated by the PAWE program (Power for the Association With Errors) for the comparison of the allele frequencies between the study groups. In the power analysis, the power was assumed to be 80 % and the level for the statistical significance 0.05. The rare allele frequency of the studied SNPs in the control group varied between 0.16 and 0.48 (Table 3 and 4). If the rare allele frequency is 0.16 in the control group, it should be 0.075 or 0.27 in the case group for the difference in the allele frequencies to be detected as being statistically significant. If the rare allele frequency is 0.48 in the control group, it should be 0.35 or 0.62 in the case group to be detected as

statistically significant. Our power to detect the observed difference in the MnSOD allele frequencies (0.46 vs. 0.56, Table 4) between the study groups to be statistically significant (with the statistical significance level of 0.05) was estimated to be 55%.

RESULTS

The characteristics of the study groups are presented in Table 1. The mean ages of the patients at the DR, DC and C groups were 59.6, 56.1 and 51.4 years, respectively. The mean duration of diabetes was 24.3 years in the DR group and 24.7 years in the DC group. The prevalence of diabetic complications and hypertension was significantly higher in the DR group than in the DC group. Smoking habits and alcohol consumption did not differ significantly between the study groups. The levels of glycated haemoglobin and plasma lipids for the study groups are shown in Table 2.

VEGF SNPs

The genotype and allele frequencies are presented in Table 3. There were no significant differences in allele and genotype frequencies between the DR and DC groups. We also analyzed VEGF alleles and genotype frequencies separately for type I and type II diabetes, but we did not find a significant difference (please see Supplemental Tables). A total of 10 haplotypes were reconstructed from the five VEGF SNPs using the THESIAS program but the haplotype frequencies did not differ significantly between study groups (data not shown). The inclusion of covariates (age, diabetes type and glycated haemoglobin) did not change the results of the haplotype analysis.

MnSOD SNP

The genotype and allele frequencies for the MnSOD Ala16Val polymorphism are presented in Table 4. We found a higher frequency of the AlaAla genotype ($p=0.03$) and the Ala allele ($p=0.04$) in the DR group when compared to the DC group. No such difference was found when the DC group was compared with the control group or when all diabetics were compared with the control group. We also analyzed MnSOD alleles and genotype frequencies separately for type I and type II diabetes, but we did not find a significant difference (please see Supplemental Tables).

Plasma and vitreous VEGF levels in VEGF and MnSOD genotypes

The plasma and vitreous fluid VEGF levels in different VEGF and MnSOD genotypes are presented in Table 5. The number of subjects in some of the groups was small, and no significant difference between plasma and vitreous VEGF and VEGF genotypes was found, except for higher plasma VEGF in the VEGF rs2010963 GG genotype than CG genotype, but there were no subjects in CC genotype at all. However, plasma and vitreous VEGF concentrations were associated with MnSOD gene, as there was a trend that the ValVal genotype was associated with a higher plasma and vitreous VEGF concentration ($p=0.0330$ and $p=0.0170$, respectively).

DISCUSSION

Diabetic retinopathy is a leading cause of new-onset blindness in the working-age population in the developed countries (5). It is considered as a microvascular complication of diabetes. In diabetic retinopathy the endothelium is damaged due to oxidative stress,

leukocyte adhesion, altered coagulation and inflammation (1), and this leads to leakage of vessels and neovascularization. The current study has examined the role of genetic polymorphisms of the VEGF and MnSOD genes in diabetic retinopathy. We found an association between MnSOD gene polymorphism and diabetic retinopathy. In this study, we selected VEGF SNPs covering all the haploblocks of the gene, and therefore, the negative results of the VEGF polymorphisms should predict the association of the whole VEGF gene due to the strong linkage disequilibrium inside the haploblocks.

Previous studies on VEGF polymorphisms have suggested that the C allele of the -634C>G (rs2010963) polymorphism is a genetic risk factor for diabetic retinopathy (14) and diabetic macular oedema at least in a Japanese population of type 2 diabetics (15). The CC genotype of the -634C>G SNP has been associated with higher serum and vitreous levels of VEGF (16), (17) but it was not correlated with proliferative retinopathy in the Slovene population or in a British population (18). A study of Polish type 2 diabetics detected an association of C allele of -634C>G polymorphism with the development of nonproliferative diabetic retinopathy (19). However, the difference in study populations may account for the difference between these previous studies and the current study, as we did not find this SNP associating with diabetic retinopathy. Although the VEGF gene itself might not be associated with diabetic retinopathy, there might be mutations in other genes regulating VEGF expression, e.g. transcription factors such as hypoxia-inducible factor 1 α (20).

Diabetic retinopathy is associated with oxidative stress and hyperglycaemia-induced mitochondrial reactive oxygen-species overproduction is a key event in the development of diabetic complications. Overexpression of MnSOD in transgenic mice has inhibited retinal

VEGF expression, protected the retina from diabetes-induced abnormalities in the mitochondria and prevented vascular histopathology (21). Recently, in a Slovenian population, a significantly higher frequency of the ValVal genotype of the Ala16Val polymorphism of the MnSOD was found in patients with diabetic retinopathy compared to those without diabetic retinopathy (22). In a Korean population of type 2 diabetics, Ala16Val polymorphism of the Mn-SOD gene was not related to the progression of diabetic retinopathy, but was associated with diabetic macular oedema in patients with a mean age 53 years (23). However, our results could not confirm these reports as we found a higher frequency of AlaAla genotype in diabetic retinopathy. This discrepancy could be due to the study population age, since previously a discrepancy between polymorphism of the MnSOD gene has been described in patients with breast cancer, in the younger group (median age 34) there was an association to ValVal (24) but in older individuals to AlaAla (25).

The strength of our study is a homogenous and well-characterized study population. The VEGF gene was also analysed thoroughly utilizing haplotypic information, in addition to using only genotypes of single SNPs. The limitation of the current study is a rather small study population. However, with the current sample size a major locus for diabetic retinopathy should be detected as specified by the power analysis. It is possible that a minor effect of VEGF gene on the risk of diabetic retinopathy is missed.

In conclusion, in the current study we found a significant association between MnSOD Ala16Val polymorphism and diabetic retinopathy. However, the studied VEGF SNPs studied were not associated with the risk of diabetic retinopathy and therefore it is unlikely that VEGF gene is a major locus determining the risk of diabetic retinopathy

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Table 1. Clinical characteristics of the study groups.

	DR	DC	C
Sex (n (%))			
Men	79 (60)	50 (51)	259 (49)
Women	52 (40)	48 (49)	267 (51)
Age (years)	59.6 (13.5)	56.1 (16.4)	51.4 (6.0)
BMI (kg/m ²)	29.0 (5.9)	27.1 (5.1)	26.4 (4.0)
Diabetes			
Duration (years)	24.3 (10.0)	24.7 (7.3)	
Type (n (%))			
I	59 (45)	48 (49)	
II	72 (55)	50 (51)	
Medication (n (%))			
Insulin	114 (87)	78 (80)	
Oral diabetes medication	55 (42)	33 (34)	
Family occurrence (n (%)) of diabetes			
None	12 (13)	13 (14)	
Parent(s)	28 (31)	31 (33)	
Sibling(s)	14 (15)	17 (18)	
Parent(s) and sibling(s)	18 (20)	13 (14)	
Unknown	19 (21)	21 (22)	
Diabetic complications (n (%))			
Nephropathy	44 (34)	14 (15)	
Neuropathy	51 (40)	15 (16)	
Retinal laserphotocoagulation (n (%))	124 (95)	1 (1)	
Hypertension (n (%))	106 (82)	51 (52)	0 (0)
Smoking (n (%))			
Never	70 (53)	49 (51)	250 (48)
Ex-smoker	36 (28)	32 (33)	100 (19)
Smoker	25 (19)	15 (16)	176 (34)
Alcohol consumption (n (%))			
Never	55 (42)	22 (23)	
Occasionally	65 (50)	63 (66)	
Regularly	9 (7)	11 (12)	
Daily	1 (1)	0 (0)	

Values shown are expressed as means (standard deviations) or numbers of subjects (percentages). DR, diabetic retinopathy, DC, diabetic control group, and C, controls.

Table 2. Glycemic control and plasma lipids, creatinine and plasma VEGF in the study groups.

	DR	DC	C
	n=131	n=98	n=526
Plasma cholesterol (mmol/l)	4.6 (1.1)	4.6 (0.9)	5.7 (1.1)
HDL cholesterol (mmol/l)	1.3 (0.4)	1.5 (0.5)	1.4 (0.4)
LDL cholesterol (mmol/l)	2.8 (0.9)	2.6 (0.7)	3.5 (1.0)
Triglycerides (mmol/l)	1.7 (1.4)	1.1 (0.7)	1.4 (0.8)
Fasting plasma glucose (mmol/l)	9.0 (3.9)	8.5 (2.9)	
Glycated haemoglobin (%)	9.2 (1.9)	8.3 (1.3)	
Creatinine (μ mol/l)	106.4 (91.5)	72.6 (20.4)	
Plasma VEGF (pg/ml)	145.0 (104.5)		
Vitreous VEGF (pg/ml)	1040.3 (1276.5)		

The data are expressed as mean (SD). DR, diabetic retinopathy, DC, diabetic control group, and C, controls.

Table 3. Allele and genotype frequencies of the five VEGF SNPs studied.

	DR	DC	C	P-values (DR vs. DC)	
rs699947					
Genotype frequencies					
AA	38 (33)	27 (28)	173 (30)	AA and AC vs. CC	0.1744
AC	58 (48)	38 (40)	250 (46)		
CC	30 (19)	31 (32)	101 (24)		
Allele frequencies					
A	134 (57)	92 (48)	596 (53)	A vs. C	0.2926
C	118 (43)	100 (52)	452 (47)		
rs2010963					
Genotype frequencies					
CC	10 (6)	5 (5)	32 (8)	CC and CG vs. GG	0.5846
CG	42 (32)	39 (41)	170 (33)		
GG	74 (61)	52 (54)	322 (59)		
Allele frequencies					
C	62 (22)	49 (26)	234 (25)	C vs. G	0.8259
G	190 (78)	143 (74)	814 (75)		
rs2146323					
Genotype frequencies					
AA	19 (15)	13 (13)	106 (21)	AA vs. AC and CC	0.8479
AC	61 (48)	44 (45)	223 (44)		
CC	47 (37)	40 (41)	178 (35)		
Allele frequencies					
A	99 (39)	70 (36)	435 (43)	A vs. C	0.5560
C	155 (61)	124 (64)	579 (57)		
rs3025033					
	n (%)	n (%)	n (%)		
Genotype frequencies					
AA	88 (69)	71 (73)	363 (71)	AA vs. AG and GG	0.5550
AG	34 (27)	22 (23)	135 (26)		
GG	5 (4)	4 (4)	15 (3)		
Allele frequencies					
A	210 (83)	164 (85)	861 (84)	A vs. G	0.6107
G	44 (17)	30 (15)	165 (16)		
rs3025039					
Genotype frequencies					
CC	88 (70)	72 (75)	368 (70)	CC vs. CT and TT	0.4514
CT	35 (27)	21 (22)	140 (28)		
TT	3 (3)	3 (3)	16 (2)		
Allele frequencies					
C	211 (84)	165 (86)	876 (84)	C vs. T	0.5952
T	41 (16)	27 (14)	172 (16)		

The data are expressed as number of genotypes and alleles (percentages). DR, diabetic retinopathy, DC, diabetic control group, and C, controls.

Table 4. Allele and genotype frequencies of the MnSOD Val16Ala polymorphism.

	DR	DC	C	P-values (DR vs. DC)	
Genotype frequencies					
AlaAla	40 (32)	18 (19)	115 (23)	AlaAla vs. AlaVal and ValVal	0.0316
AlaVal	56 (44)	49 (51)	246 (50)		
ValVal	30 (24)	29 (30)	132 (27)		
Allele frequencies					
Ala	136 (54)	85 (44)	476 (48)	Ala vs. Val	0.0449
Val	116 (46)	107 (56)	510 (52)		

The data are expressed as number of genotypes (percentages). DR, diabetic retinopathy, DC, diabetic control group, and C, controls.

Table 5. VEGF levels of plasma and vitreous fluid in different genotype groups shown for all the genotyped SNPs.

	n (%)	Plasma VEGF (pg/ml)	Vitreous VEGF (pg/ml)
VEGF rs699947			
AA	15 (38)	193.4 (139.0)	1388.3 (1802.3)
AC	19 (49)	109.3 (63.6)	825.7 (842.9)
CC	5 (13)	128.2 (35.8)	811.4 (419.4)
AA and AC vs. CC		p = 0.9812	p = 0.4427
VEGF rs2010963			
CC	0 (0)		
CG	15 (39)	100.0 (46.4)	690.1 (397.8)
GG	24 (61)	171.2 (120.0)	1259.1 (1570.4)
CC and CG vs. GG		p < 0.0001	p = 0.0799
VEGF rs2146323			
AA	5 (13)	148.7 (167.8)	925.4 (934.0)
AC	22 (56)	152.3 (90.2)	1288.2 (1602.7)
CC	12 (31)	128.7 (107.8)	633.6 (314.8)
AA vs. AC and CC		p = 0.9878	p = 0.6126
VEGF rs3025033			
AA	27 (69)	159.4 (101.4)	1118.3 (1480.1)
AG	11 (28)	115.2 (113.8)	890.7 (658.8)
GG	1 (3)	97.9 ()	577.5 ()
AA vs. AG and GG		p = 0.9419	p = 0.3512
VEGF rs3025039			
CC	27 (69)	159.4 (101.4)	1118.3 (1480.1)
CT	11 (28)	115.2 (113.8)	890.7 (658.8)
TT	1 (3)	97.9 ()	577.5 ()
CC vs. CT and TT		p = 0.9419	p = 0.3512
MnSOD			
AlaAla	12 (31)	122.3 (76.3)	457.4 (253.3)
AlaVal	21 (54)	147.3 (105.6)	1158.1 (928.1)
ValVal	6 (15)	178.5 (148.4)	1793.5 (2691.4)
AlaAla vs. AlaVal and ValVal		p = 0.0330	p = 0.0170

The data are expressed as number of genotypes (percentage) or mean (SD).