

Functional Vascular Endothelial Growth Factor –634G>C SNP Is Associated With Proliferative Diabetic Retinopathy

A case-control study in a Brazilian population of European ancestry

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OBJECTIVE — The purpose of this study was to evaluate the effect of the single nucleotide polymorphism (SNP) –634G>C at the 5' regulatory region of the vascular endothelial growth factor (VEGF) in the risk of proliferative diabetic retinopathy (PDR) in the Brazilian population of European ancestry with type 2 diabetes.

RESEARCH DESIGN AND METHODS — A case-control study was conducted in 501 type 2 diabetic patients of European ancestry. Patients underwent a standardized clinical, ophthalmological, and laboratory evaluation. Of these, 167 patients had PDR (case patients), and 334 were considered as control subjects (patients without PDR) for PDR. A reference population (110 individuals of European ancestry) was also evaluated.

RESULTS — No evidence of association between –634G>C/VEGF and the presence of diabetic retinopathy or type 2 diabetes was observed ($P > 0.05$). However, CC homozygous for the SNP –634G>C was significantly more frequent in patients with PDR (37 of 167; 22.2%) than in the corresponding control group (40 of 334; 12%) in accordance with a recessive model ($P = 0.003$). This effect was further observed when creatinine, BMI, sex, duration of type 2 diabetes, HDL cholesterol, and systolic blood pressure were taken into account (odds ratio 1.9 [95% CI 1.01–3.79], $P = 0.04$).

CONCLUSIONS — The presence of the allele –634C/VEGF in homozygosity is an independent risk factor for the development of PDR in type 2 diabetic patients of European ancestry.

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Abbreviations: AAO, American Academy of Ophthalmology; NPDR, nonproliferative diabetic retinopathy; PDR, proliferative diabetic retinopathy; SNP, single nucleotide polymorphism; VEGF, vascular endothelial growth factor.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

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Diabetic retinopathy is a common microvascular complication in patients with diabetes, constituting a major cause of blindness in this group. Although the risk of development of this complication increases with poor glycemic control, there are several indications suggesting that the occurrence or progression of diabetic retinopathy also depends on genetic factors (1–3).

Proliferative diabetic retinopathy (PDR), characterized by increased vascular permeability, tissue ischemia, and neovascularization, affects 10–20% of diabetic patients (4). This process depends on the local production of angiogenic factors and components of the extracellular matrix, which will be substrates for endothelial migration. Vascular endothelial growth factor (VEGF), a potent activator of angiogenesis, enhances collateral vessel formation and increases the permeability of the microvasculature (5,6). VEGF expression is induced by high glucose levels and hypoxia and plays an important role in normal and abnormal angiogenesis (7–9). Its levels have been found to be markedly increased in the vitreous and aqueous fluids in the eyes of patients with PDR (10–12).

Several polymorphisms at the VEGF 5' regulatory region have been characterized and evaluated as risk alleles for the susceptibility or progression of both diabetic retinopathy and diabetic nephropathy through case-control studies (13–16). The single nucleotide polymorphism (SNP) –634G>C was found to be associated with nonproliferative diabetic retinopathy (NPDR) and diabetic macular edema in type 2 diabetic patients (13,16). Ray et al. (15) detected an association between the SNP –460C>T but not the SNP –634G>C and PDR in diabetic patients. Therefore, association of the SNP –634G>C still remains controversial.

These data thus suggest that polymorphisms located at the 5' regulatory region of VEGF might represent at-risk alleles to diabetic retinopathy or to its progression. Considering the importance of validating

this hypothesis, this work was undertaken to evaluate the effect of the SNP $-634G>C$ /VEGF in the risk of diabetic retinopathy in Brazilian patients of European ancestry with type 2 diabetes, a nationally representative sample of our population. The allele $-634C$ /VEGF, with a frequency of at least 10% in Asian and European populations (13–17), is associated with increased VEGF transcription and translation (16–18).

RESEARCH DESIGN AND METHODS

A total of 501 type 2 diabetic patients were included. Of these, 359 patients belonged to a cohort being followed at the Federal University of Rio Grande do Sul, and a detailed description can be found elsewhere (19). Briefly, patients with type 2 diabetes were identified from a multicenter study that started recruiting patients in southern Brazil in 2002. The aim of that project was to study risk factors for chronic complications of diabetes. It included four centers located at general hospitals in the state of Rio Grande do Sul, namely Grupo Hospitalar Nossa Senhora da Conceição, Hospital São Vicente de Paula, Hospital Universitário de Rio Grande, and Hospital de Clínicas de Porto Alegre. The remaining 142 patients were ascertained at Hospital das Clínicas, University of São Paulo Medical School, São Paulo. All of these 501 patients were of European ancestry (defined as descendants of Portuguese, Spanish, Italians, and Germans). The ethnic groups were defined on the basis of self-classification and subjective classification (skin color, nose and lip shapes, hair texture, and information about family ancestry). Those who defined themselves as having mixed or other ancestry were not included. The study was approved by the ethics committees of the Institute of Biosciences, University of São Paulo, the Hospital das Clínicas, University of São Paulo, and the Federal University of Rio Grande do Sul. Blood was drawn only after the informed consent was obtained.

Patients underwent a standardized evaluation consisting of a questionnaire, physical examination, and laboratory tests. Diagnosis of type 2 diabetes was based on the guidelines of the report of the Expert Committee of the American Diabetes Association (20). Weight, without shoes and in light outdoor clothes, and height were measured, and BMI was calculated as weight in kilograms divided by the square of height in meters. Hypertension was defined as blood pressure

$\geq 140/90$ mmHg or use of antihypertensive medication (21).

Diabetic retinopathy

Fundus examination was performed in all patients by a trained ophthalmologist using direct and indirect ophthalmoscopy through dilated pupils. Retinopathy was classified as absent, nonproliferative (microaneurysms, hemorrhage, and hard exudates), or proliferative (newly formed blood vessels and/or growth of fibrous tissue into the vitreous cavity). Patients with panphotocoagulation were classified as presenting PDR. The severity of diabetic retinopathy was graded on the basis of the worst eye. In two patients in whom the presence of media opacities due to vitreous hemorrhage (one patient) and cataract (one patient) prevented funduscopy in one eye, the contralateral eye was used to classify diabetic retinopathy. No patient was excluded as a result of unreadable funduscopy tests in both eyes. The diagnosis of PDR on the basis of funduscopy performed by an ophthalmologist was used to classify the patients. For a subset of 240 patients, selected for reasons of convenience, stereoscopic color fundus photographs of seven standard fields (22) were obtained to analyze the agreement between the classification of retinopathy using this method and ophthalmoscopy performed by the physicians. Initially, two ophthalmologists, who were unaware of the patients' clinical data, classified the fundus photographs independently according to the criteria of the American Academy of Ophthalmology (AAO) (23). The agreement of diabetic retinopathy classification performed by ophthalmoscopy and stereoscopic fundus photographs was then analyzed to validate the ophthalmoscopy procedure used to classify studied patients.

The κ coefficient was used to assess the agreement between diabetic retinopathy classification by different ophthalmologists and by different methods (stereoscopic fundus photographs and ophthalmoscopy) and to evaluate the agreement of diabetic retinopathy classifications performed by the same ophthalmologist on two separate occasions in the subset patients. The agreement of diabetic retinopathy classification by stereoscopic fundus photographs performed by the different ophthalmologists was 95.3% ($\kappa = 0.774$; $P < 0.001$) when they used the simplified diabetic retinopathy classification (presence or absence of PDR) and 88.8% ($\kappa = 0.771$; $P < 0.001$) when they

used the AAO classification (23). Moreover, the agreement of diabetic retinopathy classification performed by ophthalmoscopy and by stereoscopic fundus photographs was 95.1% ($\kappa = 0.735$; $P < 0.001$) for the simplified classification and 84.3% ($\kappa = 0.698$; $P < 0.001$) for the AAO classification (23).

Considering that the allele $-634C$ /VEGF is associated with increased expression levels of VEGF and possibly associated with PDR, we would expect an increased frequency of this allele among patients with PDR. Therefore, patients with PDR were considered case patients and patients without PDR with at least 10 years of disease were considered control subjects. This strategy was used because epidemiological and familial studies suggested that PDR has a genetic background. This approach was also used by other authors (24–26).

Reference population

As a reference group for the allele frequencies of the specific polymorphism, 110 healthy blood donors of European ancestry who did not have type 2 diabetes or a family history of the disease (mean \pm SD age 52.00 ± 17.06 years) were included. The first consecutive samples from the DNA bank of blood donors that fulfilled these criteria were included.

Laboratory analysis

Glucose was determined by a glucose oxidase method, creatinine was determined by the Jaffé reaction, A1C was determined by an ion-exchange high-performance liquid chromatography procedure (reference range 2.7–4.3%; Merck-Hitachi L-9100 glycosylated hemoglobin analyzer; Merck, Darmstadt, Germany), and triglyceride and cholesterol levels were determined by enzymatic methods. Albuminuria was measured by immunoturbidimetry (Sera-Pak immuno microalbuminuria; Bayer, Tarrytown, NY) (mean intra- and interassay coefficients of variance 4.5 and 7.6%, respectively). Total cholesterol, HDL cholesterol, and triglycerides were measured by standard enzymatic methods.

Analysis of the SNP VEGF $-634G>C$

Genomic DNA was extracted from peripheral blood using standard protocols (27) and was PCR amplified using primers and conditions as reported previously (13). The SNP VEGF-634G>C was detected using single nucleotide primer ex-

Table 1—Clinical characteristics of type 2 diabetic patients of European ancestry according to diabetic retinopathy classification

	Control	Case	P value
Sex (male:female)	201:133	67:100	
Duration of diabetes (years)	12.81 ± 7.48	15.22 ± 8.39	0.001
Age (years)	48.51 ± 10.05	45.17 ± 11.08	
BMI (kg/m ²)	29.05 ± 5.26	27.79 ± 4.60	0.02
LDL cholesterol (mg/dl)	132.77 ± 44.08	141.45 ± 49.52	
HDL cholesterol (mg/dl)	45.30 ± 12.03	42.03 ± 10.44	0.008
Cholesterol (mg/dl)	210.63 ± 49.69	212.18 ± 48.35	
Creatinine (mg/dl)	1.11 (0.7–1.4)	2.27 (1.8–3.3)	0.0001
Triglycerides (mg/dl)	189.03 ± 131.7	194.62 ± 124.30	
A1C (%)	7.23 ± 2.26	7.92 ± 2.60	
Systolic blood pressure (mmHg)	141.84 ± 22.76	149.12 ± 23.10	0.007
Diastolic blood pressure (mmHg)	86.18 ± 14.45	86.02 ± 12.89	
GG/GC/CC genotypes	41.6/46.4/12	34.1/43.7/22.2	0.01
G/C alleles	0.65/0.35	0.56/0.44	0.24

Data are means ± SD, median (range), or percent.

tension (primer: 5' GTCACCTCACTTTGC CCCTGTC 3') and MegaBACE (single nucleotide primer extension; Amersham Biosciences, Piscataway, NJ).

Statistics

Continuous clinical data were compared by unpaired Student's *t* tests. Allele frequencies were determined by gene counting, and departures from Hardy-Weinberg equilibrium were verified using a χ^2 test. Allele and genotype distributions among groups were evaluated using a χ^2 test or Fisher's exact test. The level of significance adopted was $P < 0.05$. Logistic regression analysis was performed to assess the independent role of the VEGF genotype and other variables, including BMI, systolic blood pressure, diastolic blood pressure, A1C, duration of diabetes, and the presence of microalbuminuria. Statistical analyses were done with SSPS, version 10.0.

RESULTS

Characterization of the patient sample

The main clinical features of the patients are depicted in Table 1. The mean duration of diabetes was 13.78 ± 7.78 years. Case patients (PDR) differed from control subjects (NPDR and nondiabetic retinopathy) for diabetes duration, sex, BMI, serum HDL cholesterol and serum creatinine levels, and systolic blood pressure.

SNP –634G>C/VEGF

Genotypic distribution for the SNP –634G>C was in Hardy-Weinberg equilibrium in all groups ($P > 0.05$). The ge-

notypic and allelic frequencies did not differ statistically between the reference population group (44 GG [40%], 57 GC [51.8%], and 9 CC [8.2%]: –634C = 0.66 and –634G = 0.34) and patients with type 2 diabetes ($P = 0.13$), suggesting that this SNP is not influenced by the presence of type 2 diabetes. LDL cholesterol ($P = 0.63$), HDL cholesterol ($P = 0.71$), total cholesterol ($P = 0.11$), creatinine serum levels ($P = 0.95$), diabetic nephropathy ($P = 0.45$), BMI ($P = 0.08$), systolic blood pressure ($P = 0.63$), and diastolic blood pressure ($P = 0.73$) in type 2 diabetic patients are not associated with the GG, GC, or CC genotypes of the SNP –634G>C/VEGF.

SNP –634G>C/VEGF and diabetic retinopathy

For the case-control analysis, 167 patients with type 2 diabetes and PDR were considered as case patients and 334 patients with NPDR ($n = 55$) or without diabetic retinopathy ($n = 279$) were considered as control subjects. The CC genotype was more frequent in case patients than in control subjects of the European ancestry group ($\chi^2 = 9.27$; $P = 0.01$) (Table 1). Assuming a dominant model (CC + CG vs. GG), the –634C allele was not associated with PDR ($P = 0.12$; $P = 0.36$). However, in a recessive model (CC vs. GC + GG), the CC genotype was significantly more frequent in case patients (37 CC [22.2%], 130 CG + GG [77.8%]) than in control subjects (40 CC [12.0%], 294 CG + GG [88.0%]) and was significantly associated with PDR ($P = 0.003$), with an odds ratio (OR) of 1.85 (95% CI 1.2–2.8). Logistic regression including diabetes du-

ration, systolic arterial blood pressure, serum HDL cholesterol and creatinine levels, BMI, and sex showed that the CC genotype was independently associated with PDR ($P = 0.04$, OR adjusted 1.96 [1.01–3.79]). These results suggest that the CC genotype is associated with the severity but not with the occurrence of retinopathy.

CONCLUSIONS — In the present article we have evaluated whether there is an association between the SNP –634G>C/VEGF and PDR in Brazilian type 2 diabetic patients of European ancestry. Our data suggest that the –634C allele when in homozygosis increases by 1.9 times the chance of an individual of European ancestry developing the proliferative form of diabetic retinopathy. This association was also observed after we controlled for other possible risk factors: serum creatinine, BMI, sex, duration of diabetes, HDL cholesterol, and systolic blood pressure ($P = 0.04$; OR adjusted 1.96 [95% CI 1.01–3.79]). Therefore, our results suggest that the –634CC genotype is an independent risk factor for the development diabetic retinopathy in patients of European ancestry.

The SNP –634G>C is in linkage disequilibrium with the SNPs –460C>T, –2.578C>A, and –1.154G>A, which have been studied in type 2 diabetes and diabetic retinopathy (13,15,16). Awata et al. (13) observed an association between the SNP –634G>C and susceptibility for diabetic retinopathy but not with progression of diabetic retinopathy in Japanese patients, in opposition to our results. Ray et al. (15) did not find an association

between the SNP -634G>C and diabetic retinopathy or PDR, but they found an association between the SNP -460C>T/VEGF and diabetic retinopathy in a group comprising both of type 2 and type 1 diabetic patients. These findings suggest that the SNPs at the 5' regulatory region of VEGF are involved with diabetic retinopathy or its progression, and their involvement might vary among populations. It is worth noting that linkage disequilibrium at the 5' region is not complete, and, therefore, discordant results may reflect haplotype differences among distinct populations. Alternatively, these differences may be related to small sample size and diabetes heterogeneity: Awata et al. (13) included 70 patients with PDR and 80 with NPDR, whereas our study included 207 patients with PDR and 74 with NPDR. On the other hand, Ray et al. (15) included 69 patients with PDR and 198 without PDR with type 1 or type 2 diabetes, in contrast to our sample and that of Awata et al. (13) who included only type 2 diabetic patients. We observed that the age of onset of the disease in patients with PDR was earlier than in the respective control subjects, whereas the duration of the disease was significantly longer in case patients compared with control subjects (Table 1). These data, together with those from the literature (13,15), suggest that the PDR group is indeed etiologically distinct.

Genetic association of an isolated SNP with a given phenotype is inherently weak, and other SNPs in linkage disequilibrium with the one under study could also be responsible for the result obtained. Our findings should be interpreted very cautiously. However, functional studies have shown that the -634C allele is associated with increased transcriptional levels of VEGF, both in vitro and in vivo (16). This allele is also related to a higher activity at ribosome site B (IRESB) (17,28,29), suggesting that it also influences VEGF translation. The GG genotype is embedded in haplotypes with lower VEGF expression (haplotypes -2.578A/-1.154A/-634G and -2.578A/-1.154G/-634G), whereas the CC genotype is present in most of the haplotypes with the highest VEGF transcription levels (17,18). Therefore, the positive association between the -634C alleles and PDR is in accordance with its higher transcriptional and translational activities; that is, individuals with higher VEGF levels are more prone to develop the proliferative form of diabetic retinopathy.

In summary, our results show that the -634CC genotype/VEGF is independently associated with PDR in patients of European ancestry according to a recessive model. This finding is supported by the increased VEGF plasma levels only in those with the CC but not the CG genotype (13). Considering the fact that there are other SNPs at the 5' regulatory region, it is possible that this SNP is not the cause of the association, but still another SNP may be implicated.

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