Association of Vascular Endothelial Growth Factor Polymorphisms with Nonproliferative and Proliferative Diabetic Retinopathy

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Context: Vascular endothelial growth factor (VEGF) is a potent angiogenic and vascular permeability factor, and its polymorphisms are associated with proliferative diabetic retinopathy (PDR) and macular edema.

Objective: We investigated the contributions of VEGF gene polymorphisms to nonproliferative diabetic retinopathy (NPDR) as well as PDR.

Design, Setting, and Subjects: In this study we compared VEGF gene variants in a sample of Korean type 2 diabetes patients with and without diabetic retinopathy (DR) and in healthy controls. Of the diabetes patients, 145 had PDR, 108 had NPDR, and 134 had no retinopathy (noDR). They were all duration matched. Samples were genotyped for rs699947, rs1570360, and rs2010963 polymorphisms.

Results: We found a significant association between the A allele at rs699947 with DR (odds ratio = 1.84 (95% confidence interval = 1.28–2.66); $P = 0.001$ *vs.* noDR). Patients with NPDR, as well as PDR, had increased incidence of the A allele. The AGG haplotype was more frequently found in patients with DR than in patients with noDR (odds ratio = 4.79 (95% confidence interval = 1.42–16.16); $P = 0.006$). PDR and NPDR patients exhibited an increased incidence of the AGG haplotype.

Conclusions: VEGF polymorphisms might be a useful predictive marker for the development and progression of DR at an earlier stage of diabetes. **(*J Clin Endocrinol Metab* 95: 3547–3551, 2010)**

Diabetic retinopathy (DR), one of the most prominent pathological microvascular complications of type 2 diabetes (T2D), often leads to blindness in patients. Hyperglycemia has been recognized as the primary pathogenic factor in the development and progression of diabetic vascular complications (1), activating several biochemical pathways (2, 3). Nonetheless, even in the context of poor glycemic control, about 20% of the patients who go on to develop T2D do not exhibit significant retinal changes of the type that can easily be observed in patients with nonproliferative DR (NPDR) and proliferative DR (PDR) (1, 4). In contrast, others may develop PDR at a relatively early stage of diabetes despite good glycemic control. Therefore, DR might be a complex mul-

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Abbreviations: CI, Confidence interval; DR, diabetic retinopathy; NPDR, nonproliferative DR; PDR, proliferative DR; OR, odds ratio; SNP, singe-nucleotide polymorphism; T2D, type 2 diabetes; VEGF, vascular endothelial growth factor.

tifactorial disorder, resulting from an interaction of genetic as well as environmental etiologies. To indentify candidates who may benefit from prevention strategies, it would be useful to identify molecular markers that may help to predict the development of DR at earlier stages of diabetes.

Vascular endothelial growth factor (VEGF), a 45-kDa homodimeric glycoprotein, is a potent angiogenic and vascular permeability factor (3) and is strongly implicated in the development of several complications in patients with T2D. Specifically, several studies have shown that VEGF is critically involved in the progression of DR, and local concentration of VEGF might be a most important mediator in the development of DR (5–7), because VEGF is abundant in the eyes of patients with NPDR and even more so in those with PDR (8). Several single-nucleotide polymorphisms (SNPs) have been described in the VEGF gene, some of which have been reported to be associated with differential expression of VEGF *in vitro* (9). A series of previous studies reported association of VEGF polymorphisms with PDR and macular edema (8, 10). In the present study, we applied the duration-matched comparison method to study the association of three SNPs (rs699947, rs1570360, and rs2010963) at the VEGF gene, either separately or in combination, with different progression stages of DR. We focused on patients with NPDR to identify any possible missed associations between NPDR and VEGF polymorphisms.

Patients and Methods

Patients

A total of 387 patients with T2D (152 males and 235 females) and 260 healthy controls were recruited from the Korea National Diabetes Program, a prospective natural history study of T2D. Diagnosis and classification of diabetes were based on the guidelines of the Expert Committee Report of the American Diabetes Association (11). Among 387 diabetic patients, 145 patients had PDR, 108 patients had NPDR, and 134 patients did not have any DR lesions (noDR). Because the phenotype of DR may differ according to the duration of the diabetes, diabetic duration was matched in each of the subgroups, PDR, NPDR, and noDR (Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). Written consent was obtained from each patient before enrollment in the study. This study was approved by the Ethical Committee of Hanyang Medical University Hospital. As a baseline exam for clinical characteristics, patient's age, sex, body mass index, and systolic and diastolic blood pressure were obtained and analyzed. Blood pressure was measured by mercury sphygmomanometer. The serum levels of total cholesterol, triglyceride, creatinine, and glycosylated hemoglobin were also measured from blood samples. In addition, creatinine clearance and 24-h urine excretion for microalbumin were measured from 24-h urine collection.

Phenotype and measurements

All diabetic patients underwent a complete ophthalmological examination including corrected visual acuity, slit-lamp biomicroscopic examination, fundus examination, and fundus photography using fundus lenses with a slit lamp. A retina specialist evaluated and graded the funduscopic findings. The diagnosis of DR was confirmed by fluorescein angiography, and progression of DR was classified as PDR and NPDR. NPDR denoted signs of venous abnormality, microaneurysm, hemorrhage, hard exudates, macular edema, soft exudates, and peripheral ischemia on fluorescein angiography. PDR was identified by the presence of neovascularization elsewhere, vitreous hemorrhage, and fibrovascular proliferation (12).

Determination of the VEGF genotype

DNA was isolated from peripheral blood leukocytes by standard procedure. Genotyping of each polymorphism was carried out using the TaqMan assay and the ABI 7900HT sequence detection system (Applied Biosystems, Foster City, CA). The details of probes and primers used are provided in Supplemental Table 2. For the PCR assay, initial extension was at 95 C for 10 min, followed by 40 cycles consisting of 95 C for 15 sec and 60 C for 1 min.

Statistical analyses

All clinical data are summarized as mean ± SD. Continuous clinical data between two groups were compared using Student's *t* test. Distribution of genotypes and alleles were evaluated by χ^2 or Fisher's exact test, as appropriate. We tested each SNP in the control group for Hardy-Weinberg equilibrium. We used SPSS for Windows software for statistical analysis (version 12.0; SPSS, Chicago, IL). For SNPs that were found to be significant, the strength of association was tested by odds ratio (OR) estimates at a 95% confidence interval (CI). $P < 0.05$ was considered statistically significant. Haplotype frequencies were estimated using the expectation-maximization algorithm using Haploview version 4.1.

Results

Two SNPs (rs699947 and rs1570360) are located in the VEGF promoter (13), and one SNP (rs2010963) is located in the 5′ untranslated region of the gene (10). Allele and genotype frequencies of the three SNPs were in Hardy-Weinberg equilibrium, and these were tightly linked to each other (Lewontin's coefficient D′ = 0.8923–0.9989).

When the patients with DR were compared with those without DR (noDR), T2D patients with DR, especially with PDR, had high systolic pressure, high serum creatinine concentration, decreased creatinine clearance, and increased level of microalbumin excretion in the urine. All other clinical characteristics were not significantly different. There were no significant differences in these parameters between NPDR and noDR patients (Supplemental Table 1).

Table 1 shows distributions of genotype and allele frequencies of each of the three SNPs of the VEGF gene in

TABLE 1. Genotype and allele distributions of VEGF gene polymorphisms in healthy controls and T2D patients with and without retinopathy

Genotype or allele	Healthy controls (n = 260), %	noDR (n = 134), %	Retinopathy								
			All DR (n = 253)			NPDR (n = 108)			PDR (n = 145)		
			%	OR (95% CI)	*P*	%	OR (95% CI)	*P*	%	OR (95% CI)	*P*
rs699947											
CC	60.0	68.7	48.6	0.43 (0.28–0.67)	1.0×10^{-4}	48.1	0.42 (0.25–0.72)	0.001	49.0	0.44 (0.27–0.71)	8.0×10^{-4}
CA	32.7	26.9	45.5	2.27 (1.44–3.58)	4.0×10^{-4}	43.5	2.10 (1.22–3.60)	0.007	46.9	2.40 (1.46–3.97)	5.0×10^{-4}
AA	7.3	4.4	5.9	1.35 (0.51–3.58)	0.55	8.3	1.94 (0.67–5.63)	0.22	4.1	0.92 (0.29–2.93)	0.89
C	76.3	82.1	71.3	0.54 (0.38–0.74)	0.001	69.9	0.51 (0.33–0.78)	0.002	72.4	0.57 (0.38–0.86)	0.007
A	23.7	17.9	28.7	1.84 (1.28–2.66)	0.001	30.1	1.97 (1.29–3.02)	0.002	27.6	1.75 (1.17–2.62)	0.007
rs1570360											
GG	72.3	60.5	65.6	1.25 (0.81–1.92)	0.32	68.5	1.42 (0.84–2.43)	0.19	63.5	1.14 (0.70–1.84)	0.61
GA	23.9	34.3	30.8	0.85 (0.55–1.33)	0.48	26.9	0.70 (0.40–1.22)	0.21	33.8	0.98 (0.60–1.60)	0.92
AA	3.8	5.2	3.6	0.67 (0.24–1.84)	0.43	4.6	0.88 (0.27–2.86)	0.83	2.8	0.52 (0.15–1.80)	0.29
G	84.4	77.6	81.0	1.23 (0.86–1.77)	0.26	81.9	1.31 (0.84–2.05)	0.24	80.3	1.18 (0.74–1.78)	0.43
A	15.6	22.4	19.0	0.81 (0.57–1.17)	0.26	18.1	0.76 (0.49–1.20)	0.24	19.7	0.85 (0.56–1.28)	0.43
rs2010963											
GG	33.5	32.1	33.6	1.07 (0.68–1.67)	0.76	34.3	1.10 (0.64–1.89)	0.72	33.1	1.05 (0.63–1.73)	0.94
CG	48.5	51.5	49.4	0.92 (0.61–1.40)	0.70	48.1	0.88 (0.53–1.45)	0.61	50.3	0.96 (0.60–1.53)	0.61
CC	18.1	16.4	17.0	1.04 (0.59–1.83)	0.89	17.6	1.09 (0.55–2.13)	0.81	16.6	1.01 (0.54–1.90)	0.55
G	57.7	57.8	58.3	1.02 (0.76–1.38)	0.90	58.3	1.02 (0.71–1.47)	0.91	58.2	1.02 (0.73–1.43)	0.92
C	42.3	42.2	41.7	0.98 (0.73–1.32)	0.90	41.7	0.98 (0.68–1.41)	0.91	41.8	0.98 (070–1.38)	0.92

The genotype and allele frequencies of each SNP were compared between T2D patients with DR and without retinopathy (noDR). *P* values *vs.* T2D patients without retinopathy (noDR) are shown.

TABLE 2. Distribution of probable haplotypes of VEGF polymorphisms in T2D patients with and without DR

		Retinopathy								
		All DR (n = 253)			NPDR (n = 108)			PDR (n = 145)		
Haplotype	noDR (n = 134), %	%	OR (95% CI)	*P*	%	OR (95% CI)	*P*	%	OR (95% CI)	*P*
H1: CGC	41.6	40.8	0.96 (0.63–1.46)	0.837	41.0	0.96 (0.57–1.60)	0.869	40.6	0.96 (0.57–1.60)	0.852
H2: CGG	33.6	29.5	0.83 (0.53–1.31)	0.426	28.9	0.80 (0.46–1.38)	0.416	29.9	0.86 (0.52–1.43)	0.562
H3: AAG	15.5	17.9	1.16 (0.66–2.05)	0.599	18.1	1.22 (0.62–2.40)	0.557	17.8	1.12 (0.59–2.11)	0.724
H4: AGG	2.4	9.9	4.79 (1.42–16.16)	0.006	11.4	5.46 (1.50–19.87)	0.006	8.7	4.30 (1.20–15.44)	0.019
H5: CAG	6.3	1.1	0.19 (0.05–0.73)	0.019	0	1.06 (1.02–1.02)	0.009	1.9	0.33 (0.86–1.28)	0.126
Other	0.6	0.8	1.06 (0.10–11.80)	1.000	0.6	1.24 (0.08–20.11)	1.000	1.1	0.92 (0.08–14.92)	1.000

The frequencies of each haplotype were compared between T2D patients with DR and without retinopathy (noDR). Order of SNPs in a haplotype is as follows: rs699947, rs1570360, and rs2010963. *P* values *vs.* T2D patients without retinopathy (noDR) are shown. Haplotype frequencies were estimated using the expectation-maximization algorithm using Haploview version 4.1.

patients with PDR, NPDR, noDR, and healthy controls. When the genotype and allele frequencies in patients with DR were compared between patients without DR (noDR) and healthy controls, only those at rs699947 differed significantly. A positive association of the A allele at rs699947 with DR was found [OR = 1.84 (95% CI = 1.28–2.66); $P = 0.001$ *vs.* noDR]. We did not detect any associations between the AA genotype and DR. The CC genotype at rs699947 was significantly less frequent in patients with DR [OR = 0.43 (95% CI = 0.28–0.67); $P = 0.0001$]. Similar distributions were found in NPDR as well as PDR patients. Patients with NPDR had increased incidence of the A allele [OR = 1.97 (95% CI = 1.29–3.02); $P = 0.0002$] and decreased CC genotype [OR = 0.42 (0.25–0.72); $P = 0.0001$]. The distribution of alleles and genotypes in healthy controls did not differ from those patients with noDR.

As shown in Table 2, the AGG haplotype was frequently found in patients with DR compared with those with noDR [OR = 4.79 (95% CI = 1.42–16.16); $P = 0.006$]. The AGG haplotype was also associated with NPDR and PDR. In contrast, the CAG haplotype decreased significantly in patients with DR compared with patients with noDR [OR = 0.19 (95% CI = 0.05–0.73); $P = 0.019$].

Discussion

In the present study, we detected an association between VEGF polymorphisms and DR, including NPDR in a Korean T2D cohort. We evaluated the differential influence of VEGF polymorphisms on the development of DR in all spectra of DR development, including noDR, NPDR, PDR, and healthy controls. Given that a longer duration of diabetes increases the chances of exposure to hyperglycemia and aggravates the progression of DR, we applied a duration-matched comparison method of comparing each subgroup. We identified the rs699947 SNP and two haplotypes composed of three SNPs in the VEGF gene that were associated with DR. We confirmed this association in the NPDR stage of DR as well as in PDR. Even in NPDR stage, the incidence of the A allele increased, and the C allele was less frequent, whereas the AGG haplotype was more frequent and the CAG haplotype less frequent compared with noDR patients. This deviated distribution was also consistently found in PDR.

The A/C polymorphism at rs699947 was also found to be associated with PDR in a Japanese sample (14). However, this association was observed only in PDR, which prompted us to study the genetic influence of VEGF polymorphism in the development of DR in all stages of DR. We found that the association spared the noDR stage but started as early as NPDR and included PDR, suggesting that this polymorphism may predict the early development of DR. In our study, we found the CC genotype at rs699947 was significantly less frequent in patients with DR compared with noDR patients. This suggests that carriage of the C allele at rs699947 is potentially protected from the development of DR. Because we had too few subjects with the AA genotype in our Korean sample, we could not detect any associations of the AA genotype with DR, in contrast to the Japanese study.

We also found that the genotype and allele distributions at rs699947 in patients with DR differed compared with healthy controls (Supplemental Table 3). However, we observed a weaker deviation between DR and healthy controls compared with that between DR and noDR patients. This is due to the fact that healthy controls may develop diabetes and DR as they grow older.

It has been reported that the increased mRNA of VEGF in the vitreous fluid enhanced the development and progression of DR (15). Several SNPs have been found to be associated with differential expression of VEGF *in vitro* (9). Among them, the rs699947 SNP has been shown to functionally affect the mRNA level of VEGF (16). In a separate study, we also found that the rs699947 SNP reg-

ulates the development of DR through the differential expression of VEGF (unpublished observation). Because the level of VEGF in aqueous humor has been reported to reflect the vitreous level (17), we measured the aqueous level of VEGF from 27 patients with PDR. VEGF concentrations in the aqueous fluid of subjects with the genotype CC at rs69947 were lower than those with the genotype CA. Only one subject had the AA genotype at rs69947, so we were unable to compare the relationship between this genotype and VEGF in the aqueous fluid. The same patient happened to have the haplotype AGG. Although we found increased VEGF concentration in this patient with AGG haplotype, it may be possible that VEGF differential expression may be governed by genetic control other than the influence of the haplotype. Further investigation will be needed to clarify the association between allele A or the AGG haplotype and VEGF levels in the vitreous fluid.

Given the high linkage disequilibrium of three SNPs (rs699947, rs1570360, and rs2010963) in the VEGF gene, there is evidence of conserved haplotypes in the promoter and 5′ untranslated region. We could not find any significant associations between major haplotypes and DR, but one minor haplotype, H4 (AGG), was prevalent among the subjects with DR. The haplotype H4 was also prevalent in NPDR as well as PDR. Although the frequency of haplotypes may differ in different human populations, the influence of high-risk haplotypes transcends geographical diversity. However, more deliberate functional studies would allow further investigation of the role of the AGG haplotype and rs699947 in VEGF production and the development or progression of DR.

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