Brief Genetics Report

A Common Polymorphism in the 5′-Untranslated Region of the VEGF Gene Is Associated With Diabetic Retinopathy in Type 2 Diabetes

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Vascular endothelial growth factor (VEGF), a major mediator of vascular permeability and angiogenesis, may play a pivotal role in mediating the development and progression of diabetic retinopathy. In the present study, we examined the genetic variations of the VEGF gene to assess its possible relation to diabetic retinopathy in type 2 diabetic patients. Among seven common polymorphisms in the promoter region, 5′-untranslated region (UTR) and 3′UTR of the VEGF gene, genotype distribution of the C(−634)G polymorphism differed significantly ($P = 0.011$) between patients with ($n = 150$) and without ($n = 118$) retinopathy, and the C allele was significantly increased in patients with retinopathy compared with those without retinopathy ($P = 0.0037$). The odds ratio (OR) for the CC genotype of C(−634)G to the GG genotype was 3.20 (95% CI 1.45–7.05, $P = 0.0046$). The −634C allele was significantly increased in patients with nonproliferative diabetic retinopathy (non-PDR) ($P = 0.0026$) and was insignificantly increased in patients with proliferative diabetic retinopathy (PDR) ($P = 0.081$) compared with patients without retinopathy, although frequencies of the allele did not differ significantly between the non-PDR and PDR groups. Logistic regression analysis revealed that the C(−634)G polymorphism was strongly associated with an increased risk of retinopathy ($P = 0.0018$). Furthermore, VEGF serum levels were significantly higher in healthy subjects with the CC genotype of the C(−634)G polymorphism than in those with the other genotypes. These data suggest that the C(−634)G polymorphism in the 5′UTR of the VEGF gene is a novel genetic risk factor for diabetic retinopathy. *Diabetes* 51:1635–1639, 2002

Most diabetic patients, especially those with poor glycemic control, develop diabetic retinopathy, which remains the major cause of new-onset blindness among diabetic adults. Diabetic retinopathy is characterized by vascular permeability and increased tissue ischemia and angiogenesis. Vascular endothelial growth factor (VEGF), a 45-kDa homodimeric glycoprotein (1), has initially drawn much attention as an important mediator of retinal ischemia–associated intraocular neovasclarization (2). VEGF is produced from many cell types within the eye, and past studies have shown that VEGF levels are markedly elevated in vitreous and aqueous fluids in the eyes of individuals with proliferative diabetic retinopathy (PDR) (3,4). In addition, VEGF induction of vascular permeability may contribute to the development of nonproliferative diabetic retinopathy (non-PDR) (2). The observation of increased retinal VEGF expression early in diabetic retinopathy (5,6) and the finding in nondiabetic animals that exogenous intraocular VEGF administration can elicit retinal abnormalities resembling diabetic retinopathy (7) suggest that VEGF may also play a role in the development of the earliest stages of retinopathy. Taken together, VEGF appears to present an attractive candidate susceptibility gene for diabetic retinopathy. Although previous studies reported an association between diabetic retinopathy and several candidate genes, including aldose reductase (8,9), HLA-DQB1 (10), β_3-adrenoreceptor (11), paraoxonase (12), and $\alpha 2\beta 1$ integrin (13), most of the data appear to be inconclusive and require further confirmation. Therefore, in the present study, we examined the VEGF gene to assess its possible role in diabetic retinopathy.

Using a screening study, we identified six polymorphisms of the VEGF gene: G(−1,877)A, T(−1,498)C, G(−1,190)A, and G(−1,154)A in the promoter region and C(−634)G and C(−7)T in the 5′-untranslated region (UTR). Among them, G(−1,877)A appeared to be rare and was not analyzed further. The remaining five polymorphisms and C936T and G1612A polymorphisms in the 3′UTR were found to be common in Japanese individuals and were further genotyped by PCR restriction fragment–length polymorphism (RFLP) in type 2 diabetic patients to assess their possible relation to diabetic retinopathy. In

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LPS, lipopolysaccharide; non-PDR, nonproliferative diabetic retinopathy; OR, odds ratio; PBMC, peripheral blood mononuclear cell; PDR, proliferative diabetic retinopathy; RFLP, restriction fragment–length polymorphism; UTR, untranslated region; VEGF, vascular endothelial growth factor.

TABLE 1
Clinical characteristics of type 2 diabetic patients

	Retinopathy (−)	Retinopathy (+)		
		All (*P*)	Non-PDR (*P*)	PDR (*P*)
n	118	150	80	70
Sex (M:F)	61:57	74:76 (NS)	39:41 (NS)	35:35 (NS)
Age (years)	54.0 ± 15.1	58.4 ± 11.7 (0.0074)	61.0 ± 11.4 (<0.0001)	55.5 ± 11.3 (NS)
Age at onset (years)	46.7 ± 14.6	45.6 ± 12.3 (NS)	48.0 ± 12.5 (NS)	42.8 ± 11.5 (NS)
Duration of disease (years)	7.3 ± 6.8	12.9 ± 7.9 (<0.0001)	13.0 ± 7.1 (<0.0001)	12.7 ± 8.7 (<0.0001)
BMI (kg/m^2)	24.6 ± 5.3	23.3 ± 3.5 (0.015)	23.4 ± 3.4 (NS)	23.2 ± 3.7 (0.048)
Systolic BP (mmHg)	134.7 ± 20.1	144.8 ± 25.8 (0.0007)	141.7 ± 24.9 (0.034)	148.3 ± 26.6 (0.0001)
Diastolic BP (mmHg)	77.2 ± 15.8	78.3 ± 15.7 (NS)	77.5 ± 16.0 (NS)	79.2 ± 15.4 (NS)
HbA_{1c} (%)	8.9 ± 2.4	8.8 ± 2.2 (NS)	9.2 ± 2.1 (NS)	8.4 ± 2.3 (NS)
Cholesterol (mg/dl)	194.2 ± 41.8	198.7 ± 54.2 (NS)	192.5 ± 48.6 (NS)	205.8 ± 59.5 (NS)
Triglyceride (mg/dl)	155.0 ± 104.1	159.0 ± 110.1 (NS)	143.2 ± 99.0 (NS)	177.3 ± 119.8 (NS)
Insulin therapy	31.4	58.0 (<0.0001)	56.3 (0.0007)	60.0 (0.0001)

Data are *n*, means ± SD, or %. BP, blood pressure. Clinical data of patients were collected during hospitalization. *P* values versus patients without retinopathy are shown.

the present study, we studied 118 patients without retinopathy, 80 patients with non-PDR, and 70 patients with PDR (Table 1). Genotype and allele frequencies were compared between patients with retinopathy (all retinopathy, non-PDR, or PDR) and patients without retinopathy (Table 2). T(−1,498)C and G(−1,190)A were in complete concordance and are shown together. In each group, there was no significant deviation from Hardy-Weinberg equilibrium for any genotype. It was revealed that genotype distribution of the C(−634)G polymorphism differed significantly between patients without retinopathy and with any retinopathy (non-PDR or PDR) ($P = 0.011$), and the C allele was significantly associated with the presence of retinopathy ($P = 0.0037$); the OR for the CC genotype of C(−634)G to the GG genotype was 3.20 (95% CI 1.45–7.05, $P = 0.0046$). The −634C allele was significantly increased in the non-PDR group ($P = 0.0026$) and was insignificantly increased in the PDR group ($P = 0.081$) compared with patients without retinopathy, although frequencies of the allele did not differ significantly between the non-PDR and PDR groups. We also observed that the −634 GG genotype was significantly decreased in the non-PDR group ($P = 0.0009$), whereas −634CC was significantly increased in the PDR group ($P = 0.021$). In addition, the 936T allele was increased in patients with retinopathy compared with those without retinopathy with marginal significance ($P = 0.035$), but the genotype distribution did not differ significantly. Increased frequency of the 936T allele was observed in both the non-PDR and PDR groups, but the differences were not significant. Finally, it was revealed that genotype and allele frequencies of the VEGF polymorphism in 184 control subjects were not significantly different from the frequencies in the total type 2 diabetic patients (data not shown), suggesting that the VEGF polymorphisms, including C(−634)G and C936T, are not associated with type 2 diabetes itself.

We further assessed the relation between the VEGF genotypes of C(−634)G or C936T and diabetic retinopathy by logistic regression analysis, including the VEGF genotype and several clinical features as independent variables (Table 3). In addition to duration of diabetes, systolic blood pressure, and therapy with insulin, the C(−634)G genotype had a significant increased risk of retinopathy (OR 2.16, 95% CI 1.33–3.50, $P = 0.0018$), suggesting that the VEGF C(−634)G genotype is an independent risk factor of retinopathy, with the −634C allele increasing the risk; the observed lack of association between HbA_{1c} level and retinopathy must be interpreted with caution because the current HbA_{1c} level cannot be regarded as representative of the patients' long-term glycemic control. In contrast, by logistic regression analysis, the C936T genotype was not significantly associated with retinopathy ($P = 0.240$). Thus, in the present study, we provided evidence for the first time by case-control and logistic regression studies that the VEGF gene is associated with an increased risk of diabetic retinopathy.

Next, we studied VEGF serum levels and VEGF genotypes in 64 healthy subjects to assess possible functional relevance of the VEGF polymorphisms. As shown in Fig. 1, fasting serum VEGF levels were significantly higher in healthy subjects with the CC genotype of the C(−634)G polymorphism than in those with the other genotypes ($P = 0.021$); no significant association was found between the other polymorphisms and serum VEGF levels. Based on a putative important role of VEGF in retinopathy, the correlation of the VEGF −634CC genotype with higher VEGF production is consistent with the genetic association of the −634C allele (the occurrence of retinopathy as described above), although it is not certain why VEGF levels did not differ between subjects with CG and GG genotypes. Watson et al. (14) recently reported that the C(−634)G polymorphism (+405 polymorphism in their report, with transcription start = +1) was significantly correlated with lipopolysaccharide (LPS)-stimulated VEGF production from peripheral blood mononuclear cells (PBMCs) of 21 healthy subjects, but the highest production was observed for the GG genotype, the intermediate production for the CG genotype, and the lowest production was observed for the CC genotype. This apparent inconsistency between studies may be partly caused by methodological differences (the measurement of fasting serum in our study versus the measurement of culture medium of LPS-stimulated PBMCs in their study), which might result in a difference in the major origin of VEGF, i.e., leukocytes, aggregated plate-

TABLE 2
Genotype and allele distribution of VEGF gene polymorphisms in type 2 diabetic patients with and without retinopathy

	Genotype	Retinopathy (−) n = 118	Retinopathy (+) All (P) n = 150	Retinopathy (+) Non-PDR (P) n = 80	Retinopathy (+) PDR (P) n = 70	Allele	Retinopathy (−) n = 236	Retinopathy (+) All (P) n = 300	Retinopathy (+) Non-PDR (P) n = 160	Retinopathy (+) PDR (P) n = 140
T(−1498)C-G(−1190)A										
	TT-GG	44.1	52.7 (NS)	52.5 (NS)	52.9 (NS)	T-G	68.2	72.0 (NS)	73.1 (NS)	70.7 (NS)
	TC-GA	48.3	38.7	41.3	35.7	C-A	31.8	28.0	26.9	29.3
	CC-AA	7.6	8.7	6.3	11.4					
G(−1154)A										
	GG	74.6	76.0 (NS)	80.0 (NS)	71.4 (NS)	G	86.9	86.7 (NS)	89.4 (NS)	83.6 (NS)
	GA	24.6	21.3	18.8	24.3	A	13.1	13.3	10.6	16.4
	AA	0.8	2.7	1.3	4.3					
C(−634)G										
	CC	10.3	20.7* (0.011)	18.8 (0.0026)	22.9† (NS)	C	35.2	47.7 (0.0037)	50.6 (0.0026)	44.3 (NS)
	CG	50.0	54.0	63.8	42.9 (0.061)	G	64.8	52.3	49.4	55.7 (0.081)
	GG	39.8	25.3‡	17.5§	34.3					
C(−7)T										
	CC	66.1	70.0 (NS)	68.8 (NS)	71.4 (NS)	C	80.9	84.0 (NS)	82.5 (NS)	85.7 (NS)
	CT	29.7	28.0	27.5	28.6	T	19.1	16.0	17.5	14.3
	TT	4.2	2.0	3.8	0.0					
C936T										
	CC	72.0	62.0 (NS)	61.3 (NS)	62.9 (NS)	C	85.2	77.7 (0.035)	78.1 (NS)	77.1 (NS)
	CT	26.3	31.3	33.8	28.6 (0.064)	T	14.8	22.3	21.9 (0.081)	22.9 (0.052)
	TT	1.7	6.7	5.0	8.6					
G1612A										
	GG	78.0	77.3 (NS)	73.8 (NS)	81.4 (NS)	G	87.3	87.3 (NS)	86.3 (NS)	88.6 (NS)
	GA	18.6	20.0	25.0	14.3	A	12.7	12.7	13.8	11.4
	AA	3.4	2.7	1.3	4.3					

Data are %. T(−1498)C and G(−1190)A polymorphisms are in complete linkage disequilibrium in patients and are shown together. P values versus patients without retinopathy are shown (P values between 0.05 and 0.1 are shown in parentheses). *P = 0.029, †P = 0.021, ‡P = 0.012, §P = 0.0009 vs. patients without retinopathy.

TABLE 3
ORs adjusted by logistic regression analysis for the association with retinopathy among type 2 diabetic patients

Variable	OR (95% CI)	*P*
Female sex	0.96 (0.52–1.78)	NS
Age at onset	1.00 (0.98–1.03)	NS
Duration of disease	1.11 (1.06–1.16)	<0.0001
BMI	0.94 (0.87–1.01)	NS
Systolic BP	1.03 (1.01–1.05)	0.0003
HbA_{1c}	0.98 (0.84–1.14)	NS
Cholesterol	1.00 (0.99–1.01)	NS
Insulin therapy	2.46 (1.31–4.63)	0.0053
VEGF C(−634)G genotype	2.16 (1.33–3.50)	0.0018

BP, blood pressure.

TABLE 4
Distribution of probable haplotypes of VEGF polymorphsms in type 2 diabetic patients with and without retinopathy

Position					Without retinopathy	With retinopathy	
−1498	−1190	−1154	−634	−7	*n* = 236	*n* = 300	*P*
T	G	G	C	C	34.3	47.0	0.0035
T	G	G	G	C	31.8	23.3	0.031
C	A	G	G	T	17.8	14.7	NS
C	A	A	G	C	12.7	13.0	NS
Other or ambiguous haplotypes					3.4	2.0	NS

Data are %.

lets, and vascular endothelial cells versus activated leukocytes for our study versus their study, respectively (15,16). Watson et al. (17) also reported that the C(−634)G polymorphism was included in a potential binding site of the MZF1 transcription factor, but we could not confirm that using the MatInspector Online Tool to search for a potential transcription factor binding site. To directly evaluate the functional relevance of the C(−634)G polymorphism, we are now conducting assays of the transcriptional promoter activity using the luciferase reporter system.

Possible linkage disequilibrium was evaluated by *t* values among seven VEGF gene polymorphisms (three in the promoter region, two in the 5′UTR, and two in 3′UTR) in type 2 diabetic patients and control subjects. Significant linkage disequilibrium was observed among five polymorphisms in the promoter region or 5′UTR, and the presence of four major haplotypes was estimated. As shown in Table 4, the −1498T/–1190G/–1154G/–634C/–7C haplotype was significantly increased in patients with retinopathy compared with those without retinopathy ($P = 0.0035$), whereas the −1498T/–1190G/–1154G/–634G/–7C haplotype was significantly decreased in patients with retinopathy ($P = 0.031$). Because these haplotypes differ only in the −634 polymorphism, it is suggested that the C(−634)G polymorphism is primarily associated with retinopathy among these polymorphisms. However, further genetic studies will be useful to confirm the present findings and to clarify whether the C(−634)G polymorphism primarily contributes to the occurrence of diabetic retinopathy or, alternatively, whether other neighboring functional polymorphisms are involved in the disease development. In this regard, it may be interesting to note that the seven VEGF polymorphisms studied in the present study appear to be common in Caucasians (14,18,19, and our data) and that there are several other common polymorphisms in the promoter region of the VEGF gene (18).

Initially, we prospected that some VEGF polymorphism(s) might be associated with a severe stage of retinopathy with neovascularization, i.e., PDR, because VEGF is a strong angiogenic factor. However, the C(−634)G polymorphism was not specifically associated with PDR, or rather the association was more evident in non-PDR in the present study. Accordingly, we now suspect that VEGF-induced vascular permeability as well as angiogenesis may be the underlying rationale for the observed association. Additional association studies with a large sample size may clarify this issue. Finally, because disregulation of VEGF has been implicated in the pathogenesis of a variety of disorders, such as metastasis of tumors, rheumatoid arthritis, Graves' disease, psoriasis,

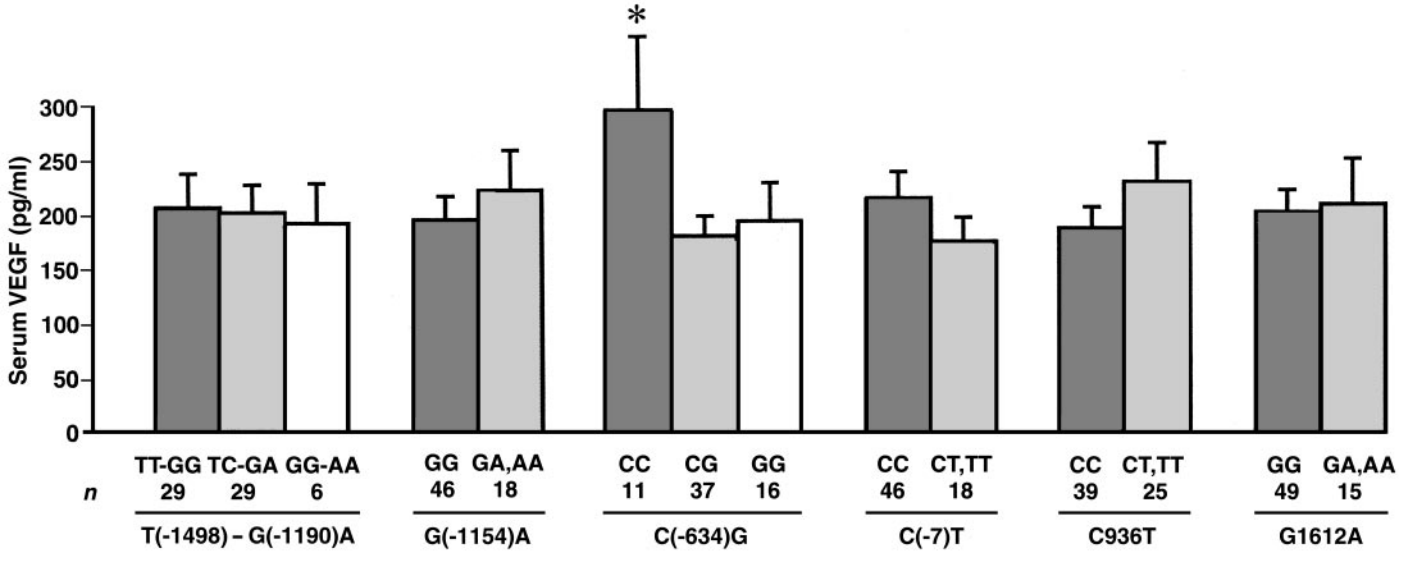

FIG. 1. Comparison of VEGF serum levels of 64 healthy subjects with VEGF genotypes. Rare genotypes (less than five subjects) −1154AA, −7TT, 936TT, and 1612AA were combined with heterozygous genotypes. Bars indicate mean values ± SE. *$P = 0.021$ (CC vs. CG plus GG).

cardiovascular disease, and peripheral limb disease (1), it may be worthwhile to investigate the possible association of VEGF gene polymorphisms with these diseases.

RESEARCH DESIGN AND METHODS

Subjects. We studied 268 type 2 diabetic patients and 184 control subjects for the association with diabetic retinopathy; all were unrelated Japanese individuals. Hospitalized patients with type 2 diabetes (aged 15–86 years) were recruited from Saitama Medical School Hospital in Saitama prefecture. The diagnosis and classification of diabetes was based on clinical features; laboratory data, including anti-GAD antibody and C-peptide levels (serum and urine); and the guidelines in the recent Expert Committee Report of the American Diabetes Association (20). Diabetic retinopathy was diagnosed in a masked manner by independent ophthalmologists using 50° color fundus photographs and was classified as retinopathy, non-PDR, and PDR. Retinopathy denotes no signs of diabetic retinopathy; non-PDR denotes signs of microaneurysm, intraretinal hemorrhage, exudates, macular edema, venous dilatation, soft exudates, peripheral ischemia on fluorescein angiography, intraretinal microvascular abnormalities, and diffuse intraretinal hemorrhage; and PDR denotes signs of neovascularization at the optic disc, neovascularization elsewhere, vitreous hemorrhage, fibrovascular proliferation, and rubeosis iridis. Control subjects (aged 18–47 years) were healthy volunteers living in Saitama prefecture. We also recruited 64 healthy subjects (aged 22–30 years) for the measurement of serum VEGF. Informed consent was given by each participant, and the study was approved by the Ethical Committee of Saitama Medical School and was conducted in accordance with the principles of the Declaration of Helsinki.

Screening of VEGF gene variations. Genomic DNA was extracted from peripheral blood of each individual. We first screened for variations in the promoter region and 5′UTR of the VEGF gene (nucleotide position −2,361 ~+9; translation start site = +1) (21) in 16 Japanese patients with type 2 diabetes. The promoter region and the 5′UTR were PCR-amplified with five sets of overlapping primers, and the PCR products were directly sequenced using a dRhodamine Terminator Cycle Sequencing Kit or a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Tokyo, Japan).

Genotyping of the VEGF gene polymorphisms. Genotyping of each polymorphism was carried out by PCR-RFLP analysis. The PCR primers for T(−1,498)C, G(−1,190)A, G(−1,154)A, C(−634)G, and C(−7)T were 5′-GTGTGTGCGTGTGGGGTTG*GC*GG-3′ (forward with mismatch nucleotides italicized) and 5′-CGACCCCCACCAAGGTTCACAG-3′ (reverse); 5′-TCCT GCTCCCTCCTCGCCAATG-3′ (forward) and 5′-TCCACAGTGATTTGGG GAAGTAGA-3′ (reverse); 5′-TCCTGCTCCCTCCTCGCCAATG-3′ (forward) and 5′-GGCGGGGACAGGCGAGCCTC-3′ (reverse); 5′-TTGCTTGCCATTC CCCACTTGA-3′ (forward) and 5′-CCGAAGCGAGAACAGCCCAGAA-3′ (reverse); and 5′-GAGGAGGGGGAGGAGGAAGAA (forward) and AAGACA GCAGAAAGTTCATGGT*C*TC (reverse with a mismatch nucleotide italicized), respectively. The −1498C, −1190A, −1154G, −634G, and −7T alleles result in the gain of a Fnu4HI, DdeI, MnlI, BsmFI, and DdeI site, respectively. After digestion by an appropriate restriction enzyme, PCR products were electrophoresed on a 2.5~4% agarose gel and visualized by ethidium bromide staining. The C936T and G1612A polymorphisms were genotyped by PCR-RFLP as described by Renner et al. (19).

Measurement of serum VEGF concentrations. Venous blood samples were taken from 64 healthy subjects before breakfast. Blood samples were centrifuged after full clotting at room temperature, and serum were stored at −20°C until analysis. VEGF serum levels were measured using an enzyme immunoassay (human VEGF Quantikine; R&D Systems, Minneapolis, MN).

Statistics. Distribution of genotypes, alleles, and haplotypes was compared by χ^2 test or Fisher's exact test. Linkage disequilibrium was assessed by t values (22). Continuous clinical data were compared by unpaired Student's t test, and categorical clinical data were compared by Fisher's exact test. Logistic regression analysis was performed to assess the independent role of the VEGF genotype and other variables, including sex, age at onset, duration of diabetes, BMI, systolic blood pressure, HbA_{1c} level, cholesterol level, and treatment of diabetes (categorical variable: insulin therapy or no therapy). VEGF serum levels were compared by unpaired Student's t test. Significance was considered at $P < 0.05$.

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