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RETINAL DISORDERS

Impact of variants in the VEGF gene on progression of proliferative diabetic retinopathy

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Abstract
Background The development of diabetic retinopathy is associated with the duration of diabetes and HbA1c levels. However, the familial aggregation of diabetic retinopathy is consistent with genetic susceptibility. Recently, a –634C/G polymorphism in the vascular endothelial growth factor (VEGF) gene was shown to be associated with diabetic retinopathy. To clarify the contribution of the VEGF gene in the development of diabetic retinopathy we analyzed variants in this gene among 469 Japanese patients with type 2 diabetes.

Methods DNA from each patient was typed for –634C/G and –2578C/A polymorphisms using conventional polymerase chain reaction techniques. The vitreous fluid samples were obtained from 40 patients with PDR for measurement of VEGF levels.
Results We found a significantly higher frequency of the A allele in the group with proliferative diabetic retinopathy (PDR) than in the control group at –2578C/A polymorphism ($p=0.036$). Moreover, if the subjects were grouped according to the duration of diabetes and status of diabetic retinopathy (a first group consisting of subjects with longer duration (>20 y) of diabetes without any retinopathy ($n=102$), and a second group of those with shorter diabetes (<15 y) but having retinopathy ($n=35$), the genotype distribution at -2578 C/A polymorphism was again significantly higher in the second group ($p=0.005$) and differed significantly ($p=0.002$) in a recessive model. The risk of the AA for PDR was 7.7 (95%, CI: 1.8–30.9).
Conclusions The AA genotype at –2578C/A polymorphism in the VEGF gene is associated with proliferative diabetic retinopathy. No significant association with –634 C/G polymorphism was confirmed.

Keywords VEFG · Polymorphism · Diabetic retinopathy

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Introduction

Retinopathy is a serious ocular complication of diabetes that causes loss of vision in adults. An earlier study, the DCCT, demonstrated that the duration of diabetes is probably the strongest predictor of the development and progression of retinopathy in patients with type 1 diabetes [1]. The UKPDS showed that improving control of blood glucose reduced development of retinopathy [2]. Hypergly-

cemia has been recognized as the primary pathogenic factor in the development and progression of diabetic retinopathy [3]. Several biochemical pathways are supposed to be activated secondary to hyperglycemia [4]. These abnormal pathways may, in turn, influence several vasoactive factors that are likely instrumental in creating functional and morphological changes in the retinas of patients with diabetes [5, 6]. For example, vascular endothelial growth factor (VEGF) plays a pivotal role in the retinal microvascular complications of patients with diabetes. Various studies have shown that the level of VEGF in vitreous is correlated with the severity of proliferative diabetic retinopathy (PDR) [7].

There are subgroups of patients with diabetes in whom retinopathy does not develop despite poor long-term control of their disease, while others exercising fairly good control develop retinopathy. This is consistent with a genetic susceptibility to diabetic retinopathy. In addition, familial predisposition to retinopathy has also been noted in diabetes [8].

Recently, it has been reported that the –634 C/G polymorphism is associated with diabetic retinopathy [9, 10]. Another VEGF polymorphism found in individuals of Italian descent, the –2578 AA genotype, is associated with type 1 diabetes [11]. In addition to the –634 G/C polymorphism, the present study examined the –2578C/A polymorphism in the VEGF gene for an association with diabetic retinopathy in Japanese patients with type 2 diabetes [9]. We also measured the VEGF levels in vitreous fluid and plasma of patients with PDR and compared them with the patients' VEGF genotypes.

Materials and methods

Patients

We enrolled 469 unrelated patients with type 2 diabetes (male/female 265:204) in this study. These patients were identified through the Diabetes Center of Tokyo Women's Medical University and affiliated hospitals. Diagnosis of type 2 diabetes was based on 1985 World Health Organization criteria (WHO Tech Rep Ser 1985) [12]. This study was approved by the Ethical Committee of Tokyo Women's Medical University and was carried out in accordance with the principles of the Declaration of Helsinki. Informed consent was obtained from all subjects before participation.

Phenotype and measurements

All patients underwent a complete ophthalmological examination that included corrected visual acuity, slit-lamp biomicroscopic examination, funduscopic examination, and fundus photography. Funduscopic findings were evaluated and graded by trained retinal specialists. Diabetic retinopathy was classified as non-PDR or PDR. Non-PDR denoted signs of microaneurysm, hemorrhage, hard exudate, macular edema, venous abnormality, soft exudate, peripheral ischemia on fluorescein angiography, or intraretinal microvascular abnormality (IRMA). PDR denoted signs of new vessels on or within 1 DD of the disc, new vessels elsewhere, vitreous hemorrhage, fibrovascular proliferation, and rubeosis iridis. Neovascularization was considered to be active if there were perfused, multi-branched iridic or preretinal capillaries, and inactive if previously documented active proliferation had regressed fully or if only nonperfused, gliotic vessels or fibrosis were present [13].

We also determined the duration of diabetes, HbA1c, BMI systolic and diastolic pressure, and current therapy. The serum levels of total cholesterol and triglyceride (TG) were measured from blood samples. All parameters were obtained from the clinical record closest to the date of eye examination.

DNA analysis

DNA was extracted from peripheral lymphocytes by a standard procedure. An ABI 3700 sequencer (Applied Biosystems, Foster City, CA, USA) was used to type –634C/G and –2578C/A polymorphisms in the VEGF gene. The primer designs and annealing temperature were, forward 5'TACTGGGGAGGTAACCTAGCAC-3', reverse 5'GGAAAAATTCCTGGCTGGTT-3' with an annealing temperature of 58°C for single nucleotide polymorphism (SNP) –2578; and for SNP –634 forward 5'TTGCTTGC CATTCCCCACTTGA-3', reverse 5'CCGAAGCGAGAA CAGCCCAGAA-3' at an annealing temperature of 58°C.

Measurement of VEGF levels in the vitreous fluid and plasma

Undiluted vitreous fluid samples were harvested from the patients with type 2 diabetes and PDR at the start of vitrectomy. The vitreous fluid samples were obtained from 40 eyes of 40 additional patients with PDR. Vitrectomy was performed for the following reason: 26 had vitreous and preretinal haemorrhage, 14 had retinal detachment. These subjects were in addition to the primary subjects shown in Table 1. The samples were frozen rapidly to –80°C. Plasma samples were also collected from these 40 patients, which were placed immediately on ice and centrifuged at 3,000×g for 5 min at 4°C, after which the plasma was frozen rapidly to –80°C. The VEGF concentration was measured by an enzyme-linked immunosorbent assay (ELISA) for human VEGF (R&D Systems, Minneapolis, MN, USA)[14].

Statistical analyses

All clinical data are summarized as mean ± SE. Differences between the controls and the cases were tested for significance by ANOVA. We used SPSS for Windows software for statistical analysis (version 11.0; SPSS, Chicago, IL). Fisher's exact, Chi square and the Mann-Whitney test were used to evaluate associations between SNP and retinopathy. $P<0.05$ was considered statistically significant. Haplotype frequencies were estimated using the expectation-maximization (EM) algorithm[15].

Results

Clinical background of the patients

We compared subjects with type 2 diabetes and PDR to those without retinopathy but having type 2 diabetes as controls. There were significant differences between the two groups in the age at diagnosis of type 2 diabetes mellitus, duration of disease, BMI, systolic and diastolic pressure, HbA1c, serum levels of total cholesterol and TG, and the proportion in each group that was taking injected insulin (Table 1).

Diabetic retinopathy and polymorphism in the VEGF gene

The typing results of SNPs–634C/G and –2578C/A in the VEGF gene (Table 2) were in Hardy-Weinberg Equilibrium. The genotype and allele frequencies for two SNP (–634, –2578) were compared between the two groups (Table 3). We found a positive association between PDR and the A allele at SNP –2578 ($p=0.036$). However, we could not confirm a positive association between proliferative diabetic retinopathy and SNP-634.

Given that the risk of developing of retinopathy increases with the duration of diabetes, as reported in DCCT and UKPDS, we next focused on the genetic contribution of the VEGF gene to PDR by adjusting for the influence of duration. We compared subjects with a shorter duration (<15 years) of diabetes who developed PDR to those with longer illness (>20 years) who were free of diabetic retinopathy. As shown in Table 4, the mean durations of disease within the >20-year and <15-year groups were 24.6± 5.8 years and 10.1±3.2 years ($p<0.001$), respectively. The mean illness duration (24.6 years) in the >20-year group was considered long enough for diabetic retinopathy to develop. The age at diagnosis of the disease was similar between the two groups (Table 4). We found that the genotype frequencies of SNP-2578 again differed significantly between these two groups ($p=0.005$) (Table 3). The AA genotype was significantly associated with the presence of PDR ($p=0.002$). The odds ratio (OR) of PDR for the AA genotype of –2578C/A to CA + CC was 7.5 (95% CI: 1.8–30.9, $p=0.002$) in the recessive model (Table 3). No significant association was found at SNP –634C/G.

Both SNP in the promoter region were used to estimate haplotype frequencies. The difference in haplotype frequencies between patients with PDR and control subjects were tested by Chi square. No significant difference in haplotype frequencies was detected (data not shown).

Variants in the VEGF gene and VEGF levels in vitreous fluid and plasma

We measured VEGF levels in the vitreous fluid of patients with PDR and compared them with VEGF genotypes at SNP –634 and –2578 in 40 subjects to assess the possible functional relation of genotype to VEGF concentration. VEGF concentrations in the vitreous fluid of subjects with genotype CC at SNP-2578 ranged from 178 to 3,790 g/mL (930±951 g/mL), for genotype CA at SNP-2578 VEGF ranged from 15.6 to 3,270 g/mL (991±914 g/mL). Only one subject had an AA genotype at SNP-2578 so we were unable to compare the relationship between this genotype and VEGF in the vitreous fluid (VEGF level for this patient with genotype AA was 570 g/mL). Further investigation will be needed to clarify the association between Allele A and VEGF levels in the vitreous fluid. For SNP –634 we found no association between polymorphism at SNP –634 and VEGF levels both either vitreous fluid or plasma, demonstrating an opposite result to those of Awata et al. [9].

Table 1 Clinical characteristics of subjects with type 2 diabetes without retinopathy (no retinopathy) and with proliferative retinopathy (PDR)

	Controls No retinopathy	Cases PDR
Number	292	177
M/F	168:124	97:80 (NS)
Age at onset (y)	45.2±13.0	34.1±13.6
Duration (y)	16.7±7.5§	22.7±8.9
BMI (kg/m^2)	23.2±3.6	24.0±3.7
Systolic BP (mmHg)	134.2±18.9§	142.1±23.3 (<0.001)
Diastolic BP (mmHg)	75.1±10.8	76.5±13.1
HbA1c (%)	7.4±1.4	7.6±1.3
T-cholesterol (mmol/L)	5.16±0.93§	4.90±1.21
TG (mmol/L)	1.28±0.80§	1.50±0.92 (<0.001)
On Insulin therapy (%)	33.0	77.0

Data are shown as mean±SD or %. *BP*:blood pressure. Clinical data on the patients were collected at the most recent visit for ophthalmological examination.
P values versus patients without retinopathy are shown. §: $P<0.05$ vs. PDR

Table 2 Genotype and allele frequencies at SNP –634G/C and –2578C/A in the VEGF gene for subjects with type 2 diabetes

Polymorphism	–634G/C				–2578C/A			
		Case PDR (+)	Control DR (-)			Case PDR (+)	Control DR (-)	
Genotype	GG	63 (35.8%)	84 (29.0%)	P=0.309	CC	85 (48.0%)	163 (55.9%)	P=0.115
	CG	79 (44.9%)	146 (50.5%)		CA	70 (39.5%)	107 (36.6%)	
	CC	34 (19.3%)	59 (20.4%)		AA	22 (12.5%)	22 (7.5%)	
Total		*176 (100%)	*289 (100%)			177 (100%)	292 (100%)	
Allele	G	205 (58.2%)	314 (54.3%)	P=0.244	C	240 (68.6%)	433 (74%)	P=0.036
	C	147 (41.8%)	264 (45.7%)		A	114 (31.4%)	151 (26%)	
Total		352 (100%)	578 (100%)			354 (100%)	584 (100%)	

The genotype and allele frequency of each polymorphisms were compared between diabetic patients with PDR and without retinopathy (DR-) using the Chi square and Fisher's exact. Data are given as percentages.
* The typing rate for SNP-634 was 0.99

Discussion

We found that the SNP-2578C/A in the VEGF gene was associated significantly with PDR in the Japanese population. Although this was the largest sample ever published, we could not duplicate a positive association at SNP –634C/G. In an allele table the allele A was prevalent significantly in the group with PDR (p =0.036) compared to those without retinopathy. As demonstrated in the UKPDS, the prevalence of diabetic retinopathy increases with the duration of diabetes.

From a Japanese study it is known that about 80% of subjects with type 2 diabetes for more than 20 years had retinopathy (pers. comm.). Therefore, we conducted a sub analysis to adjust for the effect of the duration of diabetes to evaluate the genetic effect more clearly. Separating groups at durations of 10 or 5 years, provided samples that were too small for adequate comparison. We observed a more striking positive association of the AA genotype at SNP-2578 with diabetic retinopathy weighted for duration in the sub-analysis (OR=7.5, 95% CI: 1.8–30.9, p=0.002).

A positive association at SNP –2578 in the VEGF gene has been reported with many diseases, such as lung carcinoma, atherosclerosis, Alzheimer's disease, peritoneal membrane function, graft survival, and breast cancer [16–21], but not previously in diabetic retinopathy including PDR. , Banyasz et al. found a positive association between retinopathy of prematurity (ROP) with prevalence for the A allele at –2578 in the VEGF gene [22]. However, this association was observed only in boys, therefore gender bias remains an important point of discussion. Awata et al. compared the frequency of the SNP –2578C/A polymorphism genotype and allele with DR- and DR (NPDR + PDR)

Table 3 Genotype and allele table for all samples, and duration-weighted sub analyses of SNP -634G/C and –2578C/A of VEGF gene in type 2 diabetes

SNP		For all samples PDR vs. No retinopathy	For duration-weighted sub analyses PDR vs. No retinopathy
–634 Risk allele=C	Allele table (C: G)	P=0.244 (0.42:0.58 vs. 0.54:0.55)	P=0.724 (0.42:0.58 vs. 0.54:0.46)
	Dominant model (CC + CG: GG)	P=0.774 (0.64:0.36 vs. 0.71:0.29)	P=0.641 (0.72:0.28 vs. 0.70:0.30)
	Recessive model (CC: CG + GG)	P=0.130 (0.19:0.81 vs. 0.20:0.80)	P=0.893 (0.23:0.77 vs. 0.19:0.71)
	Genotype table (CC: CG: GG)	P=0.309 (0.19:0.45:0.36 vs. 0.20:0.51:0.29)	P=0.897 (0.23:0.49:0.28 vs. 19.1:51.1:29.8)
–2578 Risk allele=A	Allele table (A: C)	P=0.036* (0.31:0.69 vs. 0.26:0.74)	(0.43:0.57 vs. 0.40:0.60)
	Dominant model (AA + CA: CC)	P=0.078 (0.51:0.49 vs. 0.44:0.56)	P=0.752 (0.43:0.57 vs. 0.40:0.60)
	Recessive model (AA:s CA + CC)	P=0.101 (0.11:0.89 vs. 0.08:0.92)	P=0.002*§ (0.20:0.80 vs. 0.03:0.97)
	Genotype table (AA: CA: CC)	P=0.115 (11.4:40.0:48.6 vs. 7.5:36.6:55.8)	P=0.005* (0.20:0.23:0.57 vs. 0.03:0.37:0.60)

The genotype and allele frequency of each SNP were compared between diabetic patients with PDR and without retinopathy (no retinopathy) using the Chi square and Fisher's exact test. Data are given as percentages
§: The odds ratio (OR) of PDR for the AA genotype of -2578C/A to CA + CC was 7.5 (95% CI: 1.8–30.9, p=0.002) in the recessive model

Table 4 Clinical characteristics of patients with type 2 diabetes

	Controls (duration >20) (no retinopathy)	Cases (duration <15) (with PDR)	p-value
Number	102	35	
E at diagnosis (years)	44.7±10.0	42.9±13.9	NS
Duration (years)	24.6±5.8	10.1±3.2	<0.001
BMI (kg/m^2)	21.8±2.9	24.9±4.7	<0.001
Systolic BP (mmHg)	133.7±19.0	143.2±26.8	NS
Diastolic BP (mmHg)	74.2±9.9	77.8±15.2	NS
HbA1c (%)	7.3±1.3	7.6±1.6	NS
T-cholesterol (mmol/l)	5.27±0.96	4.93±1.42	0.037
TG (mmol/l)	1.13±0.75	1.58±1.01	<0.001

Those subjects are subgroup of type 2 diabetic patients with PDR in (Table 1)

with and without macular edema [23]. They did not find any association at SNP –2578 in the VEGF gene with DR. Therefore, this is the first report in which the SNP –2578C/A is shown to be associated with PDR. In addition, we were unable to confirm any prior positive associations between diabetic retinopathy and SNP at –634.

The discrepancy between our results and those of Awata et al. may be explained partially by differences in allele frequencies at SNP-634. The frequency of allele C was 0.45 in all cases and 0.46 in our controls. Awata found respective frequencies of 0.48 and 0.35, which demonstrate a distinct difference in allele frequency between their study and ours in the control samples. Moreover, in an Indian population, Balasubbu et al. identified that the CG genotype of the SNP-634 region was significantly associated with DR (OR=2.33, 95% CI:1.24–4.36, P=0.008), whereas Awata et al. found that the CC genotype at SNP-634 was associated significantly with DR (OR=3.20, 95% CI:1.45–7.05, P=0.0046 in a Japanese population [9,24].

The duration of diabetes has been recognized as an important factor in the development and progression of diabetic retinopathy. In our study, the controls without retinopathy had about 16.7 years duration of diabetes, whereas the controls without retinopathy studied by Awata et al. had about 7.3 years duration of diabetes. The duration of diabetes affected influence the incidence and development of diabetic retinopathy.

Furthermore, although they showed that serum levels of VEGF were associated with VEGF polymorphism at SNP –634, we could not replicate this observation. We were unable to perform a similar regression analysis for SNP –2578 because there were too few subjects with the AA genotype.

The functional property of SNP –2578, which is located in the promoter region of this gene, has been shown to affect mRNA levels [19]. Studies have found a significantly lower expression of VEGF mRNA in the peritoneal dialysis effluent of patients with the CC genotype compared to those with the CA/AA genotype at SNP –2578 [25]. Koukouakis et al. also reported results suggesting that the allele A at SNP –2578 is associated significantly with increased VEGF expression through a dominant mode in lung cancer cells [16]. It was reported further that SNP-2578AA is associated both with type 1 diabetes and acceleration of its onset [11]. Therefore, we suspected that the SNP –2578 might show a correlation with the progression of diabetic retinopathy through VEGF expression.

A recent study has shown that the concentration of VEGF in the vitreous fluid of patients with PDR is higher than in patients without diabetes, and that the serum levels of VEGF were not attributed to serum diffusion across the blood–retinal barrier [26]. Furthermore, VEGF levels in the vitreous fluid were also reported to be increased in patients with diabetic retinopathy even at an early stage, implying that the genetic predisposition to VEGF levels in the vitreous fluid, in addition to retinal hypoxia, promotes development of proliferative diabetic retinopathy [27].

A very recent observation in murine models found that intravitreous injection of a small volume of RNAi for the VEGF gene reduced the activity of experimental ocular neovascularization and choroidal neovascularization. This showed clearly that increased VEGF mRNA levels in the vitreous fluid directly enhances neovascularization [28, 29]. These findings strongly support the proposition that mRNA levels of the VEGF gene in the vitreous fluid directly enhances the development and/or progression of diabetic retinopathy. Therefore, finding a genetic predisposition to diabetic retinopathy would suggest a benefit to therapy based on individual genetic background at an early stage to efficiently prevent progression to PDR.

From our observations and prior reports we speculate that the AA genotype at SNP-2578 in the VEGF gene may be associated with PDR by up-regulating mRNA levels of the VEGF gene through increasing the intraocular synthesis of VEGF. To clarify this hypothesis we analyzed the relationship between VEGF levels in the vitreous fluid and plasma, and the genotypes at SNP-2578 and -634 of the VEGF gene, using an additional series of 40 subjects with diabetes and PDR. Only one individual had the AA genotype at SNP-2578, so our power to detect was insufficient and the association was inconclusive. There was no association between the genotype at SNP-634 and VEGF levels in either vitreous fluid or plasma.

In summary, we found the first evidence that the VEGF-2578 AA genotype is associated with PDR in Japanese subjects with type 2 diabetes, regardless of the duration of diabetes. In addition, the positive association with SNP –634 reported previously was not reproduced in this study that employed a larger sample. Although the mechanism for the development and progression of diabetic retinopathy

was not clarified fully, multiple genetic reports support the hypothesis that the AA genotype at SNP-2578 affects transcription of the VEGF gene.

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