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Candidate gene association study for diabetic retinopathy in Chinese patients with type 2 diabetes

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Purpose: To investigate whether variants in a set of eight candidate genes are associated with diabetic retinopathy (DR) in a cohort of Chinese patients with type 2 diabetes mellitus (T2DM).

Methods: Case-control study. Patients with T2DM were recruited from the Desheng community in urban Beijing and assigned into a DR group or diabetic without retinopathy (DWR) group, based on the duration of diabetes and grading of fundus images. Twenty-six single-nucleotide polymorphisms (SNPs) within eight candidate genes, including PPARγ, vascular endothelial growth factor (VEGF) and its receptor kinase insert domain receptor (KDR), erythropoietin, aldose reductase, protein kinase C-β, angiotensin-converting enzyme, and intercellular adhesion molecule 1, were analyzed using the MassARRAY genotyping system.

Results: A total of 500 patients with T2DM (216 with DR and 284 with DWR) were enrolled in the study. Significant associations of DR were noted with genotypes of four SNPs—rs699947 ($p<0.001$), rs833061 ($p=0.001$), rs13207351 ($p<0.001$), and rs2146323 ($p=0.006$)—in the VEGF gene and one variant, rs2071559, in the KDR gene ($p=0.034$). After adjustment for covariates, significant association of DR remained with the homozygous genotype of the minor allele for the SNPs rs699947 (odds ratio [OR] = 3.54, 95% confidence interval [CI]: 1.12–11.19), rs833061 (OR = 3.72, 95% CI: 1.17–11.85), rs13207351 (OR = 3.76, 95% CI: 1.21–11.71), and rs2146323 (OR = 2.8, 95% CI: 1.46–5.37) in the VEGF gene as well as the SNP rs2071559 (OR = 1.62, 95% CI: 1.08–2.41) in the KDR gene. However, only rs699947 and rs13207351 in the VEGF gene remained statistically significant after Bonferroni correction. No associations were found in other genes tested.

Conclusions: These data expanded previous observations on the association of DR with variants in the VEGF gene in Chinese patients with T2DM. Moreover, a possible association between DR and KDR polymorphisms is suggested.

Diabetic retinopathy (DR) is one of the most common complications of diabetes and the leading cause of irreversible visual loss in people of working age in the United States [1]. The etiology of DR is complex and largely unknown. Hyperglycemia and long duration of diabetes along with hypertension are widely recognized as major risk factors for its development [2,3]; however, these factors seem to explain only a part of the risk of DR [4,5]. In clinical practice, for example, some patients with poorly controlled diabetes or a long duration of diabetes do not develop retinopathy, whereas others with relatively good glycemic control have advanced retinopathy [6]. Accumulated evidence has suggested that genetic factors may play a role in the development and progression of DR, with heritability estimated to be as high as 27% [7] for DR and 50% for proliferative diabetic retinopathy (PDR) [8].

Several studies have used a genome-wide association approach [9-12] and multipoint sib-pair analysis [13,14] to detect regions of the genome that potentially contain genes involved in the etiology of DR. While different regions of the genome have been implicated, the reported regions were not consistently identified among the studies. In addition, many studies have taken a candidate gene approach to investigate the genetic etiology of DR, implicating the potential candidate genes in DR etiology, which include genes for PPARγ [15], vascular endothelial growth factor (VEGF) [16-19], erythropoietin (EPO) [20,21], aldose reductase (AKR1B1) [22-25], protein kinase C (PKC)-β [26,27], angiotensin-converting enzyme (ACE) [28], and intercellular adhesion molecule 1 (ICAM-1) [29-31]. The validity of these results, however, remains unconfirmed, with few of the reported susceptibility variants having been consistently replicated [25].

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Kinase insert domain receptor (KDR), also called VEGF receptor (VEGFR) 2, is believed to be responsible for the majority of the angiogenic and permeability-enhancing effects of VEGF [32,33]. An increase expression of KDR has been reported in the retinas from diabetic rats [34,35]. One study investigated the fibrovascular tissues obtained from 22 PDR patients and suggested the co-expression of KDR and neuropilin-1 facilitated fibrovascular proliferation in DR [36]. Moreover, microvascular expression of KDR was found to be associated with leaky vessels in diabetic retina from eyes of diabetic donors as compared to the retina from eyes of nondiabetic control donors [37]. Taken together, these findings imply that KDR might be a major contributor in the pathogenesis of DR. Furthermore, the KDR gene has been found to be associated with other pathogenic conditions featured for vascular permeability and angiogenesis, such as age-related macular degeneration (AMD) [38], cancer [39], and coronary heart/artery disease [40,41]. However, genetic association studies with DR have not been performed for the KDR gene. In this study, we tested whether the candidate genes, including PPARγ, VEGF, EPO, AKR1B1, PKC-β, ACE, and ICAM-1 genes as well as the KDR gene, were associated with DR in our independent cohort of Chinese patients with T2DM.

METHODS

Subjects and clinical evaluation: The study protocol was approved by the Ethics Committee of Beijing Tongren Hospital (TRECKY200907) and adhered to the tenets of the Declaration of Helsinki. Written informed consent was obtained from all participants before their enrollment. Patients with T2DM were recruited between November 2009 and September 2011 from the Desheng Community of urban Beijing. Diabetes was defined as a self-reported history of physician-diagnosed T2DM treated with insulin, oral hypoglycemic agents, or diet only; or by a fasting plasma glucose (FPG) concentration of 7.0 mmol/l (126 mg/dl) or more in at least two previous examinations; or a random plasma glucose concentration of ≥11.1 mmol/l (200 mg/dl). All subjects underwent a standardized evaluation consisting of a questionnaire, ocular and anthropometric examinations, and a laboratory test. The questionnaire elicited basic information (age, sex, ethnicity, income, education), lifestyle information (such as smoking and alcohol intake), health status information (such as the use of insulin therapy and any history of systemic diseases), and family history of diseases. Anthropometric parameters included body weight and height, waist and hip circumference, and three measurements, 5 min apart, of blood pressure in a resting state. Body mass index (BMI, kg/m^2) was calculated according to the height and weight of the participant, and waist-to-hip ratio was calculated. A comprehensive ophthalmological examination included corrected visual acuity, slit-lamp biomicroscopy, and dilated fundus photography. Seven fields of 30°color fundus photographs with stereoscopic images of the optic disc and macula were taken through the dilated pupils of each patient, using a digital fundus camera (Zeiss Visucam Pro; Oberkochen, Germany).

Based on the duration of diabetes and grading of fundus photographs, patients were assigned to the diabetic-without retinopathy (DWR) group if they had more than 10 years of T2DM with no signs of DR (microaneurysms, hemorrhages, exudates) or if they had more than 15 years of T2DM with fewer than five microaneurysms. Patients with five or more microaneurysms in at least one eye were assigned to the DR group. Patients who did not meet the DWR or DR criteria were excluded from this study. The duration of diabetes was defined as the interval between the first diagnosis of diabetes and the time of enrollment in the present study.

Laboratory assays: Fasting venous blood samples were collected for measurement of FPG, glycosylated hemoglobin A1c (HbA1c), creatinine, uric acid, and lipid profile (levels of total cholesterol, triglycerides, and high-density and low-density lipoprotein cholesterol), which were measured in an automated system with reagents for routine biomarkers. HbA1c was assessed by the enzymatic method using a Hitachi analyzer 7080 (Hitachi, Ibaraki, Japan). A first-void, midstream, morning, spot urine sample was collected, and albuminuria was measured by immunonephelometry with a Roche/Cobas C501 analyzer (Ibaraki). High albuminuria was defined as ≥20 mg/l.

Candidate genes and single-nucleotide polymorphisms selection: Eight candidate genes that are involved in the pathogenesis of DR or previously identified as being associated with DR were tested, including the PPARγ, VEGF and its receptor (KDR), EPO, AKR1B1, PKC-β, ACE and ICAM-1 genes. Twenty-six single-nucleotide polymorphisms (SNPs) in these genes were chosen initially for availability of assays, informativeness, and spacing across the gene. More information about the candidate genes is given in Table 1. It should be noted that the VEGF gene has been reported in previous literature [16], and the present study repeated the association between the VEGF gene and DR in an expanded larger sample size.

Genotyping: Blood samples were collected from all participants and stored at −80 °C before DNA extraction. Genomic DNA was extracted from venous blood leukocytes using a genomic DNA extraction and purification kit (TIANamp

Table 1. Description of candidate genes investigated

Gene	Gene function	Locus	References
PPARγ	responsible for differentiation of fibroblasts to adipocytes and regulation of their function	3p25	Maciej et al. 2008 [13]
KDR	functions as the main mediator of VEGF-induced endothelial permeability, proliferation, survival, migration, tubular morphogenesis and sprouting	4q11-q12	Galan et al. 2010 [46]
VEGF	a critical angiogenic and vasopermeability factor that is upregulated in various conditions involving retinal ischemia	6p12	Churchill et al. 2008 [15] Al-Kateb et al. 2007 [16]
EPO	been linked to angiogenesis, vasculogenesis, and neuroprotection	7q21–7q22	Tong et al. 2008 [18] Abhary et al. 2010 [19]
AKR1B1	the first and rate-limiting enzyme in the polyol pathway	7q35	Richeti, F et al. 2007 [22] Abhary et al. 2009 [23]
PKC-β	PKC family members phosphorylate a wide variety of protein targets and are known to be involved in diverse cellular signaling pathways	16p11.2	Uthra et al. 2010 [24] Ikeda et al. 2004 [25]
ACE	involved in the conversion of angiotensin I to angiotensin II (ATII).	17q23	Kondo et al. 2009 [26]
ICAM-1	a member of the immunoglobulin superfamily of adhesion molecules, involved in blood-retina barrier breakdown, capillary nonperfusion and endothelial cell damage and death	19p13	Kamiuchi, et al. 2002 [21] L. Liu, et al. 2006 [22] Petrovic, et al. 2008 [23]

Locus: the chromosomal region of gene; p: long arm of chromosome; q: short arm of chromosome

Swab DNA Kit; Tiangen Biotech, Beijing, China). Study participants were genotyped for the SNPs using Sequenom MassARRAY technology (Bioyong Technologies, Beijing, China). All DNA samples passing initial quality checks were plated at a concentration of ≥5 ng/μl for processing on the platform. Quality measures taken into account for genotyped SNPs to be excluded from the subsequent analysis were minor allele frequency (MAF) <0.05, genotyping success <80%, and failed Hardy–Weinberg equilibrium (HWE) test in control samples ($p<0.001$). Quality control criteria for each SNP were implemented in our data set.

Statistical analysis: Statistical analysis was performed using the R statistical analysis package. Baseline characteristics of diabetic patients in the DR and DWR groups were compared using a *t* test for continuous variables or a chi-square test for categorical variables. The genotype frequencies of genes were checked for HWE in all groups using a chi-square test. The chi-square test was also used to analyze the distribution of genotypes and alleles. When the expected frequency was less than 5, Fisher's exact test was used. The pairwise linkage disequilibrium was calculated using Haploview, version 4.2 (Broad Institute, Cambridge, MA). Univariate and multivariate logistic regression analyses were performed to identify the association between SNPs/haplotypes and the presence of DR. Statistical results were expressed as p values, odds ratios (OR), and 95% confidence intervals (CI). The Bonferroni correction ($p<0.05$ divided by the number of SNPs analyzed) was used to account for multiple testing.

RESULTS

In total, 1,433 subjects with T2DM were enrolled from the community, of which, 500 subjects (284 in the DWR group and 216 in the DR group) participated in the genetic study based on the inclusion criteria. The distribution of demographic, behavioral, and clinical characteristics of participants are listed in Table 2. In the DR group, 59 patients were further classified as PDR. Compared with the DWR group, individuals in the DR group had a younger age of diabetic onset ($p=0.008$); higher percentage of high albuminuria ($p<0.001$); higher levels of creatinine ($p=0.027$), FPG ($p<0.001$), and HbA1c ($p<0.001$); and were more likely to use insulin ($p<0.001$). Duration of diabetes in the DWR group was longer than the DR group ($p=0.013$), which could be due to the definition of DWR in this study (more than 10 years of T2DM without retinopathy). No statistically significant differences were found between the DR and DWR groups in terms of gender, BMI, waist-to-hip ratio, blood pressure, serum levels of uric acid, or lipid profile.

Table 2. Clinical and biochemical markers for the studied groups

Clinical characteristics	DWR (n=284)	DR (n=216)	p value
Age of diabetic onset (years)	52.7±7.61	50.67±9.41	0.008
Sex (Male/Female)	111/173	103/113	0.054
Duration of diabetes (years)	14.79±4.97	13.44±7.22	0.013
BMI (kg/m^2)	25.26±3.95	25.8±4.13	0.14
WHR	0.92±0.06	0.93±0.06	0.5
High albuminuria(-/+)	240/38	143/68	<0.001
Systolic blood pressure (mmHg)	136.7±16.65	138.5±17.18	0.22
Diastolic blood pressure (mmHg)	77.65±9.36	79.23±9.69	0.066
Insulin therapy (yes/no)	92/191	118/97	<0.001
HbA1c (%)	6.97±1.40	7.84±1.70	<0.001
FPG (mmol/l)	8.09±2.43	9.22±3.21	<0.001
Creatinine (µmol/l)	67.43±17.69	73.66±49.84	0.027
Uric acid (µmol/l)	281.9±82.42	281.1±76.58	0.98
Cholesterol (mmol/l)	5.05±0.98	5.18±1.17	0.19
Triglycerides (mmol/l)	1.55±0.93	1.73±1.52	0.11
HDL cholesterol (mmol/l)	1.25±0.30	1.23±0.30	0.38
LDL cholesterol (mmol/l)	3.07±0.83	3.08±0.90	0.83

The data are expressed as mean ± standard deviation (SD) or number of subjects. Differences between diabetic retinopathy (DR) and diabetic without retinopathy (DWR) groups were compared using *t* test or Chi-square test. BMI, body mass index; WHR, waist and hip ratio; HbA1c, glycosylated hemoglobin A1c; FPG, fasting plasma glucose; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Basic information of SNPs analyzed with allelic distributions is shown in Table 3. Out of the 26 SNPs selected, rs11645239 in the PKC-β gene had a low genotyping call rate (<52%); rs1805192 in the PPARγ gene and rs1799969 in the ICAM-1 gene had a minor allele frequency of less than 5% in the studied population. These three SNPS were not studied further. The remaining 23 SNPs were tested in the DR and DWR groups for any departure from HWE. All were in HWE except rs3900008 ($p<0.001$), which failed to be genotyped in 53 individuals, indicating a possible genotyping error. The SNP rs3900008 was therefore excluded, and a total of 22 SNPs were included for subsequent analysis. For each of the SNPs evaluated, the number of participants varied slightly depending on the genotype call rate (≥94%).

Table 4 shows the genotypic distribution in the DR and DWR groups for the 22 informative SNPs. A strong association with DR was detected at three promoter variants (rs833061, rs13207351, rs699947) and one intronic SNP (rs2146323) in the VEGF gene, with the minor alleles of SNPs rs833061, rs13207351, rs699947, and rs2146323 showing a similar magnitude of risk (OR≥1.52; $p<0.003$). The ORs for the homozygote minor alleles were 3.80 (95% CI: 1.91-7.57) for rs699947, 3.6 (95% CI: 1.79- 7.22) for rs833061, 3.7 (95% CI: 1.86-7.39) for rs13207351, and 2.8 (95% CI: 1.46-5.37) for the intronic SNP rs2146323. The three promoter variants rs833061, rs13207351, rs699947 in the VEGF gene showed strong linkage disequilibrium ($r^2\geq0.98$). SNP rs2071559 in the KDR gene was also significantly associated with DR with an OR of 1.41 (95% CI: 1.07-1.86) for the minor allele and 1.57 (95% CI: 1.05-2.28) for the minor homozygote.

Table 5 presents the multivariate analyses data adjusted for variables including the age of diabetic onset, duration of diabetes, high albuminuria, insulin use, HbA1c, and creatinine levels. The association with DR remained statistically significant with the homozygous genotype of the minor allele for SNPs rs699947 (OR=3.74, 95% CI: 1.75-8.00), rs833061 (OR=3.50, 95% CI: 1.62-7.54), rs13207351 (OR=3.64, 95% CI: 1.71-7.77), and rs2146323 (OR=2.88, 95% CI: 1.4-5.93) in the VEGF gene as well as SNP rs2071559 (OR=1.88, 95% CI: 1.01-3.48) in the KDR gene. However, only rs699947 and rs13207351 in the VEGF gene remained statistically significant after Bonferroni correction for the 22 SNPs analyzed. No statistically significant association with DR was found for all other SNPs in genes for EPO, AKR1B1, PKC-β, ACE, and ICAM-1.

Haplotype analysis revealed five SNP blocks (Figure 1). Only two haplotypes in the VEGF gene showed strong evidence of an association with DR, and the others did not

Table 3. Basic information of Alleles and allele association results Between DR and DWR groups

Gene	locus	SNP	position	Minor Allele	MAF	HWpval DR	HWpval DWR	OR (CI)	P value
PPARγ	**3**	rs1805192	12,396,238	G	0				
		rs2071559	55,687,123	C	0.34	0.39	0.022	1.41(1.07,1.86)	**0.011**
KDR	**4**	rs13109660	55,665,437	A	0.31	0.63	0.49	1.06(0.8,1.41)	0.66
		rs1870378	55,661,210	A	0.48	0.67	0.33	0.92(0.71,1.21)	0.55
		rs1870377	55,667,731	A	0.48	0.4	0.73	0.95(0.73,1.23)	0.67
		rs699947	43,844,367	A	0.28	0.009	0.51	1.63(1.22,2.18)	**0.001**
		rs833061	43,845,464	C	0.28	0.021	0.33	1.53(1.14,2.04)	**0.003**
		rs13207351	43,845,772	A	0.28	0.01	0.41	1.58(1.18,2.12)	**0.001**
VEGF	**6**	rs2010963	43,846,328	C	0.44	0.41	0.9	0.87(0.67,1.13)	0.29
		rs833069	43,850,557	G	0.44	1	0.9	0.92(0.71,1.2)	0.53
		rs2146323	43,853,073	A	0.26	0.003	0.49	1.52(1.13,2.05)	**0.004**
		rs3025021	43,857,141	T	0.16	0.58	1	1.36(0.95,1.97)	0.083
		rs3025039	43,860,514	T	0.09	1	0.38	1.34(0.96,1.88)	0.077
		rs507392	100,157,872	C	0.2	0.4	0.03	0.93(0.67,1.28)	0.64
EPO	**7**	rs551238	100,159,464	C	0.2	0.39	0.045	1.04(0.75,1.45)	0.8
		rs1617640	100,155,234	G	0.19	0.1	0.14	1.18(0.85,1.66)	0.31
AKR1B1	**7**	rs759853	133,794,498	A	0.07	0.79	0.34	1.31(0.92,1.87)	0.11
		rs3900007	23,754,453	G	0.42	0.49	0.22	1.01(0.78,1.31)	0.95
		rs3900008	23,754,514	C	0.3	<0.001	<0.001	1.05(0.78,1.41)	0.75
PKC-β	**16**	rs11645239	23,754,563	G	0.36				
		rs2575390	23,754,255	G	0.06	1	1	0.95(0.53,1.69)	0.85
		rs3760106	23,753,297	A	0.06	1	1	1.02(0.57,1.84)	0.95
ACE	**17**	rs1800764	58,904,261	C	0.39	1	1	1.23(0.94,1.6)	0.12
		rs9896208	58,929,841	T	0.42	0.26	0.021	0.95(0.73,1,24)	0.71
		rs5498	10,256,683	G	0.31	0.64	0.1	1.32(1,1.75)	**0.044**
ICAM-1	**19**	rs1799969	10,255,792	A	0.003				

MAF: minor allele frequency; HWpval: p value for Hardy–Weinberg (H-W) equilibrium; OR: odds ratio; CI:95% confidence interval; Genotype distributions for SNPs were in Hardy–Weinberg (H-W) equilibrium, except rs3900008 in both groups.

show any statistically significant association (Table 6). The frequency of haplotype ACGA in the VEGF gene, defined by the minor alleles of the three SNPs rs699947, rs833061, rs13207351 at the promoter region and rs2010963 at the 5′ untranslated region, was 32.4% in the DR group and 23.2% in the DWR group (p=0.002, OR=1.58, 95% CI: 1.19–2.10). The haplotype AA defined by the minor alleles of the SNPs rs833069 and rs2146323 at intron 2 of the VEGF gene was 30.7% in the DR group and 22.5% in the DWR group (p=0.004), with an OR of 1.53 (95% CI: 1.15–2.04) for the increased risk of DR.

DISCUSSION

We analyzed the possible association between polymorphisms in eight candidate genes and DR in a well-defined cohort of Chinese patients with T2DM. Our data showed significant association between SNPs in the VEGF gene and the risk of DR after adjustment for possible confounding risk factors. However, no statistically significant association was found for other genes, including KDR, PPARγ, EPO, AKR1B1, PKC-β, ACE, and ICAM-1.

The pathogenesis of DR is complex with multifactorial biochemical causes influenced by genetic and environmental factors. Many vasoactive factors stimulated by hyperglycemia or oxidative stress have been identified [1]. Among

Table 4. Genotype frequencies of polymorphisms in the studied groups

Gene	SNP	Sample size	genotype	DR	DWR	OR (CI)	p-value
KDR	rs2071559	492	TT	84(39.3)	141(50.7)		0.034
			CT	95(44.4)	103(37.1)	1.73 (1,2.98)	
			CC	35(16.4)	34(12.2)	1.57 (1.05,2.28)	
	rs1870377	490	TT	53(24.8)	78(28.3)		0.6
			TA	113(52.8)	134(48.6)	1.24 (0.81,1.91)	
			AA	48(22.4)	64(23.2)	1.1 (0.66,1.84)	
	rs1870378	471	GG	54(26.9)	80(29.6)		0.8
			GA	97(48.3)	126(46.7)	1.14 (0.74,1.76)	
			AA	50(24.9)	64(23.7)	1.16 (0.7,1.92)	
	rs13109660	490	GG	102(47.9)	127(45.8)		0.9
			GA	93(43.7)	125(45.1)	0.93 (0.64,1.35)	
			AA	18(8.5)	25(9)	0.9 (0.46,1.73)	

Gene	SNP	Sample size	genotype	DR	DWR	OR (CI)	p-value
	rs699947	495	CC	105(48.6)	162(58.1)		<0.001
			CA	79(36.6)	104(37.3)	1.17 (0.8,1.72)	
			AA	32(18.4)	13(4.7)	3.80 (1.91,7.57)	
	rs833061	491	TT	104(49.1)	158(56.6)		0.001
			CT	78(36.8)	108(38.7)	1.1 (0.75,1.61)	
			CC	30(14.2)	13(4.7)	3.6 (1.79,7.22)	
	rs13207351	491	GG	105(48.8)	158(57.2)		<0.001
			GA	78(36.3)	105(38)	1.12 (0.76,1.64)	
			AA	32(14.9)	13(4.7)	3.7 (1.86,7.39)	
	rs2146323	490	CC	112(52.3)	167(60.5)		0.006
			CA	72(33.6)	93(33.7)	1.15 (0.78,1.7)	
			AA	30(14)	16(5.8)	2.8 (1.46,5.37)	
VEGF	rs3025021	491	TT	5(2.4)	9(3.2)		0.21
			CT	48(22.6)	81(29)	1.07 (0.34,3.37)	
			CC	159(75)	189(67.7)	1.51 (0.5,4.61)	
	rs833069	497	GG	40(18.6)	58(20.6)		0.82
			GA	105(48.8)	138(51.8)	1.1 (0.69,1.78)	
			AA	70(18.6)	86(30.5)	1.18 (0.71,1.97)	
	rs2010963	495	GG	70(32.6)	84(30)		0.47
			GC	111(51.6)	140(50)	0.95 (0.64,1.42)	
			CC	34(15.8)	56(20)	0.73 (0.43,1.24)	
	rs3025039	498	CC	137(63.7)	198(70.0)		0.15
			CT	69(32.1)	47(28.3)	2.6 (0.85,7.93)	
			TT	9(4.2)	5(1.8)	1.25 (0.85,1.84)	
	rs5498	492	AA	92(42.8)	138(49.8)		0.078
ICAM-1			GA	100(46.5)	123(44.4)	1.22 (0.84,1.77)	
			GG	23(10.7)	16(5.8)	2.16 (1.08,4.30)	

Molecular Vision 2014; 20:200-214 <http://www.molvis.org/molvis/v20/200>

Gene	SNP	Sample size	genotype	DR	DWR	OR (CI)	p-value
	rs507392	496	TT	141(65.3)	181(64.6)		0.68
			CT	65(30.1)	81(28.9)	1.03 (0.69,1.53)	
			CC	10(4.6)	18(6.4)	0.71 (0.32,1.59)	
	rs551238	494	AA	141(65.3)	182(65.5)		
EPO			CA	65(30.1)	79(28.4)	1.06 (0.72,1.58)	0.74
			CC	10(4.6)	17(6.1)	0.76 (0.34,1.71)	
	rs1617640	491	TT	146(69.2)	182(65)		
			GT	55(26.1)	82(29.3)	0.84 (0.56,1.25)	0.61
			GG	10(4.7)	16(5.7)	0.78 (0.34,1.77)	
	759853	471	GG	145(70.7)	167(62.8)		
AKR1B1			GA	54(26.3)	91(34.2)	0.68 (0.46,1.02)	0.18
			AA	6(2.9)	8(3.0)	0.86 (0.29,2.55)	
	1800764	494	TT	88(40.9)	98(35.1)		0.31
			CT	99(46.0)	134(48)	0.83 (0.56,1.23)	
			CC	28(13)	47(16.8)	0.65 (0.38,1.14)	
ACE	rs9896208	496	CC	79(36.6)	84(30)		0.056
			TC	97(44.9)	156(55.7)	0.66 (0.44,0.98)	
			TT	40(18.5)	40(14.3)	1.06 (0.62,1.82)	
	3,900,007	496	AA	69(31.9)	99(35.4)		0.36
			GA	111(51.4)	126(45)	1.26 (0.85,1.88)	
			GG	36(16.7)	55(19.6)	0.94 (0.56,1.58)	
	2575390	499	CC	193(89.4)	251(88.7)		0.95
PKC-β			CG	22(10.2)	31(11.0)	0.92 (0.52,1.64)	
			GG	1(0.5)	1(0.4)	1.3 (0.08,20.92)	
	3760106	492	GG	190(89.2)	248(88.9)		0.97
			GA	22(10.3)	30(10.8)	1.31 (0.08,21)	
			AA	1(0.5)	1(0.4)	0.96 (0.54,1.71)	

Data are expressed as number (%).The p value represents comparison between DR and diabetic without retinopathy (DWR) groups, using $\chi 2$ or Fisher's exact test.

Table 5. Association analysis of polymorphisms in multivariate models

Gene	SNP	genotype	Ajusted OR (CI)	P value
KDR	rs2071559	TT		0.05
		CT	1.57 (1.01,2.44)	
		CC	1.87 (1.01,3.48)	
	rs1870377	TT		0.64
		TA	1.13 (0.69,1.85)	
		AA	0.88 (0.48,1.6)	
	rs1870378	GG		0.79
		GA	1.1 (0.66,1.81)	
		AA	0.91 (0.5,1.66)	
	rs13109660	GG		0.93
		GA	1.01 (0.48,2.13)	
		AA	1.09 (0.7,1.68)	

Gene	SNP	genotype	Ajusted OR (CI)	P value
VEGF	rs699947	CC		0.002
		CA	1.26 (0.82,1.95)	
		AA	3.74 (1.75,8.00)	
	rs833061	TT		0.004
		CT	1.19 (0.77,1.85)	
		CC	3.50 (1.62,7.54)	
	rs13207351	GG		0.002
		GA	1.22 (0.79,1.89)	
		AA	3.64 (1.71,7.77)	
	rs2146323	CC		0.014
		CA	1.14 (0.73,1.77)	
		AA	2.88 (1.4,5.93)	
	rs3025021	TT		
		CT	0.75 (0.2,2.78)	0.22
		CC	1.14 (0.32,4.06	
	rs833069	GG		
		GA	1.09 (0.63,1.89)	0.63
		AA	1.3 (0.73,2.34)	
	rs2010963	GG		
		GC	0.89 (0.56,1.41)	0.51
		CC	0.7 (0.38,1.28)	
	rs3025039	CC		0.23
		CT	1.23 (0.79,1.92)	
		TT	2.8 (0.76,10.27)	
ICAM-1	rs5498	AA		0.091
		GA	1.44 (0.94,2.22)	
		GG	2.04 (0.94,4.44)	

Gene	SNP	genotype	Ajusted OR (CI)	P value
	rs507392	TT		0.88
		CT	1.02 (0.65,1.61)	
		CC	0.8 (0.32,2.02)	
	rs551238	AA		0.94
EPO		CA	1.07 (0.67,1.68)	
		CC	0.92 (0.36,2.36)	
	rs1617640	TT		
		GT	0.85 (0.53,1.36)	0.79
		GG	0.94 (0.36,2.41)	
	759853	GG		0.11
AKR1B1		GA	0.62 (0.39,0.99)	
		AA	0.59 (0.17,2.05)	
	1800764	TT		0.33
		CT	0.87 (0.56,1.37)	
		CC	0.62 (0.33,1.16)	
ACE	rs9896208	CC		
		TC	0.62 (0.39,0.97)	0.097
		TT	0.88 (0.47,1.63)	
	3900007	AA		0.82
		GA	1.14 (0.72,1.8)	
		GG	1.17 (0.64,2.14)	
	2575390	CC		0.78
		CG	0.89 (0.46,1.71)	
PKC-β		GG	2.73 (0.1,73.85)	
	3760106	GG		0.81
		GA	2.67 (0.1,71.35)	
		AA	0.92 (0.48,1.77)	

Multiple logistic regression analysis was used to calculate odds ratio (OR) and 95% confidence interval (CI) by comparing diabetic retinopathy (DR) group with diabetic without retinopathy (DWR) group, which include the most significant SNP at kinase insert domain receptor gene (KDR) gene (rs2071559), vascular endothelial growth factor (VEGF) gene (rs833061, rs13207351, rs699947, rs2146323). Adjusted OR represents data after adjustment for covariates including age of diabetic onset, duration, high albuminuria, insulin use, glycosylated hemoglobin A1c (HbA1c), fasting plasma glucose and creatinine levels.

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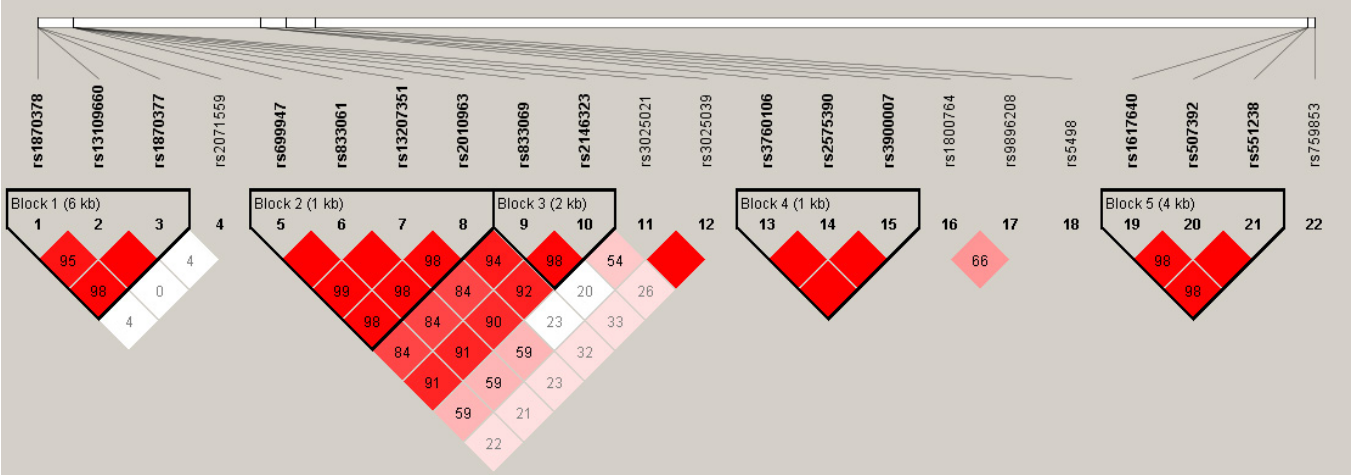

Figure 1. Linkage disequilibrium plot generated by Haploview 4.2 software. Five haplotype blocks (bold) were identified for single-nucleotide polymorphisms (SNPs) in the kinase insert domain receptor, vascular endothelial growth factor gene, protein kinase C-β gene, and in erythropoietin gene. Linkage disequilibrium (LD) is displayed as the pairwise D' value multiplied by 100 and given for each SNP combination. Shading represents the magnitude and significance of the pairwise LD, with a red-to-white gradient reflecting higher-to-lower LD values. Red diamond without a number corresponds to a D' value of 1.0.

them, VEGF has been characterized as a critical angiogenic and vasopermeability factor that is upregulated in various retinal ischemic disorders, such as retinopathy of prematurity, AMD, and DR [32], whereas anti-VEGF therapy has been shown to be effective for patients with AMD [42] or diabetic macular edema [43]. The human VEGF gene is highly polymorphic and has a large number of individual SNPs examined in relationship with DR [16-19,44]. In our previous study, three SNPs (rs833061, rs13207351, rs699947) at the promoter region of the VEGF gene showed a strong association with the risk of DR [16]. This current study with an expanded sample size confirmed our previous report. Discussions of the three promoter SNPs have been described in detail in our previous literature [16]. Moreover, the association of DR with an intronic SNP rs2146323 in the VEGF gene has been suggested in this current study. Although the intronic region of VEGF is believed to contain binding sites for transcription factors that regulate VEGF production [45], there is no functional data to date on the intronic SNPs.

There are three high-affinity VEGF tyrosine kinase receptors: VEGFR-1, VEGFR-2 (KDR), and VEGFR-3 that are expressed mainly in the vascular endothelial cells [32,33]. KDR is the primary responder to the VEGF signal regulating endothelial cell migration and proliferation [32,33]. Several lines of evidence have implicated that KDR plays a role in the etiology of DR [34-37]. Although it became insignificant after Bonferroni correction, the present study suggested a possible association between the SNP rs2071559 in the KDR gene and DR. Further studies with a larger sample size to confirm this observation would be warranted. The present study failed to find an association of DR with other previously reported genes, including ACE, AKR1B1, PKC-β, PPARγ, ICAM-1, and EPO. Discrepancies between our study and previous reports could be due to different ethnic origin, diagnostic criteria, or sample size.

Strengths of the current study include the enrollment of participants from a relatively homogeneous population of a single community, collection of detailed information on a variety of risk factors, and the standardized fundus photograph and assessment of DR severity. Additionally, previous genetic association studies for DR have been limited to studies of one or a modest number of candidate genes [25,46]. To our knowledge, this is the first and largest study in the Chinese population to address the question of whether multiple candidate genetic polymorphisms are associated with DR.

In summary, the present study confirmed our previous observation on the association between DR and VEGF polymorphisms independent of other clinical factors in the Chinese population. Moreover, a possible association between DR and KDR polymorphisms is suggested and further studies are warranted.

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Table 6. Haplotype association analysis between polymorphisms and risk of diabetic retinopathy in patients with type 2 diabetes mellitus

Haplotype blocks	DR group (%)	DWR group (%)	OR (95%CI)	Chi Square	p value
Block 1					
AAG	48.7	47.2	1.07 (0.82, 1.39)	0.26	61
TGA	29.5	31.2	0.93 (0.69, 1.23)	0.28	0.59
TGG	20.7	20.8	0.99(0.72–1.37)	0.001	0.98
Block 2					
ACGA	32.4	23.2	1.58 (1.19, 2.10)	9.87	0.002
CTCG	42.1	44.6	0.90 (0.69, 1.16)	0.68	0.41
CTGG	25.2	31.6	0.73(0.55, 0.97)	4.8	0.03
Block 3					
AA	30.7	22.5	1.53 (1.15, 2.04)	8.39	0.04
AC	25.8	32.4	0.72 (0.55, 0.96)	5.16	0.02
GC	43.4	44.8	0.94 (0.73, 1.21)	0.23	0.63
Block 4					
CGA	57.3	57.9	0.97(0.75, 1.26)	0.044	0.83
CGG	37.1	36.3	1.04(0.80, 1.35)	0.068	0.79
GAG	5.6	5.8	0.97(0.57, 1.68)	0.009	0.92
Block 5					
CCG	17.5	20	0.86(0.62, 1.20)	0.79	0.37
TAT	80.6	79.4	1.16(0.84, 1.61)	0.79	0.37

Data were given as frequency of each haplotype within diabetic retinopathy (DR) group or diabetic without retinopathy (DWR) group. Block 1 included rs1870378, rs13109660, and rs1870377 in kinase insert domain receptor gene. block 2 included promoter SNPs rs699947, rs833061, rs13207351 and rs2010963 at 5′ UTR, and Block3 included SNPs two intronic SNPs of rs833069 rs2146323 in vascular endothelial growth factor gene, Block4 included SNPs rs3760106 rs2575390 rs3900007 in protein kinase C-β gene, block 5, rs1617640 rs507392 rs551238 in erythropoietin gene. Only haplotypes with frequency over 3% were included in this table. OR indicates odds ratio and CI refers to confidence interval.

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