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JPH2 is a novel susceptibility gene on chromosome 20q associated with diabetic retinopathy in a Taiwanese population

Yu-Chuen Huang[a,b], Hsin-Yi Lin[a], Hui-Ju Lin[b,c], Shih-Yin Chen[a,b], Shih-Ping Liu[d,e], Wen-Ling Liao[f,g], Jane-Ming Lin[b,c], Yung-Hsiang Chen[g], Fuu-Jen Tsai[a,b,h,i,*]

[a] Genetics Centre, Department of Medical Research, China Medical University Hospital, Taichung 404, Taiwan
[b] School of Chinese Medicine, College of Chinese Medicine, China Medical University, Taichung 404, Taiwan
[c] Department of Ophthalmology, China Medical University Hospital, Taichung 404, Taiwan
[d] Centre for Neuropsychiatry, China Medical University Hospital, Taichung 404, Taiwan
[e] Graduate Institute of Basic Medical Science, College of Medicine, China Medical University, Taichung 404, Taiwan
[f] Personalized Medicine Centre, China Medical University Hospital, Taichung 404, Taiwan
[g] Graduate Institute of Integrated Medicine, College of Chinese Medicine, China Medical University, Taichung 404, Taiwan
[h] Department of Pediatrics, China Medical University Hospital, Taichung 404, Taiwan
[i] Department of Medical Genetics, China Medical University Hospital, Taichung 404, Taiwan

*Corresponding author, e-mail: d0704@mail.cmuh.org.tw

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ABSTRACT: A number of genes on human chromosome 20 have been implicated in susceptibility to diabetic retinopathy (DR) in type 2 diabetes (T2D) patients. This study investigated the association between genetic variants on chromosome 20 and DR development in T2D patients in a Taiwanese population. Unrelated subjects with T2D, without DR ($n = 575$) and with DR ($n = 174$), were genotyped for single nucleotide polymorphisms (SNPs) using Illumina BeadChips and genotypes compared between these 2 groups. Seven SNPs on chromosome 20 demonstrated associations with DR, with p-values $< 1 \times 10^{-6}$. After controlling for diabetic duration and haemoglobin A1C, rs761207 and rs6031415, in *junctophilin 2* (*JPH2*), remained associated to DR and increased the risk for DR development 1.43-fold (95% confidence interval (CI) = 1.04–1.98) and 1.42-fold (95% CI = 1.02–1.97), respectively. These SNPs were also associated with non-proliferative DR. The results implicate that genetic variants of *JPH2* are associated with the pathogenesis of DR, particularly in the earlier non-proliferative phase. Given that JPH2 is an essential regulator of calcium flux and that vascular endothelial growth factor, which has previously been implicated in DR, is a mediator of calcium entry, calcium release, and endothelial permeability, our finding indicates that *JPH2* is a plausible new candidate gene for DR development.

KEYWORDS: diabetic complication, junctophilin 2, single-nucleotide polymorphism, haplotype, linkage disequilibrium

INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder that has reached epidemic proportions worldwide, and its incidence is increasing rapidly. Over 95% of DM cases are attributed to type 2 diabetes mellitus (T2D), which is characterized by abnormal hepatic glucose output, insulin resistance, and impaired insulin production[1]. Genetic factors are also thought to strongly influence the development of T2D[2].

The severe complications of diabetes mostly involve macro- and micro-vascular disease. Diabetic retinopathy (DR) is the most common micro-vascular complication of diabetes and is a leading cause of blindness in working-age individuals[3–5]. DR involves 2 stages, viz., an earlier non-proliferative (NPDR) and a later proliferative retinopathy (PDR) stage. In PDR, new, abnormal vessels develop in the retina and lead to neovascularization within the retina and vitreous gel.

Previous studies indicate that there are many risk factors for the development of DR, which include poor glycaemic control, longer diabetic duration, hypertension, hyperlipidemia, and albuminuria[6–10]. Although the underlying mechanisms of DR have not been

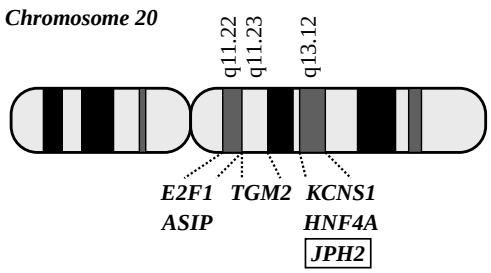

Fig. 1 Potential candidate genes for diabetic retinopathy on the long arm of chromosome 20. These include *E2F1*, *ASIP*, *TGM2*, *KCNS1*, and *HNH4A*. In this study, *JPH2* was implicated in the pathogenesis of diabetic retinopathy.

clarified, the pathogenesis of the condition is believed to be complex and multifactorial. In addition, there is increasing evidence implicating genetic factors in the susceptibility to DR, which are independent of known risk factors, and which may contribute to variation in the onset and severity of DR [11–13].

To date, genome-wide linkage studies have suggested the presence of potential candidate genes for DR on the long arm of chromosome 20 [14]. Moreover (Fig. 1), the genes *KCNS1* (encoding the potassium voltage-gated channel, delayed-rectifier, subfamily S, member 1), on chromosome 20q13.12, and *TGM2* (encoding transglutaminase 2), on the chromosome 20q11.23, are known to be involved in retinal biology or retinal disease [15, 16]. Other genes in the region, including *E2F1* (encoding E2F transcription factor 1), *ASIP* (encoding agouti signalling protein), on chromosome 20q11.22, and *HNF4A* (encoding hepatocyte nuclear factor-4), located on chromosome 20q13.12 and which has been implicated in type 1 maturity-onset diabetes of the young, may be associated with various aspects of insulin resistance or T2D [17, 18]. In addition, the putative non-insulin-dependent diabetes mellitus 3 (NIDDM 3) locus has also been mapped to chromosome 20q12-13.1 [19–21].

Given that so much evidence indicates the presence of more than one DR susceptibility gene on chromosome 20 [19, 22], we assessed whether chromosome 20 was also associated with DR development in T2D Taiwanese subjects.

MATERIALS AND METHODS

Subjects

Subjects in this study were recruited from the China Medical University Hospital (CMUH), Taichung, Taiwan. The study was approved by the CMUH institutional review board, and informed consent was obtained from all participants in the study. In total, 749 unrelated individuals, over the age of 20 years, with T2D were recruited for the study. Subjects were diagnosed using the American Diabetic Association Criteria and individuals with type 1 diabetes, gestational diabetes, or maturity-onset diabetes of the young, were excluded.

All T2D subjects underwent a complete ophthalmologic examination, including corrected visual acuity, fundoscopic examination, and fundus photography. Retinopathy status was obtained from the treating ophthalmologist and graded according to the scales for severity of clinical diabetic retinopathy proposed by the American Academy of Ophthalmology [23]. A detailed questionnaire was designed to collect information regarding gender, age at diagnosis of diabetes, and ocular history. For each subject, systolic and diastolic blood pressure, waist and hip circumferences, body mass index, and haemoglobin A1C (HbA1c) levels were determined.

DNA extraction and genotyping

Genomic DNA was extracted from peripheral blood mononuclear cells using a PUREGENE DNA isolation kit (Gentra Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Genotyping, using Illumina HumanHap550-Duo BeadChips (San Diego, CA, USA), was performed by deCODE genetics, Inc., Reykjavík, Iceland. The HumanHap550-Duo BeadChip contained roughly 14 269 SNPs located on chromosome 20, which were selected based on a novel tag SNP approach. Genotype calling was performed using the standard procedure implemented in BeadStudio, with default parameters suggested by the platform manufacturer.

Quality control of the genotype data was performed by examining several summary statistics. The total successful call rate and minor allele frequency in the study group were also calculated for each SNP. SNPs were excluded if they showed one of the following: (i) no polymorphism; (ii) a total call rate of $< 95\%$; or (iii) a minor allele frequency of $< 5\%$. Genotyping validation was performed using the Sequenom iPLEX assay (SEQUENOM MassARRAY system, Sequenom, San Diego, CA, USA).

Statistical analysis

Diabetic retinopathy association analysis was carried out to compare allele frequency and genotype distribution between subjects with and without DR, using three single-point methods: genotype (chi squared test or Fisher's exact test), allele (chi squared test or Fisher's exact test), and trend (Cochran-Armitage test)

Table 1 Summary of SNPs associated with diabetic retinopathy in type 2 diabetes.

dbSNP ID	Chromosome	Position (bases)	Related gene	Risk allele[a] (non-risk allele)	Risk allele frequency (with DR/without DR)	p-value[b] (best model)	$-\log_{10}(p)$
rs761207	20q	42 192 248	*JPH2*	A(G)	0.25/0.18	4.60×10^{-7} (A)	6.34
rs6031415	20q	42 202 723	*JPH2*	A(G)	0.24/0.17	2.24×10^{-7} (T)	6.65
rs761206	20q	42 208 871	*JPH2*	A(C)	0.16/0.12	9.57×10^{-7} (T)	6.02
rs6013574	20q	50 996 467	near *TSHZ2*	T(C)	0.08/0.06	7.95×10^{-8} (A)	7.10
rs6097170	20q	51 002 357	near *TSHZ2*	G(A)	0.09/0.06	8.22×10^{-8} (T)	7.09
rs715064	20q	59 084 122	near LOC100506470	C(T)	0.11/0.07	3.03×10^{-10} (T)	9.52
rs3746780	20q	60 811 731	*NTSR1*	C(T)	0.07/0.05	6.54×10^{-7} (T)	6.18

[a] Risk allele: the allele with higher frequency in subjects with DR compared with subjects without DR.
[b] p-value of the most significant statistic obtained from these 3 models: genotype (G), allele (A), and trend (T) models.

models for each SNP. The most significant test statistic obtained from these 3 models was selected. SNPs with p-values $< 10^{-6}$ ($\alpha = 0.05/14\,269 \times 3$, SNPs on the chromosome 20 times 3 models), established as a cut-off by the Bonferroni correction for multiple comparison, were considered to be significantly associated with the DR.

Characteristics and clinical data of subjects with and without DR were compared by Student's t-test, for continuous variables, and by chi squared test, for categorical variables. Odds ratios (ORs) and 95% confidence intervals (CIs) were determined by multiple logistic regression and were adjusted for diabetes duration and HbA1c level. Statistical analysis was done using the statistical package for the social sciences software package, v18.0 (SPSS Inc., Chicago, IL, USA). Linkage disequilibrium analysis (D' and r^2) between any two loci were performed using the HAPLOVIEW program, v4.1 [24]. D' and r^2 are the two most common measures of linkage disequilibrium. D' is determined by dividing the coefficient of linkage disequilibrium (D) by its maximum possible value, given the allele frequencies at the two loci; r^2 is equal to D^2 divided by the product of the allele frequencies at the two loci.

RESULTS

Characteristics and clinical profiles of the study subjects

Of the 749 T2D subjects enrolled in the study, 174 subjects were diagnosed with DR; 102 of these (59%) had non-proliferative diabetic retinopathy (NPDR) and 72 (41%) had proliferative diabetic retinopathy (PDR). The mean age at diagnosis of T2D of the subjects was 50.2 ± 9.2 years in patients without a diagnosis of DR, and 47.7 ± 9.3 years in those with DR ($p < 0.01$). The mean duration of diabetes in subjects without DR was 8.3 ± 6.5 years, but 14.8 ± 8.3 years in those with DR ($p < 0.001$). The mean HbA1c level of the subjects without DR was $7.7 \pm 1.4\%$, and that in subjects with DR was $8.3 \pm 6.5\%$ ($p < 0.001$) [25].

DR-associated SNPs on chromosome 20

The DR-associated SNPs were selected from those on chromosome 20 showing $-\log_{10}(p\text{-value}) > 6$ under the most significant test statistic obtained from any of the 3 statistical models. As shown in Table 1, of the approximately 14 269 SNPs on chromosome 20, 7 SNPs reached this threshold; these SNPs were all located on the long arm (q arm) of chromosome 20. The SNP showing the strongest association with DR (rs715064) was located on chromosome 20q.13.33 [$-\log_{10}(p\text{-value}) = 9.52$]. This SNP is located in an intergenic region near hypothetical protein LOC100506470. Another 6 associated SNPs (rs761207, rs6031415, and rs761206) are located in the *JPH2* (*junctophilin 2*) gene, while SNP rs3746780 is located in the *NTSR1* (*neurotensin receptor 1*) gene. SNPs rs6013574 and rs6097170 are both located in an intergenic region near *TSHZ2* (*teashirt zinc finger homeobox 2*) gene. The genotypic frequency of susceptibility-associated SNPs in T2D subjects with DR and without DR is presented in Table 2.

Association of DR-associated SNPs adjustment for diabetes duration and HbA1c levels

As DR can vary with the duration of diabetes and the status of glycaemic control, multiple logistic regression analysis of DR susceptibility-associated SNPs was performed in subjects with and without DR, after controlling for the diabetes duration and HbA1c levels (Table 3). After this adjustment, two SNPs in the *JPH2* gene remained significantly associated with DR under the trend model. The risk allele A of rs761207 was associated with a 1.43-fold increase in DR risk (OR, 1.43; 95% CI, 1.04–1.98), while the risk allele A of rs6031415 was associated with a 1.42-fold increase in DR risk (OR, 1.42; 95% CI, 1.02–1.97).

Table 2 Genotypic frequency of susceptibility-associated SNPs in type 2 diabetes subjects with retinopathy and without retinopathy.

dbSNP ID	Related gene	Risk allele (non-risk allele)	SNP allele 1/2	Subjects	Genotypic frequency 1/1	1/2	2/2
rs761207	*JPH2*	A(G)	A/G	DR	12 (6.9)	62 (35.6)	100 (57.5)
				without DR	17 (3.0)	171 (29.9)	384 (67.1)
rs6031415	*JPH2*	A(G)	A/G	DR	11 (6.3)	61 (35.1)	102 (58.6)
				without DR	14 (2.4)	169 (29.5)	390 (68.1)
rs761206	*JPH2*	A(C)	A/C	DR	5 (2.9)	45 (25.9)	124 (71.3)
				without DR	6 (1.0)	121 (21.2)	445 (77.8)
rs6013574	near *TSHZ2*	T(C)	T/C	DR	3 (1.7)	22 (12.6)	149 (85.6)
				without DR	0 (0)	66 (11.5)	507 (88.5)
rs6097170	near *TSHZ2*	G(A)	G/A	DR	3 (1.7)	24 (13.8)	147 (84.5)
				without DR	0 (0)	66 (11.5)	507 (88.5)
rs715064	near LOC100506470	C(T)	C/T	DR	1 (0.6)	36 (20.7)	137 (78.7)
				without DR	2 (0.3)	81 (14.1)	491 (85.5)
rs3746780	*NTSR1*	C(T)	C/T	DR	1 (0.6)	22 (12.6)	151 (86.8)
				without DR	0 (0)	58 (10.1)	515 (89.9)

Table 3 Adjusted odds ratios of diabetic retinopathy susceptibility-associated SNPs in type 2 diabetic subjects, under an adjusted additive genetic model.

Nearest gene	dbSNP ID	Risk allele (non-risk allele)	T2D subjects								
			With DR vs. without DR			With NPDR vs. without DR			With PDR vs. without DR		
			aOR[a]	(95%CI)	p value[b]	aOR	(95%CI)	p value	aOR	(95%CI)	p value
JPH2	rs761207	A(G)	**1.43**	**(1.04–1.98)**	**0.029**	**1.55**	**(1.05–2.28)**	**0.029**	1.43	(0.91–2.25)	0.121
JPH2	rs6031415	A(G)	**1.42**	**(1.02–1.97)**	**0.039**	**1.59**	**(1.07–2.38)**	**0.023**	1.36	(0.85–2.16)	0.197
JPH2	rs761206	A(C)	1.37	(0.96–2.01)	0.113	1.52	(0.97–2.41)	0.069	1.27	(0.73–2.21)	0.408
TSHZ2	rs6013574	T(C)	1.40	(0.83–2.38)	0.212	1.65	(0.91–3.02)	0.101	1.07	(0.48–2.40)	0.864
TSHZ2	rs6097170	G(A)	1.49	(0.88–2.51)	0.140	1.79	(0.99–3.22)	0.054	1.07	(0.48–2.40)	0.864
LOC100506470	rs715064	C(T)	1.50	(0.94–2.41)	0.092	**1.93**	**(1.13–3.30)**	**0.016**	1.09	(0.54–2.22)	0.809
NTSR1	rs3746780	C(T)	1.23	(0.70–2.19)	0.474	1.08	(0.52–2.23)	0.844	1.42	(0.67–3.04)	0.361

[a] aOR: Adjusted odds ratio after controlling diabetic duration and HbA1c.
[b] Adjusted p-values after controlling diabetic duration and HbA1c.

Association of DR-associated SNPs in DR subjects with non-proliferative DR or proliferative DR

We subsequently classified the T2D subjects with DR into NPDR and PDR, according to the DR severity scales (Table 3). We used the same trend model to analyse the DR susceptibility-associated SNPs in subjects with NPDR versus those without DR, or with PDR versus those without DR. The results showed that 3 SNPs significantly associated with DR in the NPDR group, but none did so in the PDR group. Two of the significant SNPs were those associated with DR per se above, located in the *JPH2* gene, while one was located in an intergenic region near the hypothetical protein LOC100506470. In the NPDR group, the risk alleles (A in both cases) of rs761207 and rs6031415 in *JPH2* were associated with a 1.55-fold (95% CI, 1.05–2.28) and 1.59-fold (95% CI, 1.07–2.38) increase, and the risk allele C of rs715064, near LOC100506470, was associated with 1.93-fold (95% CI, 1.13–3.30) increase.

Haplotype analysis of SNPs in the *JPH2* gene

Subsequently, we performed haplotypes analysis of these 3 SNPs, rs761207, rs6031415, and rs761206, in the *JPH2* gene (Table 4). This demonstrated that the latter 2 SNPs form a single haplotype block. These SNPs were in strong linkage disequilibrium with each other ($D' = 0.94$; $r^2 = 0.558$). Three *JPH2* haplotypes, comprising rs6031415 and rs761206, with frequencies of more than 1%, accounted for approximately 99% of all haplotypes in both subjects with DR and those without DR. As shown in Table 4, the frequency of the A-A haplotype (rs6031415-rs761206) was significantly higher in subjects with DR than in those without DR. Compared with the most common G-C haplotype, the A-A haplotype exhibited a 1.53-fold increase in DR risk (OR = 1.53, 95% CI = 1.08–2.26).

DISCUSSION

Here, we report the results of a study designed to identify genetic variants on chromosome 20 that influence DR development in Taiwanese subjects with

Table 4 Distribution of *JPH2* haplotype frequencies in T2D subjects with DR and without DR.

Haplotype[a]	T2D subjects		OR	(95% CI)
	with DR (%)	without DR (%)		
G-C	75.7	82.2	1.00	(reference)
A-A	15.5	11.0	**1.53**	**(1.08–2.26)**
A-C	8.4	6.2	1.46	(0.93–2.29)

[a] Order of SNPs comprising the *JPH2* haplotypes: rs6031415-rs761206.

T2D. We identified 7 SNPs on this chromosome that showed significant association with DR. We also showed, for the first time, that a strong association exists between *JPH2* and DR, particularly in the early NPDR phase, which is independent of diabetic duration and glycaemic control status in the multiple logistic regression models.

SNP rs3746780 is located in a gene that encodes NTSR1; this G-protein-coupled receptor is a high affinity neurotensin receptor with 7 transmembrane-spanning regions that activates a phosphatidylinositol-calcium second messenger system[26,27]. Two SNPs, rs6013574 and rs6097170, are located in an intergenic region near the *TSHZ2* gene. *TSHZ2* encodes a Teashirt-family zinc finger protein, which is a transcriptional regulator and is involved in developmental processes[28,29]. Nevertheless, these SNPs are not significantly associated with DR after controlling for diabetes duration and HbA1c levels; thus the role of these genes in DR pathogenesis awaits further clarification.

Two SNPs remained significantly associated with DR, independent of diabetic duration and HbA1c levels, viz., rs761207 and rs6031415; these SNPs are both located in *JPH2* on chromosome 20q13.12. JPH2 is a member of the junctophilin family and is the predominant isoform of this protein in cardiac tissue, but is also expressed, along with JPH1, in skeletal muscle[30]. JPH2 plays a key role in the organization of junctional membrane complexes (JMCs), which are a common feature of all excitable cell types, by linking the membrane of the endoplasmic/sarcoplasmic reticulum to the plasma membrane; thus it mediates cross-talk between the cell surface and intracellular ion channels[31]. JPH2 is believed to keep the plasma membrane and endoplasmic/sarcoplasmic reticulum at a fixed distance within the JMC, which is essential for proper Ca^{2+}-induced Ca^{2+}-release during cellular signalling. Moreover, previous studies have shown that knock-out of *JPH2* in mice causes embryonic lethality, due to the wider gap size of the JMCs and deficient $[Ca^{2+}]_i$-transients in cardiomyocytes[31].

Another link between Ca^{2+} flux and DR has recently been noted, with the finding that vascular endothelial growth factor (VEGF)-induced vascular permeability is dependent on Ca^{2+} influx. Diabetic microvascular changes in the retina lead to hypoxia, which stimulates production of VEGF, a regulator of angiogenesis and microvascular permeability[32] that is believed to play a significant role in the development of DR[33]. VEGF stimulates angiogenesis[34], through endothelial cell migration, tube formation, and proliferation, which is mediated, at least in part, through VEGF-receptor-mediated Ca^{2+}-influx into the endothelial cell[35]. Given that VEGF is a mediator of Ca^{2+}-entry and -release in endothelia, modifying vascular permeability[36,37], it seems reasonable that *JPH2*, an essential regulator of Ca^{2+}-transients, is a plausible candidate gene for DR development.

One other SNP, rs715064, was found to be associated only with NPDR; it is located in an intergenic region around the hypothetical protein LOC100506470. However, it is uncertain whether this locus is, in fact, involved in DR, or whether this association is merely a reflection of linkage disequilibrium between this SNP and other functional loci.

In the present study, we did not confirm the previously reported list of potential candidate genes for DR on chromosome 20q[14], perhaps because of differences in ethnicity or analysis strategies between the studies. However, it is interesting that *JPH2* is located in the region of chromosome 20q13.12, near *KCNS1* and *HNF4A*, which were previously implicated in DR (Fig. 1). This suggests that the long arm of chromosome 20 may harbour a number of genes conferring susceptibility to DR. Here, subjects without DR had a shorter mean duration of diabetes and a lower mean HbA1c level compared with those with DR. This may be considered to be a limitation to our study. However, by adjusting for these factors in the multiple regression analyses, any influences these factors may have had on the results has been accounted for. The other limitation of this study is the sample size of proliferative DR subjects. The similarities in the odds ratios between patients with non-proliferative DR and proliferative DR suggest that the apparent differences in association between these two groups might result from the smaller number of patients in the proliferative DR group. Future studies with a larger number of subjects are needed to confirm these findings.

In conclusion, we have shown for the first time that the *JPH2* gene influences the risk of developing DR in the Taiwanese population. Although this finding requires further study for confirmation in a

different cohort or a larger sample size, it is interesting to speculate that our findings may indicate a novel pathway in the pathogenesis of DR.

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