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

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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 junctionophilin 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, junctionophilin 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
Potential candidate genes for diabetic retinopathy on the long arm of chromosome 20. These include E2F1, ASIP, TGM2, KCNS1, and HNF4A. 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 DR11–13.

To date, genome-wide linkage studies have suggested the presence of potential candidate genes for DR on the long arm of chromosome 2014. 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 disease15,16. 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 T2D17,18. In addition, the putative non-insulin-dependent diabetes mellitus 3 (NIDDM 3) locus has also been mapped to chromosome 20q12-13.119–21.

Given that so much evidence indicates the presence of more than one DR susceptibility gene on chromosome 2019,22, 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 Ophthalmology23. 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., Reykjavik, 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.

<table>
<thead>
<tr>
<th>rsSNP ID</th>
<th>Chromosome</th>
<th>Position (bases)</th>
<th>Related gene</th>
<th>Risk allelea (non-risk allele)</th>
<th>Risk allele frequency (with DR/without DR)</th>
<th>p-valueb (best model)</th>
<th>(-\log_{10}(p))</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs761207</td>
<td>20q</td>
<td>42 192 248</td>
<td>JPH2</td>
<td>A(G)</td>
<td>0.25/0.18</td>
<td>6.40 \times 10^{-7}</td>
<td>6.34</td>
</tr>
<tr>
<td>rs6031415</td>
<td>20q</td>
<td>42 202 723</td>
<td>JPH2</td>
<td>A(G)</td>
<td>0.24/0.17</td>
<td>2.24 \times 10^{-7}</td>
<td>6.65</td>
</tr>
<tr>
<td>rs761206</td>
<td>20q</td>
<td>42 208 871</td>
<td>JPH2</td>
<td>A(C)</td>
<td>0.16/0.12</td>
<td>9.57 \times 10^{-7}</td>
<td>6.02</td>
</tr>
<tr>
<td>rs6013574</td>
<td>20q</td>
<td>50 996 467</td>
<td>near TSH2</td>
<td>T(C)</td>
<td>0.08/0.06</td>
<td>7.95 \times 10^{-8}</td>
<td>7.10</td>
</tr>
<tr>
<td>rs6097170</td>
<td>20q</td>
<td>51 002 357</td>
<td>near TSH2</td>
<td>G(A)</td>
<td>0.09/0.06</td>
<td>8.22 \times 10^{-8}</td>
<td>7.09</td>
</tr>
<tr>
<td>rs715064</td>
<td>20q</td>
<td>59 084 122</td>
<td>near LOC100506470</td>
<td>C(T)</td>
<td>0.11/0.07</td>
<td>3.03 \times 10^{-10}</td>
<td>9.52</td>
</tr>
<tr>
<td>rs3746780</td>
<td>20q</td>
<td>60 811 731</td>
<td>NTSR1</td>
<td>C(T)</td>
<td>0.07/0.05</td>
<td>6.54 \times 10^{-7}</td>
<td>6.18</td>
</tr>
</tbody>
</table>

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 (\(\alpha = 0.05/14 \times 20 \times 3 = 3,\) 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 (\(D^2\) and \(r^2\)) between any two loci were performed using the HAPLOVIEW program, v4.1. \(D^2\) and \(r^2\) are the two most common measures of linkage disequilibrium. \(D^2\) 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 ± 1.4%, and that in subjects with DR was 8.3 ± 6.5% (\(p < 0.001\)).

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,3.3 [\(-\log_{10}(p\text{-value}) = 9.52\)]. This SNP is located in an intergenic region near the 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 some 20q.

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.

<table>
<thead>
<tr>
<th>dbSNP ID</th>
<th>Related gene</th>
<th>Risk allele (non-risk allele)</th>
<th>SNP allele</th>
<th>Subjects</th>
<th>Genotypic frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs761207</td>
<td>JPH2</td>
<td>A(G)</td>
<td>A/G</td>
<td>1/1</td>
<td>12 (6.9) 62 (35.6) 100 (57.5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1/2</td>
<td>without DR</td>
</tr>
<tr>
<td>rs761206</td>
<td>JPH2</td>
<td>A(C)</td>
<td>A/C</td>
<td>1/2</td>
<td>DR</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1/2</td>
<td>without DR</td>
</tr>
<tr>
<td>rs6013574</td>
<td>near TSHZ2</td>
<td>T(C)</td>
<td>T/C</td>
<td>1/2</td>
<td>DR</td>
</tr>
<tr>
<td>rs6097170</td>
<td>near TSHZ2</td>
<td>G(A)</td>
<td>G/A</td>
<td>1/2</td>
<td>without DR</td>
</tr>
<tr>
<td>rs715064</td>
<td>near LOC100506470</td>
<td>C(T)</td>
<td>C/T</td>
<td>1/2</td>
<td>DR</td>
</tr>
<tr>
<td>rs3746780</td>
<td>NTSR1</td>
<td>C(T)</td>
<td>C/T</td>
<td>1/2</td>
<td>without DR</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Nearest gene</th>
<th>dbSNP ID</th>
<th>Risk allele (non-risk allele)</th>
<th>With DR vs. without DR aOR (95%CI) p value</th>
<th>With NPDR vs. without DR aOR (95%CI) p value</th>
<th>With PDR vs. without DR aOR (95%CI) p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>JPH2</td>
<td>rs761207</td>
<td>A(G)</td>
<td>1.43 (1.04–1.98) 0.029</td>
<td>1.55 (1.05–2.28) 0.029</td>
<td>1.43 (0.91–2.25) 0.121</td>
</tr>
<tr>
<td>JPH2</td>
<td>rs6031415</td>
<td>A(G)</td>
<td>1.42 (1.02–1.97) 0.039</td>
<td>1.59 (1.07–2.38) 0.023</td>
<td>1.36 (0.85–2.16) 0.197</td>
</tr>
<tr>
<td>JPH2</td>
<td>rs761206</td>
<td>A(C)</td>
<td>1.37 (0.96–2.01) 0.113</td>
<td>1.52 (0.97–2.41) 0.069</td>
<td>1.27 (0.73–2.21) 0.408</td>
</tr>
<tr>
<td>TSHZ2</td>
<td>rs6013574</td>
<td>T(C)</td>
<td>1.40 (0.83–2.38) 0.212</td>
<td>1.65 (0.91–3.02) 0.101</td>
<td>1.07 (0.48–2.40) 0.864</td>
</tr>
<tr>
<td>LOC100506470</td>
<td>rs715064</td>
<td>G(A)</td>
<td>1.49 (0.88–2.51) 0.140</td>
<td>1.79 (0.99–3.22) 0.054</td>
<td>1.07 (0.48–2.40) 0.864</td>
</tr>
<tr>
<td>NTSR1</td>
<td>rs3746780</td>
<td>C(T)</td>
<td>1.50 (0.94–2.41) 0.092</td>
<td>1.93 (1.13–3.30) 0.016</td>
<td>1.09 (0.54–2.22) 0.809</td>
</tr>
</tbody>
</table>

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
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 domains. Its activation by neurotensin, a neuropeptide released from the intestinal tract, is believed to play a significant role in the development of diabetes and vascular diseases. The *JPH2* gene encodes a Teashirt-family zinc finger protein, which is a transcriptional regulator and is involved in developmental processes. 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. *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. *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$^{2+}$],-transients in cardiomyocytes.

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, that is believed to play a significant role in the development of DR. VEGF stimulates angiogenesis, 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. Given that VEGF is a mediator of Ca$^{2+}$-entry and -release in endothelia, modifying vascular permeability, 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 20q14, 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

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

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>T2D subjects with DR (%)</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>without DR (%)</td>
<td></td>
</tr>
<tr>
<td>G-C</td>
<td>75.7</td>
<td>82.2</td>
</tr>
<tr>
<td>A-A</td>
<td>15.5</td>
<td>11.0</td>
</tr>
<tr>
<td>A-C</td>
<td>8.4</td>
<td>6.2</td>
</tr>
</tbody>
</table>

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