Impact of Genetic Loci Identified in Genome-Wide Association Studies on Diabetic Retinopathy in Chinese Patients With Type 2 Diabetes

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Purpose. Diabetic retinopathy (DR) is a common microvascular complication of type 2 diabetes (T2DM). Genome-wide association studies (GWAS) had identified novel DR-susceptibility genetic variants in various populations. We examined the associations of these DR-associated single nucleotide polymorphisms (SNPs) with severe DR in a Chinese T2DM cohort.

Methods. Cross-sectional case-control studies on sight-threatening DR (STDR) and proliferative DR (PDR) were performed. We genotyped 38 SNPs showing top association signals with DR in previous GWAS in 567 STDR cases, including 309 with PDR and 1490 non-DR controls. Multiple logistic regression models with adjustment for conventional risk factors, including age, sex, duration of diabetes, and presence of hypertension, were employed.

Results. The strongest association was found at *INSR* rs2115386, an intronic SNP of *INSR*: $P_{adjusted} = 9.13 \times 10^{-4}$ (odds ratio [OR],1.28; 95% confidence interval [95%CI], 1.11–1.48) for STDR, and $P_{adjusted} = 1.12 \times 10^{-4}$ (OR [95%CI],1.44 [1.20–1.74]) for PDR. rs599019 located downstream of *COLEC12* ($P_{adjusted} = 0.019$; OR [95%CI],1.19 [1.03–1.38]) and rs4462262 located at an intergenic region between *ZWINT* and *MRPS35P3* ($P_{adjusted} = 0.041$; OR [95%CI],1.38[1.01–1.89]) also were significantly associated with STDR, but not with PDR alone. On the other hand, *MYT1L-LOC729897* rs10199521 ($P_{adjusted} = 0.022$; OR [95%CI],1.25 [1.03–1.51]) and *API5* rs899036 ($P_{adjusted} = 0.049$; OR [95%CI],1.36 [1.00–1.85]) showed significant independent associations only with PDR. Similar results were obtained when hemoglobin A1c also was included in the adjustment models.

Conclusions. We demonstrated the significant and independent associations of several GWAS-identified SNPs with DR in Chinese T2DM patients with severe DR. The findings on *INSR* rs2115386 are supportive of the role of insulin resistance, or the compensatory hyperinsulinemia, in the pathogenesis of DR.

Keywords: diabetic retinopathy, sight-threatening diabetic retinopathy, proliferative diabetic retinopathy, genome-wide association studies, genetic variants, *INSR*

Type 2 diabetes mellitus (T2DM) is a chronic disease currently posing huge burdens on individual and public health worldwide. This disease has been considered as a major source of morbidity and mortality attributable to its associated acute or chronic complications. Diabetic retinopathy (DR) is one of the most common chronic microvascular complications of T2DM and is the leading cause of irreversible loss of vision among working populations in developed countries.[1] Prevalence of DR in the Caucasian populations has been estimated to range from 19% to 30% among subjects with T2DM.[2–5] A recent large-scale systematic screening study for DR, using the English National Screening Program grading standard,[6] reported a high prevalence of DR in 39% of the Hong Kong Chinese diabetic population.[7] The prevalence of the more advanced stage of DR, sight-threatening DR (STDR), which is defined as the presence of either preproliferative DR, proliferative DR (PDR), or maculopathy, was found to have reached 9.8%.[7]

Over the past few decades, extensive epidemiologic studies have identified important risk factors for DR, such as duration of diabetes, hypertension (HT), and inadequate control of

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glycemia. However, clinical studies reveal a substantial variation in the onset and severity of retinopathy, which is not fully explained by these known risk factors.[8,9] Indeed, some patients with poor control of glycemia or blood pressure (BP) may be found after long diabetes duration to have no DR.[10] These data suggest that genetic factors also have a role in the development of DR. The heritability of DR has been estimated to be approximately 25%.[11,12] Understanding the genetic basis of DR will help to identify the underlying pathophysiologic mechanisms. This genetic information also may contribute to the risk profiling of DR in diabetic patients, thus facilitating their early diagnosis and disease management. Robust associations of the DR-susceptibility variants would serve as the basis for their use as genetic markers to supplement the prediction by conventional clinical predictors for more accurate risk stratification. Advancing from the traditional candidate gene approach, which focused only on a small number of potential candidates implicated in the pathogenesis of DR, the genome-wide association studies (GWAS) have provided an alternative strategy for detecting novel genetic loci for DR. Several GWAS in various ethnic groups have identified novel susceptibility genetic variants for DR in T1DM and T2DM cohorts.[13–16] So far, only a few replication studies have been conducted to validate these novel associations.[17–20] Whether these DR-associated variants identified in previous GWAS all show an impact on the development of DR in the Chinese population has not been systematically investigated. The primary objective of the present study was to evaluate the associations of the GWAS identified DR-associated single nucleotide polymorphisms (SNPs) with severe DR in Hong Kong Chinese patients with T2DM.

Subjects and Methods

Subjects

Two cross-sectional case-control studies on STDR and PDR were performed in a total of 2057 Southern Chinese patients with T2DM. This study involved 1490 non-DR controls and 567 STDR cases, including 309 subjects who had PDR. Type 2 diabetes mellitus was defined as fulfilling at least one of the following criteria, according to the American Diabetes Association 2008 diagnostic criteria:[21] fasting plasma glucose (FPG) $\geq$ 7 mmol/l, 2-hour glucose during oral glucose tolerance test (OGTT) $\geq$ 11.1 mmol/l, showing symptoms of hyperglycemia and a causal plasma glucose $\geq$ 11.1 mmol/l, or on antidiabetic treatment (insulin or other glucose-lowering medications). All non-DR controls ($n = 1490$) were recruited from the Hong Kong West Diabetes Registry (HKWDR).[22] Unrelated T2DM patients, who were on regular follow-up at the diabetes clinics of the Queen Mary Hospital, were invited to participate in the HKWDR, which was commenced in 2008. At 12- to 18-month intervals, the participants underwent extensive medical assessments and laboratory examinations to assess for the presence of diabetic complications, including DR as determined by the 2-field digital fundus photographs assessed by specialist ophthalmologists. The STDR cases in this study were recruited from the HKWDR, ophthalmology clinics at Queen Mary Hospital, Tseung Kwan O Hospital, and United Christian Hospital, Hong Kong. At assessment, detailed medication, and medical and family histories were recorded using a standardized questionnaire; anthropometric (weight, height, waist circumference) and clinical (age, sex, BP, and hemoglobin A1c [HbA1c]) data were collected. Duration of diabetes was defined as the difference between the time at diagnosis and time at ascertainment of STDR. Hypertension was defined as BP $\geq$ 140/90 mm Hg or taking antihypertensive drugs. After an overnight fast of at least 8 hours, blood samples for biochemical and genetic analysis were drawn with written informed consents were obtained. Ethical approvals were obtained from the Institutional Review Boards (IRB) of the 3 hospitals (IRB references: UW 07-378; UW 13-237; KC/KE-13-0087/FR-1). All study procedures of this research were in accordance with the Declaration of Helsinki.

Phenotype Characterization

The current study included Chinese T2DM patients either without retinopathy or with STDR, determined on the basis of digital, color fundal photographs taken with fundus cameras (TRC50-DX type 1A; Topcon, Tokyo, Japan) with 2 photographic fields (45°) for each eye (one centered at the macula and the other centered at the optic disc). Visual acuity was assessed with the Early Treatment of Diabetic Retinopathy Study (ETDRS) chart using the auto-chart projector (Auto-Chart Projector ACP-7EM; Topcon). All STDR cases have been assessed systematically by specialist ophthalmologists who determined the presence and graded the severity of DR according to the English National Screening Program guidelines.[6] Cases with STDR were defined as patients with either pre-PDR (graded R2), PDR (graded R3), or showing features of maculopathy (graded M1).[7] Non-DR controls were T2DM patients without retinopathy (graded R0). Subjects with background DR (graded R1) or ungradable fundus photographs were excluded from the current study.

Genetic Analysis

We selected 38 previously reported DR-associated SNPs to examine for their associations with severe DR (STDR and PDR) in a Southern Chinese population. These SNPs have shown top association signals with $P < 5 \times 10^{-4}$ as reported in at least one of the available GWAS[13–16] published before the commencement of genotyping in June 2014. For the reported SNPs which showed strong linkage disequilibrium (LD; $r^2 > 0.9$) on the 1000 Genome Project for Southern Chinese, only one representative SNP with stronger association was selected for genotyping. Single nucleotide polymorphisms that were monomorphic or with minor allele frequency (MAF) < 1% in the Southern Chinese population were not included in the current study. Genomic DNA for genetic analysis was extracted using the ReliaPrep Blood gDNA Miniprep System (Promega, Madison, WI, USA) extraction kits according to the manufacturer's instructions. All SNPs were genotyped using the Sequenom iPLEX Gold genotyping platform at the Center for Genomic Sciences of the University of Hong Kong. Four SNPs that were incompatible with the Sequenom multiplexing design were replaced by a corresponding proxy SNP (with $r^2 > 0.8$) for genotyping (rs2038823 replaced by rs16953072, rs1970671 replaced by rs1125313, rs10910200 replaced by rs6662352, and rs11867934 replaced by rs117421492). Genotyping of rs9565164 and rs1399634 was unsuccessful and was not included in the analyses. Hardy-Weinberg Equilibrium (HWE) for each SNP was examined by the exact test using PLINK version 1.09.[23] To ensure the accuracy of genotype data, we excluded 3 SNPs (rs4470583, rs13163610, and rs6909083) that showed deviation from HWE ($P < 0.05/38$ SNPs $= 1.3 \times 10^{-3}$), and 4 SNPs (rs117421492, rs1445754, rs1125313, and rs2380261) with a call rate of less than 90%, from further analyses. After quality control, all 3 variants (rs96565164, rs1399634, and rs2380261) identified in a GWAS[16] conducted by Sheu et al.[16] were excluded for the reasons mentioned above. Thus, for the final analysis, a total of 29 SNPs that were identified from the remaining GWAS[13–15]

Table 1. Clinical Characteristics of Study Participants

Variables	Non-DR	STDR	PDR
Number	1490	567	309
Age, y	62.79 ± 12.76	62.62 ± 11.17	59.92 ± 11.16*
Diabetes duration, y†	7 (3–12)	14 (6–23)*	14 (5–23)*
Sex, male; %	56.6	57.0	58.6
HT, %	82.9	95.0*	94.8*
SBP,‡ mm Hg	144.42 ± 21.70	151.78 ± 22.04*	153.18 ± 20.92*
DBP,‡ mm Hg	78.79 ± 10.09	79.08 ± 11.33	79.97 ± 11.54
HbA1c, %†	7.1 (6.5–7.8)	7.5 (6.8–8.6)*	7.5 (6.7–8.5)*
BMI, kg/m^2	26.27 ± 4.15	25.70 ± 4.0	26.11 ± 4.24
WC, cm	M: 92.17 ± 9.88	M: 90.36 ± 9.80	M:90.66 ± 10.17
	F: 86.15 ± 11.07	F: 87.45 ± 11.06	F:88.55 ± 11.66
Dyslipidemia, %	68.9	67.0	68.9
Ever smoke, %	33.6	33.7	36.6
Ever drink, %	39.1	35.8	35.6

Hypertension was defined as BP ≥ 140/9 mm Hg or taking antihypertensive drugs. BMI, body mass index; WC, waist circumference; SBP, systolic blood pressure; DBP, diastolic blood pressure.

* Significant association ($P < 0.05$) after correction for multiple comparison.

† Natural-log transformed before analysis.

‡ SBP + 10 mm Hg and DBP + 5 mm Hg if on antihypertensive drugs. Dyslipidemia was indicated by a documented history of dyslipidemia in patients' records or taking lipid-lowering drugs.

and passed quality measures were examined. The average successful call rate of SNPs that passed quality control was 99.0% and the concordant rate was 99.98% based on 20 duplicated samples.

Statistical Analysis

All statistical analyses were conducted using PLINK version 1.09[23] and IBM SPSS Statistics 21. All continuous variables were summarized as mean ± SD or median with interquartile range as appropriate. Variables that did not follow a normal distribution as reflected by a significant P value in the Kolmogorov-Smirnov test were natural-logarithmically transformed before analyses. Continuous and categorical clinical parameters were compared between the case and control groups by 1-way ANOVA and the χ^2 test, respectively. The associations between the SNPs and severe DR were examined by the multiple logistic regression analyses with adjustment for the conventional risk factors, including age, sex, duration of diabetes and the presence of HT (Model 1), under an additive model. Hemoglobin A1c also was included as an additional covariate in Model 2. A 2-tailed $P < 0.05$ was considered as statistically significant. Bonferroni correction for multiple testing was used to correct for multiple comparison. The power of study was calculated using the Genetic Power Calculator (available in the public domain at http://pngu.mgh.harvard.edu/~purcell/gpc/).[24]

Results

Association With STDR

A total of 29 DR-associated SNPs identified in previous GWAS[13–15] was successfully genotyped in 1490 non-DR controls and 567 STDR cases, among whom 309 had PDR. Table 1 shows the clinical characteristics of the study participants. As expected, the subjects with STDR or PDR had a longer duration of diabetes, more of them also were affected with HT, and they had greater HbA1c when compared to the non-DR subjects.

Assuming an STDR prevalence of 9.8%,[7] the current sample size was sufficient to achieve over 80% power for detecting significant associations of SNPs with MAF ≥ 0.2 and effect sizes greater than 1.25, at a significance level of 0.05. On the other hand, our study was underpowered to detect associations of SNPs with modest effect sizes and lower MAF (See Supplementary Fig. S1). Table 2 shows the association analyses with STDR. Of the 29 SNPs, 13 showed consistent direction of effect with the previous reports, including the strongest association at an intronic SNP rs2115386 of the insulin receptor (*INSR*) gene. rs2115386 was significantly associated with STDR after adjustment for the conventional confounding factors including age, sex, duration of diabetes, and the presence of HT ($P_{adjusted} = 9.13 \times 10^{-4}$; odds ratio [OR], 1.28; 95% confidence interval [95%CI], 1.11–1.48). This association survived the multiple testing corrections. rs599019 located downstream of *COLEC12* ($P_{adjusted} = 0.019$; OR [95%CI], 1.19 [1.03–1.38]) and an intergenic SNP rs4462262 located at the *ZWINT-MRPS35P3* locus ($P_{adjusted} = 0.041$; OR [95%CI], 1.38 [1.01–1.89]) also showed significant associations with STDR, but were unable to survive the stringent Bonferroni correction for multiple testing. All these associations remained significantly associated with STDR when HbA1c also was included in the adjustment model (*INSR* rs2115386, $P_{adjusted} = 7.18 \times 10^{-4}$; *COLEC12* rs599019, $P_{adjusted} = 9.09 \times 10^{-3}$; *ZWINT-MRPS35P3* rs4462262, $P_{adjusted} = 0.031$). Supplementary Table S1 shows the comparison of association results between the current study and the original reports.

Association With PDR

We further examined the associations of these SNPs with PDR. Table 3 shows the association analyses with PDR. The *INRS* rs2115386 again showed a significant association with PDR even after adjustment for the confounding factors ($P_{adjusted} = 1.12 \times 10^{-4}$; OR [95%CI], 1.44 [1.20–1.73]) and this association was able to survive the correction for multiple testing. However, *COLEC12* rs599019 and *ZWINT-MRPS35P3* rs4462262, which were significantly associated with STDR, failed to show a significant association with the advanced stage PDR alone. On the other hand, an intergenic SNP rs10199521 at the *MYT1L-LOC729897* locus ($P_{adjusted} = 0.022$; OR [95%CI], 1.25 [1.03–1.51]) and an intronic variant of *API5* rs899036 ($P_{adjusted} = 0.049$; OR [95%CI], 1.36 [1.00–1.85]) only showed significant associations with PDR. When HbA1c was included in the adjustment model, *INSR* rs2115386 ($P_{adjusted} = 1.23 \times$

Table 2. Association Analysis of STDR

Nearest Gene(s)	SNP	CHR	Position	A1	A2	MAF		Model 1		Model 2	
						No-DR	STDR	OR (95%CI)	$P_{adjusted}$	OR (95%CI)	$P_{adjusted}$
IGSF21-KLHDC7A	rs3007729*	1	18795255	T	C	0.32	0.31	0.90 (0.76–1.05)	0.183	0.91 (0.77–1.07)	0.243
MYSM1	rs2811893†	1	59162148	C	T	0.34	0.35	1.02 (0.88–1.19)	0.788	0.98 (0.83–1.14)	0.762
SCYL1BP1	rs6427247‡§	1	170380480	G	A	0.25	0.26	1.03 (0.88–1.22)	0.702	1.03 (0.87–1.22)	0.715
TNFSF4-LOC730070	rs1342038*	1	173301516	C	T	0.42	0.42	0.97 (0.84–1.13)	0.734	0.97 (0.83–1.13)	0.675
KIAA1804-KCNK1	rs6662352*‖	1	233642172	T	G	0.35	0.34	0.94 (0.80–1.10)	0.423	0.93 (0.79–1.09)	0.372
AKT3-ZNF238	rs10927101*	1	244173872	C	A	0.28	0.30	1.09 (0.92–1.28)	0.318	1.09 (0.92–1.28)	0.325
AKT3-ZNF238	rs476141*	1	244176424	A	C	0.20	0.19	0.91 (0.76–1.10)	0.343	0.91 (0.75–1.10)	0.323
MYT1L-LOC729897	rs10199521*§	2	2519513	T	C	0.35	0.37	1.11 (0.95–1.29)	0.182	1.12 (0.96–1.31)	0.144
LINC01249	rs699549‡	2	4705263	T	C	0.23	0.22	0.91 (0.77–1.09)	0.315	0.93 (0.78–1.11)	0.399
HNMT	rs763970‡§	2	138636133	A	C	0.30	0.31	1.03 (0.89–1.21)	0.675	1.05 (0.89–1.23)	0.564
BFSP2	rs1197310‡	3	133128224	A	T	0.49	0.49	1.02 (0.88–1.18)	0.786	1.00 (0.86–1.16)	0.989
LEKR1-CCN1	rs13064954*§	3	156854742	A	G	0.08	0.09	0.98 (0.76–1.27)	0.874	1.04 (0.80–1.35)	0.786
KRT18P34-VEPH1	rs9866141*§	3	156950579	T	C	0.08	0.09	1.14 (0.88–1.48)	0.336	1.15 (0.88–1.50)	0.308
C5orf36	rs17376456†§	5	93557702	G	A	0.05	0.04	0.76 (0.53–1.11)	0.155	0.84 (0.58–1.22)	0.354
CAMK4	rs2300782‡	5	110788785	T	C	0.41	0.39	0.91 (0.78–1.05)	0.200	0.90 (0.77–1.04)	0.162
C6orf170	rs17083119‡	6	121402110	G	A	0.12	0.11	0.89 (0.71–1.13)	0.348	0.91 (0.72–1.16)	0.445
LOC729200-MAP3K7IP2	rs7772697*	6	149435111	C	T	0.18	0.18	1.07 (0.88–1.30)	0.481	1.05 (0.86–1.28)	0.617
CREB5	rs11765845*	7	28391142	A	G	0.27	0.26	0.95 (0.8–1.12)	0.513	0.92 (0.78–1.10)	0.360
PLXDC2-NEBL	rs12219125†§	10	20593087	T	G	0.11	0.12	1.02 (0.81–1.29)	0.863	1.08 (0.85–1.36)	0.539
ARHGAP22	rs4838605†§	10	49699957	C	T	0.10	0.10	1.02 (0.80–1.30)	0.857	1.10 (0.86–1.41)	0.438
ARHGAP22	rs11101357†	10	49723300	A	G	0.10	0.10	0.99 (0.77–1.27)	0.953	1.07 (0.83–1.38)	0.582
ZWINT-MRPS35P3	**rs4462262†**	**10**	**59189178**	**T**	**C**	**0.05**	**0.06**	**1.38 (1.01–1.89)**	**0.041**	**1.41 (1.03–1.93)**	**0.031**
API5	rs899036‡	11	41682910	C	A	0.08	0.1	1.14 (0.88–1.48)	0.310	1.14 (0.88–1.47)	0.336
CNTN5	rs10501943‡§	11	99946999	C	T	0.03	0.03	1.41 (0.90–2.20)	0.131	1.46 (0.92–2.30)	0.106
HS6ST3	rs16953072†§‖	13	96950313	T	G	0.05	0.05	0.96 (0.68–1.36)	0.827	0.99 (0.70–1.41)	0.975
FMN1	rs10519765‡§	15	33205424	A	G	0.11	0.09	0.80 (0.63–1.02)	0.070	0.82 (0.64–1.05)	0.111
COLEC12	**rs599019‡**	**18**	**294495**	**C**	**A**	**0.46**	**0.49**	**1.19 (1.03–1.38)**	**0.019**	**1.22 (1.05–1.42)**	**9.09×10^{-3}**
INSR	**rs2115386*§**	**19**	**7196565**	**C**	**T**	**0.47**	**0.53**	**1.28 (1.11–1.48)**	**$\underline{9.13 \times 10^{-4}}$**	**1.29 (1.11–1.50)**	**$\underline{7.18 \times 10^{-4}}$**
VSTM2B-POP4	rs10403021*§	19	30079604	T	C	0.28	0.28	0.98 (0.83–1.15)	0.761	1.00 (0.85–1.18)	0.991

Single nucleotide polymorphisms ranked by chromosomal position. Single nucleotide polymorphisms showed statistically significant association with STDR are highlighted in bold. Chromosomal position corresponds to human reference genome hg19. Odds ratio corresponds to the minor allele (A1). Model 1: Adjusted for age, sex, duration of diabetes and presence of HT. Model 2: Adjusted for age, sex, duration of diabetes, presence of HT and HbA1c. CHR, chromosome; A1, minor allele; A2, major allele; MAF, minor allele frequency.

* Significantly associated SNPs in Reference 14.

† Significantly associated SNPs in Reference 15.

‡ Significantly associated SNPs in Reference 13.

§ Single nucleotide polymorphisms showing consistent direction of effect as in the original reports. Single nucleotide polymorphism remained significant after Bonferroni correction for multiple testing is underlined.

‖ rs6662352 is the proxy SNP of rs10910200 ($r^2 = 0.99$) and rs16953072 is the proxy SNP of rs2038823 ($r^2 = 1$).

10^{-4}) and *MYT1L-LOC729897* rs10199521 ($P_{adjusted} = 0.016$) also remained significantly associated with PDR, but *API5* rs899036 ($P_{adjusted} = 0.058$) was unable to reach statistical significance after the adjustment.

Discussion

In the present study, we evaluated the associations of 29 previously reported DR-associated SNPs with STDR and PDR in a total of 2057 Chinese patients with T2DM. We successfully confirmed the associations of several previously reported DR-susceptibility genetic variants in our population, including *INSR* rs2115386, which remained significantly and independently associated with STDR and PDR even after the stringent Bonferroni correction for multiple testing.

Among the studied loci, the strongest association was found at the *INSR* locus. This association could be biologically relevant, since *INSR* encodes the insulin receptor which has a key role in insulin signaling in insulin-sensitive tissues, including retina.[25] In the retina, it has been shown that disrupted insulin receptor signaling leads to cell dysfunction,[25] and insulin provides trophic support for retinal neurons through a PI3-kinase/Akt-dependent pathway.[26] Animal studies also have demonstrated that diabetes progressively impaired the constitutive retinal insulin receptor/Akt pro-survival signaling.[27] On the other hand, insulin receptors on retinal endothelial cells have been shown to regulate the expression of vascular mediators, such as VEGF and have been implicated in the control of retinal endothelial cell growth, neovascularization, and DR.[28] Insulin resistance, which often is accompanied by hyperinsulinemia, has been implicated in DR[29] and shown to be an independent predictor of PDR in T2DM patients.[30] Hyperinsulinemia is a cause of endothelial dysfunction,[31] and has been suggested to contribute to the altered retinal microvascular blood flow involved in the development of DR.[32] Our findings on the significant association of *INSR*

Table 3. Association Analysis of PDR

Nearest Gene(s)	SNP	CHR	Position	A1	A2	MAF		Model 1		Model 2	
						No-DR	PDR	OR (95%CI)	$P_{adjusted}$	OR (95%CI)	$P_{adjusted}$
IGSF21-KLHDC7A	rs3007729*	1	18795255	T	C	0.32	0.30	0.86 (0.70–1.06)	0.160	0.88 (0.72–1.08)	0.228
MYSM1	rs2811893†	1	59162148	C	T	0.34	0.35	1.04 (0.86–1.26)	0.687	1.00 (0.82–1.22)	0.993
SCYL1BP1	rs6427247‡§	1	170380480	G	A	0.25	0.25	1.01 (0.82–1.24)	0.932	1.00 (0.81–1.24)	0.985
TNFSF4-LOC730070	rs1342038*	1	173301516	C	T	0.42	0.42	0.97 (0.80–1.16)	0.708	0.96 (0.79–1.16)	0.651
KIAA1804-KCNK1	rs6662352*‖	1	233642172	T	G	0.35	0.33	0.89 (0.73–1.08)	0.228	0.86 (0.70–1.05)	0.139
AKT3-ZNF238	rs10927101*	1	244173872	C	A	0.28	0.31	1.12 (0.92–1.37)	0.263	1.10 (0.89–1.35)	0.373
AKT3-ZNF238	rs476141*	1	244176424	A	C	0.20	0.19	0.95 (0.75–1.20)	0.664	0.93 (0.73–1.18)	0.534
MYT1L-LOC729897	**rs10199521*§**	**2**	**2519513**	**T**	**C**	**0.35**	**0.39**	**1.25 (1.03–1.51)**	**0.022**	**1.27 (1.05–1.54)**	**0.016**
LINC01249	rs699549‡§	2	4705263	T	C	0.23	0.25	1.07 (0.87–1.33)	0.520	1.09 (0.87–1.35)	0.461
HNMT	rs763970‡	2	138636133	A	C	0.30	0.29	0.94 (0.77–1.14)	0.512	0.95 (0.78–1.16)	0.619
BFSP2	rs1197310‡	3	133128224	A	T	0.49	0.49	1.02 (0.85–1.22)	0.862	1.00 (0.83–1.20)	0.975
LEKR1-CCN1	rs13064954*§	3	156854742	A	G	0.08	0.08	0.85 (0.60–1.19)	0.335	0.89 (0.63–1.26)	0.511
KRT18P34-VEPH1	rs9866141*§	3	156950579	T	C	0.08	0.08	1.03 (0.74–1.43)	0.871	1.00 (0.71–1.41)	0.996
C5orf36	rs17376456†§	5	93557702	G	A	0.05	0.04	0.84 (0.53–1.33)	0.453	0.95 (0.60–1.50)	0.826
CAMK4	rs2300782‡	5	110788785	T	C	0.41	0.38	0.84 (0.70–1.02)	0.071	0.83 (0.69–1.01)	0.056
C6orf170	rs17083119‡	6	121402110	G	A	0.12	0.10	0.84 (0.62–1.14)	0.270	0.89 (0.66–1.21)	0.469
LOC729200-MAP3K7IP2	rs7772697*	6	149435111	C	T	0.18	0.18	1.02 (0.80–1.29)	0.898	0.98 (0.77–1.25)	0.876
CREB5	rs11765845*	7	28391142	A	G	0.27	0.26	0.94 (0.76–1.16)	0.580	0.93 (0.75–1.15)	0.504
PLXDC2-NEBL	rs12219125†	10	20593087	T	G	0.11	0.11	0.94 (0.70–1.26)	0.677	0.99 (0.73–1.33)	0.942
ARHGAP22	rs4838605†§	10	49699957	C	T	0.10	0.11	1.05 (0.77–1.42)	0.777	1.17 (0.86–1.59)	0.316
ARHGAP22	rs11101357†	10	49723300	A	G	0.10	0.10	0.98 (0.71–1.34)	0.879	1.08 (0.79–1.48)	0.628
ZWINT-MRPS35P3	rs4462262†	10	59189178	T	C	0.05	0.05	1.10 (0.72–1.67)	0.663	1.12 (0.73–1.72)	0.595
API5	**rs899036‡**	**11**	**41682910**	**C**	**A**	**0.08**	**0.11**	**1.36 (1.00–1.85)**	**0.049**	1.35 (0.99–1.84)	0.058
CNTN5	rs10501943‡§	11	99946999	C	T	0.03	0.04	1.60 (0.95–2.71)	0.077	1.63 (0.95–2.79)	0.076
HS6ST3	rs16953072†‖	13	96950313	T	G	0.05	0.06	1.15 (0.77–1.73)	0.493	1.17 (0.77–1.76)	0.459
FMN1	rs10519765‡§	15	33205424	A	G	0.11	0.10	0.85 (0.63–1.15)	0.281	0.85 (0.62–1.16)	0.308
COLEC12	rs599019‡	18	294495	C	A	0.46	0.47	1.13 (0.94–1.36)	0.201	1.16 (0.96–1.4)	0.132
INSR	**rs2115386*§**	**19**	**7196565**	**C**	**T**	**0.47**	**0.55**	**1.44 (1.20–1.73)**	**$\underline{1.12 \times 10^{-4}}$**	**1.44 (1.20–1.74)**	**$\underline{1.23 \times 10^{-4}}$**
VSTM2B-POP4	rs10403021*§	19	30079604	T	C	0.28	0.28	0.95 (0.78–1.17)	0.634	1.01 (0.82–1.24)	0.954

Single nucleotide polymorphisms ranked by chromosomal position. Single nucleotide polymorphisms showed statistically significant association with PDR are highlighted in bold. Chromosomal position corresponds to human reference genome hg19. Odds ratio corresponds to the minor allele (A1). Model 1: Adjusted for age, sex, duration of diabetes and presence of HT. Model 2: Adjusted for age, sex, duration of diabetes, presence of HT and HbA1c. CHR, chromosome; A1, minor allele; A2, major allele; MAF, minor allele frequency.

* Significantly associated SNPs in Reference 14.

† Significantly associated SNPs in Reference 15.

‡ Significantly associated SNPs in Reference 13.

§ Single nucleotide polymorphisms showing consistent direction of effect as in the original reports. Single nucleotide polymorphism remained significant after Bonferroni correction for multiple testing is underlined.

‖ rs6662352 is the proxy SNP of rs10910200 ($r^2 = 0.99$) and rs16953072 is the proxy SNP of rs2038823 ($r^2 = 1$).

rs2115386 are supportive of a role of insulin resistance, or the compensatory hyperinsulinemia, in the pathogenesis of DR.

The association of rs2115386 with severe DR (PDR or diabetic macular edema) was first identified in a GWAS meta-analysis of two T1DM cohorts, including a total of 2829 subjects from the Epidemiology of Diabetes Intervention and Control Trial (EDIC) and the Genetics of Kidney in Diabetes (GoKinD) studies.[14] In that study, none of the reported associations had reached genome-wide significance.[14] Although not being the most significant association identified in that study, *INSR* rs2115386 was a variant that showed an association of marginal genome-wide significance ($P = 2.86 \times 10^{-6}$) with severe DR that deserved further investigation.[14] The small number of cases with PDR and different inclusion criteria for cases and controls may explain why another study, which examined only 163 cases with non-PDR or PDR and 300 controls with no DR or mild DR, was unable to replicate the association of this SNP.[18] Further replication in other independent studies with similar phenotype characterization would be essential to validate this association. rs2115386 is located at intron 2 of *INSR* and it shows no obvious functional significance (RegulomeDB score, 5).[33] It may be linked with the disease-causing variant that is yet to be identified. Fine-mapping and deep-sequencing analyses to further refine the *INSR* gene region would serve to identify the causative variants. Functional studies to elucidate the role of the insulin receptor in the pathogenesis of DR also are warranted. Our findings that this SNP was significantly associated with STDR and PDR would strongly support *INSR* as a susceptibility gene for DR. The effect of *INSR* could possibly be more evident in PDR, as demonstrated in the current study. Our successful replication of this association in a T2DM cohort has provided support for a shared underlying pathogenetic pathway for DR in T1DM and T2DM.

As with many other complex diseases, the investigations of the genetic basis for DR have benefited from the advanced microarray-based genotyping technologies in recent years. In 2010, the first GWAS for DR involving 103 moderate to severe

non-PDR and PDR cases, and 183 normal to early non-PDR controls with T2DM of Mexican-American ancestry was published.[13] Several GWAS of DR in different populations subsequently were published.[14–16] Nonetheless, the results of these GWAS have only yielded limited success with only a few variants being able to reach genome-wide significance ($P \leq 5 \times 10^{-8}$) and these associations were unable to be replicated in independent GWAS.[13–16] Notably, these GWAS generally were conducted in relatively small sample sizes, based on different definitions for cases and controls, types of diabetes, and covariates adjustments. These studies also lacked follow-up analyses, except for the study of Sheu et al.,[16] which included 2 independent replication cohorts. In the current study, we sought to systematically replicate the top association signals in a T2DM cohort with a reasonable sample size. Assuming a STDR prevalence of 9.8%[7] and a MAF of 0.2, our current sample size was sufficient to achieve 68%, 85%, and 94% power to detect a significant association for an effect size of 1.20, 1.25, and 1.30, respectively, at a significance level of 0.05. We were able to detect the significant associations of the *INSR* variant and several other variants with severe DR with the current sample size. Among these, the *INSR* rs2115386, *COLEC12* rs599019, *AIP5* rs899036, and *MYT1L* rs1019952 were previously identified in the Mexican-American or Caucasian populations. Our successful replication of these SNPs in an Asian population would provide further support of the involvement of these loci in DR. We also replicated the association of *ZWINT-MRPS35P3* rs4462262, which was detected in a Taiwan Chinese population. The unsuccessful replications of other variants could have been due to lack of study power to detect those SNPs with relatively lower allele frequency and modest effect size. Furthermore, the difference in definitions for cases and controls and the varying covariates adjustments between studies also might contribute to the unsuccessful replications. Alternatively, some of these signals could be false-positive. Further replication studies should be conducted in independent cohorts of larger sample size to validate or to reject the associations of these GWAS-identified variants.

Only a handful of genes or pathways have been shown to be robustly associated with the development of DR so far. Possibly, the genetic susceptibility to DR could be conferred by rare or low frequency variants that were not covered in the candidate gene analyses and conventional GWAS that mainly focused on the common variants. The recently developed Exome-chip or whole exome/genome sequencing have provided an excellent tool for the discovery of rare, low frequency, or even population-specific disease-susceptibility variants. The gene-based tests, which account for the correlations between variants within the same gene,[34] would provide an alternative approach to the single-variant analyses that often lack the power to detect the associations of rare variants. Large-scale meta-analyses with sufficient study power will be essential to detect more genetic variants with modest effect. Due to cost and time considerations in using conventional genotyping platforms, researchers may tend to focus only on the genome-wide significant signals for replications. Potential susceptibility variants that showed marginal associations sometimes may be overlooked for follow-up. Current array-based genotyping technologies allow analyses of much larger number of variants simultaneously at reasonable cost. Application of the array-based genotyping method in future follow-up studies to include more SNPs would facilitate the identification of more potential disease-susceptibility variants.

The strength of this study was the well-defined phenotype and clear grading of DR severity based on the well-established English National Screening Program guidelines.[6] Unlike many others, the current study only included subjects without DR (graded R0) as controls. The associations of SNPs identified in previous GWAS that were based on varying phenotype definitions have been comprehensively studied in this study by examining the more broadly defined STDR (including maculopathy, pre-PDR, and PDR), and the more specifically defined PDR. Potential confounding was taken into consideration by adjustment for the conventional risk factors. To maximize the sample size, we have not specifically imposed an inclusion criterion on the duration of diabetes for controls. Instead, we accounted for this possible confounding by adjustment for duration of diabetes in the analyses. During the preparation of this manuscript, 2 additional GWAS on DR[35,36] were published. However, the current study has not examined these newly identified DR-associated variants. Our study would have been strengthened by a more comprehensive examination of all reported DR-susceptibility variants, including the newly identified SNPs as well as those that failed to pass quality check. The current study also was limited by the fact that it was slightly underpowered to detect the associations of variants with lower allele frequencies and modest effect.

In conclusion, we have successfully confirmed the significant and independent associations of several SNPs identified in previous GWAS with severe DR (STDR and/or PDR) in our Chinese patients with T2DM and these genetic data may contribute to risk profiling in future diabetes management. Our findings indicated that the *INSR* gene is likely to be a susceptibility candidate for severe DR and supported the involvement of insulin resistance in the pathogenesis of DR.

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