Replication of Genetic Loci Implicated in Diabetic Retinopathy

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Purpose. Recent genome-wide association studies for diabetic retinopathy (DR) have identified novel single nucleotide polymorphisms (SNPs) associated with this potentially blinding disease. These markers could prove useful in risk profiling, if the association are validated by replication. To date, these associations have not been well assessed in independent cohorts. The objective of this study was to ascertain any association of these polymorphisms with advanced stages of DR.

Methods. A total of 463 patients who had either type 1 ($n = 46$) or 2 ($n = 417$) diabetes were genotyped for 24 SNPs previously implicated in DR. Cases ($n = 163$) were defined as people with severe nonproliferative DR or proliferative DR. Control participants ($n = 300$) with a confirmed duration of diabetes of at least 5 years had either no evidence of DR or only mild DR.

Results. Two SNPs (rs1073203 and rs4838605) were found to be significantly associated with DR in patients with diabetes after adjusting for covariants; rs1073203-G ($P = 0.012$, odds ratio [OR] = 0.317, 95% confidence interval [95% CI]: 0.129–0.778), rs1073203 in a dominant model ($P = 0.005$, OR = 0.251, 95% CI: 0.096–0.655), rs4838605 in an additive model ($P = 0.047$, OR = 1.650, 95% CI: 1.007–2.703), In a dominant model rs1073203 ($P = 0.027$, OR = 1.400, 95% CI: 0.101–0.857), was significantly associated with DR in type 2 diabetes after adjustment for covariants. This study was sufficiently powered to replicate previous findings.

Conclusions. This study confirmed that two variants (rs1073203 and rs4838605) are associated with advanced stages of DR in our cohort. The underlying genes in these candidate regions provide interesting future gene association targets for understanding the pathogenesis of DR.

Keywords: diabetic retinopathy, single nucleotide polymorphism, clinical management

A severe complication of diabetes mellitus is vision loss, which can occur in the advanced stages of diabetic retinopathy (DR). At these sight-threatening stages there are microvascular abnormalities that result in preretinal or vitreous hemorrhage or tractional retinal detachment that can obstruct vision.[1] With timely detection, vision loss can be prevented by using current DR treatment of laser photocoagulation and intraocular injections at the severe nonproliferative DR (NPDR)/proliferative DR (PDR) stage.[2,3] Consequently, recent efforts have been undertaken to discover genetic markers involved in DR[4–8] because current clinical measures (such as glycosylated hemoglobin [HbA1c] or lipid levels) lack sensitivity and specificity for DR risk prediction.[2,3] Heritability studies in DR have revealed familial clustering in patients with both type 1 (T1DM) and type 2 (T2DM) diabetes, not appropriated by conventional risk factors.[4,5] The polygenic variability in DR risk is estimated to be approximately 25%, suggesting that multiple genes influence the phenotype of sight-threatening DR.[6] While the pathogenesis of DR development is seemingly multifactorial with genetic, epigenetic, and environmental contributions,[7,8] these markers are not utilized in the clinical management of DR. If genetic markers for DR were identified and were replicable, they could aid in early diagnosis and risk stratification among patients with diabetes.

Recently, two genome-wide association studies (GWASs) on DR in different populations reported new loci not previously associated in DR. Grassi et al.[9] published GWAS results for retinopathy in T1DM within two Caucasian cohorts and identified a strong association ($P < 1.0 \times 10^{-7}$) with four loci (*AKT3-ZNF238*, *RBFOX1*, *KEKR1-CCNL1*, and *KRT18P34-VEPH1*).[9] Several additional single nucleotide polymorphisms (SNPs) were found to reach a suggestive level ($P < 1.0 \times 10^{-6}$) of association with DR.[9] Huang and colleagues[10] found different loci associated with DR through a GWAS of DR in an Asian-Taiwanese cohort of people with T2DM.[10] Significant associations ($P < 3.0 \times 10^{-7}$) with DR and multiple SNPs across five loci (*MYSM1*, *PLXDC2*, *ARHGAP22*, *HS6ST3*, and *C5orf36*) were identified.[10] Of these only the *ARHGAP22* locus was associated with PDR.[10] The identification of these SNP candidates for DR from both recent GWASs has provided

important insights into the underlying risk for developing DR. If replicable, these genetic markers could be utilized for diabetes risk management to detect those at risk of sight-threatening DR.

A recent replication study by Grassi et al.[11] was unable to replicate the top loci signals associated with DR using T1DM from the Wisconsin Epidemiological Study of Diabetic Retinopathy (WESDR) cohort ($n = 469$). They attributed the inability to replicate the signals to differences in the case/control DR severity cutoff levels between the discovery and replication cohorts.[11] Clearly the classification and comparison between levels of DR severity is essential when investigating risk factors for disease development.[11–15]

Since many individuals with T2DM and almost all people with T1DM develop DR, the aim of our study was to replicate recently discovered susceptibility alleles or genotypes with advance sight-threatening stages of DR. We hypothesized that the underlying genetic predisposing factors for developing DR was similar regardless of diabetes subtype. Using a well-defined clinical cohort for DR severity and demographic risk factors, we sought to ascertain the association between the candidate SNPs and advance stages of DR in an independent cohort. Herein we present our findings on the associations of these loci with DR.

Methods

Subjects were recruited between March 2009 and December 2012 as part of the Diabetes Management Project (DMP) from clinics at the Royal Eye and Ear Hospital (RVEEH), Melbourne, Australia.[16] Ethical approval was provided by the RVEEH Human Research and Ethics Committee (08/815H), and informed consent was obtained from all participants. The DMP protocol adhered to the Declaration of Helsinki, and all privacy requirements were met.

Data Collection

Each participant underwent an extensive examination, including comprehensive ocular and anthropometric measurements as well as completing socio-demographic and medical history questionnaires. Midstream urine samples were collected to assess albumin and creatinine levels. Peripheral blood samples were collected following a 12-hour fast, and measurements of HbA1c, low-density lipoprotein (LDL), high-density lipoprotein (HDL), cholesterol, triglycerides, and glucose was performed. All biochemical parameters were analyzed at Melbourne Pathology (Melbourne, Australia). DNA was also extracted from these samples using the QiaAmp Blood midi kit (Qiagen, Valencia, CA).

Phenotypic Characterization

This cohort was predominantly Caucasian of reported Northern European descent (Table 1). Participants were defined as having T1DM if they had a juvenile diagnosis and were insulin dependent from diagnosis, while those with a nonjuvenile diagnosis and those who did not require immediate insulin therapy were defined as T2DM. Severity of retinopathy was obtained with the use of two-field, 45°, digital, nonstereo, color fundus photographs from both eyes, collected using a non-mydriatic retinal camera (Canon CR6-45NM; Canon, Inc., Tokyo, Japan). Trained graders masked to clinical measures assessed images for the presence of retinopathy using a modified Airlie House classification as used in the Early Treatment of Diabetic Retinopathy Study protocol.[17–19] The retinopathy assessment was independently validated by an ophthalmologist (PPC). Classifications of retinopathy were selected based on two groups; controls were defined as those without DR or with mild NPDR, and cases were those with severe NPDR/PDR (including participants previously treated with laser photocoagulation therapy for PDR). Given the association between duration of diabetes mellitus and the development of DR, all control subjects without DR or with only mild NPDR required at least a 5-year duration of disease.

SNP Analysis

SNP selection was based on the most significant SNP associations with DR from the GWASs of Grassi et al.[8] and Huang et al.[10] Three SNPs failed quality control (QC) measures for inclusion in the multiplex design and were not included in further analysis (rs10927101, rs11867934, and rs4787008). Participants were genotyped for 24 SNPs using the Sequenom iPLEX Gold chemistry (Sequenom, San Diego, CA) on an Autoflex mass spectrometer (Sequenom, Brisbane, Australia) at the Australia Genome Research Facility (Brisbane, Australia). All genotyped SNPs had a call rate of >97%.

Statistical Analysis

A cross-sectional case–control design was used. All demographic parameters are presented as mean ± standard deviation (SD), unless otherwise specified, and compared by the Student's *t*-test. Genetic data were analyzed using PLINK v1.07.[20] The default setting in PLINK was used for assessment of Hardy-Weinberg disequilibrium.[20] Two SNPs (rs2811893 ($P = 0.041$) and rs4838605 ($P = 0.044$)) were found not to be in Hardy-Weinberg equilibrium in the control cohort. To investigate potential models of inheritance additive, dominant and recessive genotypic models were applied. A multivariate logistic regression model was also constructed to adjust for clinical traits differing between case and control groups. Power calculations were conducted using case–control for discrete traits analysis through the Genetic Power Calculator,[21] assuming a disease prevalence of 0.6% for PDR, a genotyped-to-causal variant LD of $D' = 0.9$, an identical marker–disease minor allele frequency (MAF), and an additive disease risk model. Given that we set out to confirm previous findings, we set a significance threshold for replication of $\alpha = 0.05$.

Results

The demographic characteristics of the participants are presented in Table 1. Age, sex, duration of diabetes, and hematological levels of HbA1c, fasting glucose, and serum creatinine differed significantly between patients with severe NPDR/PDR and controls. Urinary microalbumin levels and the microalbumin/creatinine ratio were found to be significantly different between groups (Table 1). To adjust for case/control differences, these variables were used for the adjusted multivariate logistic regression model. For T1DM and T2DM combined, this study was well powered to replicate variants with an MAF greater than 0.20 (Fig.). For variants with an MAF lower than 0.10 (rs6702784, rs13064954, rs9866141, rs2696835, rs17404956, and rs4470583), this study was underpowered to detect a modest association.

The analysis for allelic association revealed rs1073203-G had a suggestive association ($P_{raw} = 0.012$; $P_{corrected} = 0.288$) with severe NPDR/PDR in T1DM and T2DM following covariate adjustment (Table 2). Genotypic association analyses revealed association in the dominant genotype model for rs1073203 (Table 3), although this was not significant

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Table 1. Demographic Features and Clinical Profiles of Study Participants

	Control: Without DR/ With Mild NPDR	Case: Severe NPDR/PDR	*P* Value
No.	300	163	—
No. female (%)	115 (38.33)	40 (24.54)	0.003
Age at study, y	67.6 (10.78)	59.90 (11.12)	<0.001
Caucasian (%)	253 (84.33)	138 (84.66)	0.926
Duration of diabetes, y	16.8 (11.67)	19.94 (11.63)	0.006
On insulin therapy (%)	212 (70.67)	123 (75.46)	0.430
Type 2 diabetes (%)	279 (93.00)	138 (84.66)	0.004
HbA1c, %	7.57 (1.37)	8.34 (1.62)	<0.001
BMI, kg/m^2	30.15 (6.13)	30.92 (6.38)	0.271
Systolic blood pressure, mm Hg	137.7 (17.98)	139.75 (21.22)	0.277
Diastolic blood pressure, mm Hg	75.33 (8.7)	76.56 (10.83)	0.190
Mean arterial pressure, mm Hg	96.02 (9.5)	97.64 (11.47)	0.141
Cholesterol, mmol/L	4.56 (1.28)	4.63 (1.37)	0.584
Triglycerides, mmol/L	1.78 (1.28)	1.83 (1.42)	0.706
HDL, mmol/L	1.75 (5.56)	1.36 (0.81)	0.384
LDL, mmol/L	2.34 (1.01)	2.52 (1.18)	0.094
LDLC/HDLC ratio	1.83 (1.29)	2.04 (1.04)	0.112
Total: HDL cholesterol ratio	3.47 (1.40)	3.79 (1.35)	0.031
Fasting glucose, mmol/L	8.20 (3.22)	9.39 (4.02)	0.001
Serum creatinine	94.61 (52.43)	117.18 (92.98)	0.016
EGFR	67.76 (19.26)	62.80 (23.29)	0.082
Microalbumin, mg/L	120.875 (413.47)	510.66 (1356.29)	<0.001
Microalbumin/creatinine ratio	13.12 (40.17)	61.80 (171.79)	<0.001
Smoker	0.85 (0.76)	0.82 (0.77)	0.735
Never (%)	133 (44.33)	76 (46.62)	
Previous (%)	51 (17.00)	31 (19.02)	
Current (%)	115 (38.33)	56 (34.35)	
Not available (%)	1 (0.33)	1 (0.61)	

Data are presented as a mean (SD) unless otherwise indicated. DM, diabetes mellitus; BMI, body mass index; EGFR, estimate of glomerular filtration rate; LDLC/HDLC ratio, low-density lipoprotein cholesterol/high-density lipoprotein cholesterol ratio.

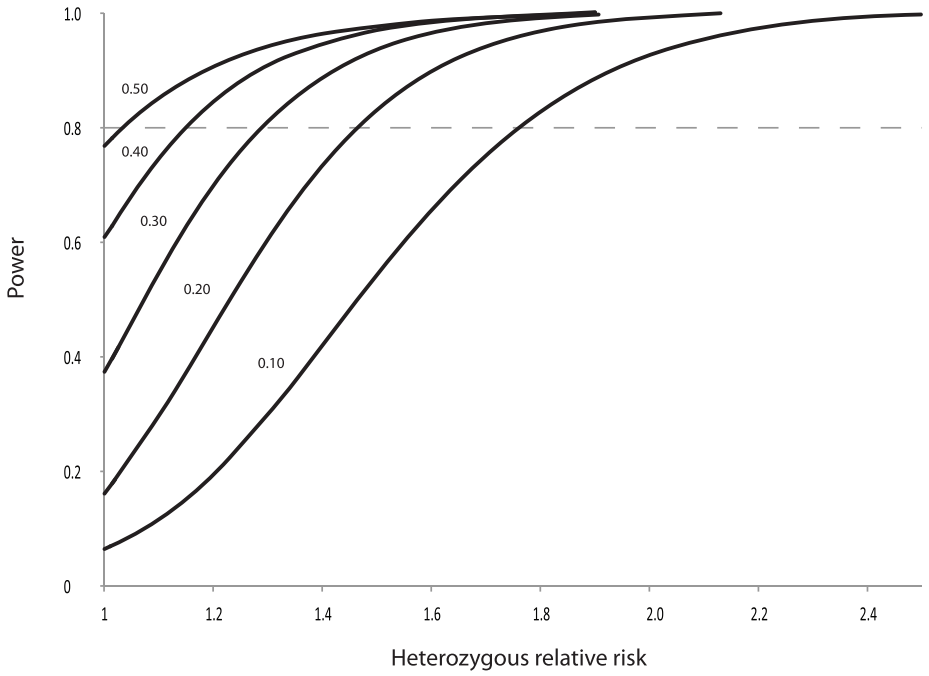

Figure. Study-wide power for detecting a significant association for replication at the $\alpha = 0.05$ level for differing risk allele frequencies (0.10–0.50) under an additive risk model.

Table 2. Allelic Associations With Diabetic Retinopathy

SNP	Minor Allele	f_{case}	$f_{control}$	Unadjusted				Adjusted‡			
				P Value	OR	95% CI	χ^2	*P* Value	OR	95% CI	*t*-stat
rs3007729*	C	0.359	0.371	0.710	0.948	0.716–1.255	0.138	0.670	0.897	0.545–1.476	−0.426
rs6702784*	C	0.071	0.082	0.563	0.859	0.513–1.438	0.334	0.255	0.535	0.183–1.568	−1.139
rs2115386*	A	0.509	0.480	0.395	1.124	0.858–1.473	0.723	0.303	1.279	0.801–2.042	1.031
rs1342038*	T	0.411	0.440	0.395	0.888	0.676–1.167	0.723	0.115	0.682	0.424–1.098	−1.574
rs476141*	A	0.448	0.470	0.519	0.915	0.698–1.199	0.417	0.053	0.610	0.370–1.006	−1.935
rs10910200*	G	0.228	0.244	0.592	0.916	0.666–1.261	0.287	0.355	0.763	0.430–1.353	−0.925
rs10199521*	A	0.193	0.210	0.546	0.901	0.643–1.263	0.365	0.142	0.615	0.321–1.178	−1.467
rs13064954*	A	0.049	0.041	0.551	1.217	0.637–2.326	0.355	0.679	1.259	0.423–3.750	0.414
rs9866141*	T	0.031	0.035	0.719	0.869	0.404–1.868	0.130	0.655	1.296	0.416–4.041	0.447
rs2696835*	C	0.009	0.027	0.072	0.338	0.098–1.168	3.228	0.198	0.312	0.053–1.842	−1.286
rs12607567*	A	0.313	0.345	0.322	0.865	0.648–1.153	0.980	0.766	0.930	0.574–1.505	−0.297
rs1073203*	G	0.086	0.119	0.123	0.697	0.440–1.105	2.378	0.012	0.317	0.129–0.778	−2.510
rs17376456*	G	0.086	0.072	0.437	1.217	0.741–1.999	0.604	0.408	1.534	0.557–4.221	0.828
rs7772697*	C	0.407	0.405	0.950	1.009	0.766–1.329	0.004	0.486	0.832	0.495–1.397	−0.697
rs11765845*	A	0.298	0.258	0.202	1.216	0.901–1.641	1.631	0.387	1.248	0.756–2.061	0.866
rs10403021*	T	0.339	0.311	0.402	1.131	0.847–1.510	0.702	0.254	1.321	0.819–2.130	1.140
rs4838605†	T	0.379	0.364	0.655	1.066	0.805–1.412	0.199	0.065	1.570	0.972–2.537	1.842
rs4462262†	T	0.407	0.341	0.046	1.328	1.005–1.755	3.985	0.231	1.354	0.825–2.223	1.198
rs1925117†	A	0.083	0.114	0.143	0.706	0.442–1.127	2.145	0.446	0.734	0.331–1.626	−0.763
rs2811893†	C	0.398	0.402	0.902	0.983	0.745–1.296	0.015	0.884	0.966	0.610–1.530	−0.146
rs4470583†	A	0.012	0.013	0.883	0.913	0.273–3.055	0.022	0.999	8.70×10^{-10}	NA	−0.002
rs17404956†	G	0.127	0.128	0.979	0.995	0.662–1.493	0.001	0.845	1.068	0.552–2.066	0.196
rs12219125†	T	0.132	0.146	0.570	0.892	0.602–1.322	0.322	0.380	1.376	0.675–2.803	0.879
rs1571942†	C	0.120	0.138	0.442	0.852	0.567–1.281	0.592	0.703	1.159	0.543–2.472	0.382

f, frequency; *t*-stat, *t*-statistic.
* Significantly associated SNPs.[9]
† Significantly associated SNPs.[10]
‡ Adjusted for age, sex, duration of diabetes mellitus, HbA1c, and microalbumin/creatinine ratio.

Table 3. Genotypic Associations With Diabetic Retinopathy

SNP	Additive Model			Dominant Model			Recessive Model		
	P Value	OR	95% CI	*P* Value	OR	95% CI	*P* Value	OR	95% CI
rs3007729*	0.891	0.964	0.572–1.624	0.446	0.763	0.380–1.531	0.822	1.117	0.479–2.923
rs6702784*	NA	NA	NA	0.255	0.535	0.183–1.568	NA	NA	NA
rs2115386*	0.298	1.286	0.801–2.063	0.273	1.556	0.706–3.429	0.550	1.258	0.595–2.673
rs1342038*	0.175	0.709	0.432–1.165	0.104	0.565	0.284–1.125	0.353	0.643	0.253–1.634
rs476141*	0.050	0.601	0.361–1.001	0.162	0.581	0.272–1.243	0.076	0.454	0.185–1.086
rs10910200*	0.467	0.744	0.336–1.650	0.403	0.746	0.376–1.481	0.539	0.611	0.127–2.944
rs10199521*	0.452	0.654	0.216–1.976	0.144	0.583	0.282–1.202	0.539	0.503	0.056–4.516
rs13064954*	NA	NA	NA	0.679	1.259	0.423–3.750	NA	NA	NA
rs9866141*	NA	NA	NA	0.655	1.296	0.416–4.041	NA	NA	NA
rs2696835*	NA	NA	NA	0.198	0.312	0.053–1.842	NA	NA	NA
rs12607567*	0.845	1.053	0.628–1.767	0.429	0.764	0.392–1.489	0.573	1.327	0.496–3.548
rs1073203*	0.706	1.357	0.277–6.642	0.005	0.251	0.096–0.655	0.582	2.408	0.105–54.990
rs17376456*	0.999	2.17×10^{4}	NA	0.444	1.504	0.529–4.273	0.999	4.36×10^{8}	NA
rs7772697*	0.495	0.824	0.473–1.436	0.574	0.816	0.402–1.657	0.579	0.752	0.274–2.062
rs11765845*	0.490	1.220	0.694–2.145	0.393	1.346	0.681–2.663	0.610	1.327	0.447–3.937
rs10403021*	0.158	1.446	0.867–2.411	0.551	1.227	0.626–2.407	0.140	2.077	0.788–5.477
rs4838605†	0.047	1.650	1.007–2.703	0.216	1.549	0.775–3.099	0.055	2.407	0.981–5.910
rs4462262†	0.376	1.279	0.742–2.206	0.196	1.571	0.793–3.114	0.605	1.310	0.471–3.646
rs1925117†	0.999	4.92×10^{-5}	NA	0.511	0.758	0.332–1.733	0.999	0.000	NA
rs2811893†	0.856	0.958	0.601–1.525	0.979	1.010	0.488–2.091	0.772	0.886	0.390–2.011
rs4470583†	NA	NA	NA	0.999	0.000	NA	NA	NA	NA
rs17404956†	0.753	1.177	0.427–3.242	0.912	1.045	0.485–2.251	0.753	1.382	0.184–10.400
rs12219125†	0.999	NA	NA	0.155	1.752	0.809–3.794	0.999	0.000	NA
rs1571942†	0.999	NA	NA	0.462	1.350	0.607–3.005	0.999	0.000	NA

* Significantly associated SNPs.[9]
† Significantly associated SNPs.[10]

following Bonferroni correction ($P_{raw} = 0.005$; $P_{corrected} = 0.120$). Genotypic analysis showed suggestive associations for rs4838605 and severe NPDR or PDR under a recessive and additive genotypic model, following covariable adjustment (Table 3); however, this was again not significant following Bonferroni correction.

For the T2DM subgroup genotypic analysis identified a similar trend association with severe NPDR/PDR for the dominant genotype of rs1073203-G, after covariable adjustment (Supplementary Table S2) ($P_{corrected} = 0.025$; odds ratio [OR] = 0.295; 95% confidence interval [95% CI]: 0.101–0.857). There were no significant allelic associations identified in the T2DM subgroup (Supplementary Table S1).

Discussion

Our study has replicated two loci (rs1073203 and rs4838605) that are nominally associated with the advance stages of DR from the SNP candidates of recently reported GWASs. The strongest association for the advance stages of DR was with rs1073203 in a dominant genotypic model, after covariable adjustment. This finding suggested that the association is related to DR and does not reflect the impact of other diabetes-related variables. The association was significant for both T1DM and T2DM combined and for T2DM independently. The ancestral G allele for rs1073203 was significantly associated with advance stages of DR. Initially this locus was identified in a T1DM cohort. Although the SNP occurs in the intronic region, it may have an impact on neighboring gene function. The rs1073203 locus encompasses the genes *RPSAP37* and *GRAMD3*, both involved in new blood vessel regulation, which is clinically observed in vision-threatening DR. The ribosomal protein SA (RPSA) 37 is transcribed from *RPSAP37*. Ribosomal protein SAs are cell surface and cytoplasm receptors expressed in vascular tissue and are crucial in the process of new blood vessel formation, termed angiogenesis, which is a key clinical feature of PDR.[22–25] *GRAMD3* has been implicated in choroid neovascularization from a recent GWAS for age-related macular degeneration,[26] suggesting it could be also be involved in choroid neovascularization in DR. rs1073203 (G allele and in a dominant genotypic model) has provided a possible genetic marker for DR clinical management, and two gene candidates for future studies investigating the roles of angiogenesis and neovascularization in the pathogenesis of DR.

Our study confirmed an association for rs4838605 with DR as identified previously in the Taiwanese DR study.[10] The allelic frequencies of this locus differ greatly with ethnicity,[10] and confirming that the association is replicable in a predominately Caucasian population suggests that this locus has potential as a genetic management marker for DR. The association of rs4838605 with DR could be biologically important in our understanding of the pathogenesis of DR because rs4838605 is located in the intron region of the *ARHGAP22* gene. This gene codes for the RHO GTPase-activating protein, which is known to be involved in insulin response mechanisms involved in endothelial cell migration and metastasis.[10,27,28]

This study lacked power to perform a subgroup analysis of patients with T1DM. While the underlying etiologies of T1DM and T2DM differ, it is acknowledged that the genetic architecture of DR could be similar. Although previous studies have combined cohorts with both types of diabetes to assess risk for DR,[29,30] future studies should separate analyses based on the type of diabetes.

Our full cohort had sufficient power to replicate a variant of modest effect size for and MAF greater than ~15% at the $\alpha = 0.05$ level. The strength of our study is our well-defined clinical cohort and clear differences in DR severity. Future studies attempting replication should ensure a similarly phenotypic classification. Negative associations found between SNPs and DR may reflect a winner's curse on the initial discovery, a relative lack of power for replication, or underlying genetic differences between study populations.[31]

It is important to note that no variant reached significance following correction for multiple testing (Bonferroni corrected P threshold = 0.002). Clearly SNP-based associations can be difficult to replicate across different study populations.[32–34] Though given that we set out to confirm previous findings, we set an initial significance threshold for replication of $\alpha = 0.05$. Recent modeling of SNP-based associations suggests variants that are not initially replicated by using conservative multiple correction thresholds could still be useful in disease risk prediction modeling.[33,34] As such, some ongoing research may be warranted into the genes encompassed by the two loci (rs1073203 and rs4838605) where we found nominal levels of association with the advance stages of DR.

Overall results indicate that these two candidate loci are likely to be markers of advance stage sight-threatening DR. The current study had adequate power to detect locus variation and a well-defined clinical phenotype. The identification of the loci as putative genetic markers of DR has implications for the future management of DR for vision preservation in diabetes. The association of markers with DR has also provided interesting gene candidates and has supplemented our understanding of the genetic contribution to the pathogenesis of DR.

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