

Multiple variants in Vascular Endothelial Growth Factor (VEGF) are risk factors for time to severe retinopathy in type 1 diabetes: The DCCT/EDIC genetics study

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Running Title: VEGF SNPs associated with Severe Retinopathy

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Abbreviations:

VEGF, Vascular Endothelial Growth Factor; DCCT, Diabetes Control and Complications Trial; EDIC, Epidemiology of Diabetes Interventions and Complications; T1D, Type 1 Diabetes; T2D, Type 2 Diabetes; DR, Diabetic Retinopathy; SR, Severe Retinopathy; PDR, Proliferative Diabetic Retinopathy; non-PDR, non-Proliferative Diabetic Retinopathy; ETDRS, Early Treatment Diabetic Retinopathy Study; LD, Linkage disequilibrium

Abstract

Objective: To determine if any common variants in the gene for Vascular Endothelial Growth Factor (VEGF) are associated with long-term renal and retinal complications in type 1 diabetes (T1D).

Research Design and Methods: 1369 white subjects with T1D from the Diabetes Control and Complications Trial (DCCT)/Epidemiology of Diabetes Interventions and Complications (EDIC) study had an average of 17 retinal photographs and 10 renal measures over 15 years. In the DCCT/EDIC, we studied 18 single nucleotide polymorphisms (SNPs) in VEGF that represent all linkage disequilibrium bins (pairwise $r^2 \geq 0.64$), and tested them for association with time to development of: severe retinopathy (SR); ≥ 3 -step progression of retinopathy; clinically significant macular edema; persistent microalbuminuria; and severe nephropathy.

Results: In a global multi-SNP test there was a highly significant association of VEGF SNPs with SR ($P = 6.8 \times 10^{-5}$) – the four other outcomes were all non-significant. In survival analyses controlling for covariate risk factors, eight SNPs showed significant association with SR ($P < 0.05$). The most significant single-SNP association was rs3025021 (Hazard Ratio = 1.37, 95% confidence interval [1.13-1.66], $P = 0.0017$). Family-based analyses of SR provide evidence of excess transmission of C at rs699947 ($P = 0.029$), T at rs3025021 ($P = 0.024$) and the C-T haplotype from both SNPs ($P = 0.035$). Multi-SNP regression analysis including 15 SNPs, and allowing for pairwise interactions, independently selected six significant SNPs ($P < 0.05$).

Conclusions: These data demonstrate that multiple VEGF variants are associated with the development of SR in T1D.

Introduction

Diabetic retinopathy (DR) affects the majority of patients with >15 years of diabetes [1-2]. Risk factors for DR include poor glycemic control (as measured by HbA1c), longer diabetes duration, earlier age at diagnosis, higher waist-to-hip ratio, fasting triglyceride, BMI, blood pressure and serum/plasma fibrinogen [3-5]. The DCCT study demonstrated familial clustering of severe retinopathy (SR), but not for any retinopathy [6], consistent with others [7-8]. The essential features of DR include capillary microaneurysms and increased vascular permeability leading to macular edema, vascular occlusion and ischemia, neovascularization, and contraction of fibrovascular proliferation in the vitreous.

Vascular endothelial growth factor (VEGF) is a mitogen that specifically acts on endothelial cells [9] and has various effects, including mediating increased vascular permeability [10], inducing angiogenesis [11-13], cell growth, migration, and inhibition of apoptosis [14]. Many retinal cells produce VEGF, including pigment epithelial [15], capillary pericytes [15], endothelial [16], Mueller [17], ganglion [18], and glial cells [19]. VEGF expression in retinal cells is increased 3- to 30-fold by hypoxia [20]. VEGF is significantly up-regulated in DR, particularly in retinal pigmented epithelial cells, glial cells, and vitreal fibroblasts [21]. Moreover, higher plasma [22] and vitreous VEGF levels [22-24] were found in individuals with proliferative diabetic retinopathy (PDR) when compared to non-diabetic controls [22] or to individuals with non-ocular diseases [23]. Phase II clinical trials have shown safety and efficacy of intraocular injections of pegaptanib, an anti-VEGF aptamer in the treatment of diabetic macular edema [25].

For these reasons, VEGF is a functional candidate gene for the predisposition to DR.

Several cross-sectional studies have investigated the genetic involvement of VEGF in DR [26-30]. Three studies tested rs833061(-460) for association with DR in patients with type 2 diabetes (T2D) [26-28]: One observed an association with the C allele and the C/C genotype [26]; another found association with the C/T genotype [27]; whereas the third one found no association [28]. Two studies investigated the association of an 18-bp deletion in the promoter with DR [29, 30] in type 1 diabetes (T1D) [30] and T2D [29], but produced conflicting results. However all of these studies [26-30] employed a case-control design with various phenotype definitions and investigated only markers in the promoter region in relatively small sample sizes of Japanese T2D patients [28] or Caucasians with a mix of T1D and T2D [30]. Covariates, if available, were measured at a single time point when the phenotype was also determined.

The current study utilizes longitudinal data for retinal and renal complications in the white T1D population from the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications (DCCT/EDIC) study to examine the role of VEGF polymorphisms [31].

Research Design and Methods

To reduce population stratification, a potential source of bias in genetic association studies, analyses were restricted to 1369 white DCCT/EDIC subjects from whom DNA was collected (see Online Appendix (OLA) supplementary text for details). The OLA also provides the distribution of baseline

characteristics at DCCT entry (OLA Table 1) and clinical measures of available parents and siblings (OLA Table 2).

Phenotypic characterization of retinal and renal outcomes

Based on repeated measurements of retinal and renal function (OLA text), we defined three retinal (≥ 3 -step progression, severe retinopathy, and clinically significant macular edema [CSME]) and two renal (persistent microalbuminuria and severe nephropathy) phenotypes. For each phenotype, a time-to-event outcome was derived as years from DCCT baseline until the event occurred or censoring. The number of events observed for all retinal and renal phenotypes is provided in Table 1.

Follow-up during DCCT/EDIC averaged 15.1 ± 2.8 (Mean \pm SD) years, during which time an average of 16.7 ± 3.7 retinal photographs were taken for each proband. Photos were scored using the Early Treatment Diabetic Retinopathy Study (ETDRS) system [32]. The ≥ 3 -step phenotype was defined as progression from DCCT baseline to ≥ 3 ETDRS steps. Criteria for severe retinopathy (SR) included an ETDRS level $53 / < 53$ or greater, or scatter laser treatment. The presence of CSME was defined according to ETDRS criteria [32]. In addition, patients who underwent focal photocoagulation for macular edema were counted as having CSME thereafter.

Renal measurements of urinary albumin excretion rate (AER) were obtained yearly in DCCT and biennially in EDIC (details in OLA). The presence of persistent microalbuminuria was defined as AER > 20.8 $\mu\text{g}/\text{min}$ on two consecutive assessments. Severe nephropathy was assigned to subjects with persistent microalbuminuria who

progressed to an AER > 208 $\mu\text{g}/\text{min}$ or renal replacement therapy (dialysis or transplant).

Genotyping methods and quality control

In total we selected 18 SNPs for genotyping across the VEGF gene (OLA Table 3). Fifteen of these represent tagSNPs, selected from the SeattleSNP Project using LDSelect (<http://pga.gs.washington.edu/VG2.html>) using genotype data for 23 European-American individuals and applying an $r^2 \geq 0.64$ cutoff [33]. rs2010963, previously named -634 [28] or +405 [34], was selected from the promoter region. Two SNPs (rs699947 [-2578] [35] and rs1547651) that lie in the 5' region were selected from the CEU data of phase I of the International HapMap project [36] since this region was not sequenced by the SeattleSNP project.

Eleven SNPs were genotyped using Taqman assays and eight using the Illumina Goldengate bead array platform [37]. Details regarding genotyping procedures, including sequences of primers and probes used in TaqMan (OLA Table 4) and detailed quality control measurements (OLA Table 3) are described in the OLA. rs3025028 was genotyped on both Illumina and Taqman platforms with a 1% genotype disagreement rate. Only genotypes that were identical between the two platforms were used in the analysis of this SNP. For the Illumina platform, the highest error rate based on duplicate plate was 4.4% for rs1547651 (OLA Table 3). The lowest mean Gencall score, a measure of genotyping quality [37], was 0.7 for rs3025007 (OLA Table 3). Among duplicate samples genotyped on Taqman, the highest error rate was 12%, observed for rs833069. rs1413711 showed

significant deviation from Hardy-Weinberg equilibrium (HWE) ($P < 0.0001$) due to the absence of rare homozygous genotypes, likely a result of technical difficulties. Therefore, rs833069 and rs1413711 were replaced by rs833068 and rs833070, respectively, since they are in the same r^2 bin. All analyses with outcomes examined the 16 SNPs (Table 3) with good quality control results. In the family data, there were zero, one, two, and six Mendelian errors at rs1547651, rs699947, rs3025010 and rs3025021, respectively. Families with Mendelian errors were excluded from the family-based analysis. Linkage disequilibrium (LD) was calculated using Haploview v3.2 [38] in 1180 subjects with complete genotype data at all SNPs.

Statistical analysis for genetic association

Primary analyses were designed to evaluate the association of VEGF SNPs with the time-to-event outcome for each retinal and renal phenotype (see OLA text). To explain variability in diabetic complications, multivariate Cox proportional hazards (PH) models were developed to account for the DCCT/EDIC design (cohort and treatment, OLA Table 5) as well as known risk factors and other potential confounding factors (Table 2 and OLA Table 6). For reasons of validity, we elected to use the start of DCCT rather than diabetes diagnosis as the time-to-event baseline, including diabetes duration along with DCCT baseline measures as explanatory covariates. DCCT participants met eligibility criteria based on disease duration and clinical progression, so are not representative of an unselected population of newly diagnosed diabetics, precluding straightforward natural-history modeling. Diabetes duration prior to entry into

DCCT as well as HbA1c at eligibility screening for DCCT were included in the multivariate Cox PH model to explain variation due to differences in duration and to capture prior glycemic exposure. Time-dependent covariates were included for updated HbA1c and hypertension to account for DCCT treatment effects. For parsimony, SNPs were coded additively representing the number of copies of the minor allele. Although this strategy is expected to be robust to non-additivity, we also examined models with both linear and quadratic genotype terms to assess this assumption.

To adjust for multiple testing and select phenotypes for further analysis, we first performed a global test including all sixteen VEGF SNPs in the multivariate Cox PH model to test association between time-to-event and overall genetic variation at the VEGF locus. The corresponding likelihood ratio test (16 df) was corrected using a Bonferroni factor of five, and only phenotypes with $p < 0.01$ were examined further. In additional single-SNP analyses, the relative risk associated with each SNP was estimated by the hazard ratio (HR) from the Cox PH model. Single-SNPs were examined in both univariate and multivariate Cox PH models. Multi-SNP selection analyses were also performed to determine the most likely etiological variant(s) and estimate HR associated with multiple SNP genotype combinations. These included multiple regression models with forward and backward selection of main effects and two-way interactions of VEGF SNPs (details in the OLA).

Martingale residuals, obtained from the multivariate Cox PH models without genotypes, were used as a continuous trait in subsequent family-based analyses of 4 SNPs genotyped in first-degree relatives (see OLA Table 7

for family structure). This approach conditions on parental genotypes to test for departures between the observed offspring genotype and that expected under Mendelian inheritance [39]. Family-based analyses were performed assuming an additive genetic model and employed the FBAT v1.7.3 software to perform single-SNP, multi-SNP and haplotype analyses [40-42].

Results

Marker-marker LD

The genomic region encompassing VEGF contains three distinct sets of SNPs that are in strong LD with each other (D' and r^2 values are shown in Figure 1). The first LD block extends from rs1547651 to rs2146323; the second contains rs3025020 and rs3025021; and the third block contains rs3025028, rs3025035 and rs3025053. The highest pairwise r^2 was observed between rs2010963 and rs833068 (0.988) in the first LD block (Figure 1).

Proportional hazards model with covariates

A number of significant ($p < 0.05$) associations were detected between DCCT baseline covariates, specifically triglycerides and HDL with certain VEGF SNPs (OLA Table 8). These were considered as possible confounders in subsequent multivariate association analyses. Prior to inclusion of SNPs, multivariate Cox PH analysis of time to SR revealed the following covariates to be significant at $P < 0.05$: secondary vs. primary cohort, gender, prior diabetes duration (joint effect of linear and quadratic terms), HbA1c at screening for DCCT eligibility, smoking (pack years) at DCCT baseline, updated mean HbA1c and updated hypertension (see Table 2, for

univariate and multivariate for other outcomes see OLA Tables 5 and 6).

Proportional hazards model for SR with SNPs

Only the severe retinopathy (SR) phenotype showed significant association in the global test which included all 16 VEGF SNPs ($P = 6.8 \times 10^{-5}$, Table 1). Therefore we focus further analyses solely on SR. (Results of single-SNP analyses of the renal phenotypes, ≥ 3 -steps progression and CSME are provided in the OLA Tables 9-12.) Single-SNP multivariate Cox PH analyses detected nominally significant ($p < 0.05$) associations between SR and each of rs699947, rs833070, rs2146323, rs3025010, rs3025021, rs3025028, rs3025007 and rs3025020 (Table 3). In contrast, only 3 SNPs (rs3025007, rs3025020 and rs3025035), achieved this criterion when examined in a single-SNP univariate model, suggesting that inclusion of covariates increases the sensitivity of the analysis (OLA Table 10 and OLA Supplementary material). The greater extremity of hazard ratio (HR) estimated in the multivariate model can be explained as a consequence of adjustment for imbalances in covariate distributions across genotypes. However, if strict Bonferroni adjustment are applied within VEGF then only rs3025021 would achieve significance ($p < 0.0031$). This SNP rs3025021 provided the highest HR estimate and the most significant association (HR=1.37, 95% CI [1.13-1.66], $P = 0.0017$). rs2010963, that lies in the promoter region, approached nominal 5% significance ($P = 0.078$). Except for rs699947 ($P = 0.074$), similar results were obtained when analyses were repeated using the subset of 1180 probands with complete genotype data for all 16 SNPs (OLA Table 12).

To assess whether a non-additive association may have been overlooked, we tested a quadratic term for the gene effect in the multivariate Cox PH model (OLA Table 13). Evidence for non-additivity was observed for rs3025030 ($p = 0.019$), likely due to the rare homozygous genotypes and to a less extent for rs833068 and rs3025007 ($P = 0.041$, and 0.042 , respectively). Given these marginal results, the additive model was applied to all subsequent analyses.

Multivariate multi-SNP analysis

To identify the most likely etiological variant(s) among the SNPs, we performed variable selection ($P < 0.05$) in multivariate Cox PH regression models for SR. Due to high LD ($r^2=0.998$) between rs2010963 and rs833068, the latter was excluded, leaving 15 SNPs for backward and forward variable selection analyses. Six SNPs were retained in the final backward model, whereas two were included in the forward selection (OLA Table 13). Of note, rs3025007 and rs3025021 were retained in both models. Application of a conservative, crude Bonferroni adjustment for 15 tests ($P < 0.0033$) to the final backward selection model suggests that rs699947, rs2146323, rs3024987, rs3025007, and rs3025021 are unlikely to be associated with SR by chance alone. The SNPs thus selected represent the first (rs699947, rs3024987 and rs2146323), and second LD blocks (rs3025021) as well as the region between these two blocks (rs3025007, Figure 1). While global tests for two-way SNP interactions in exploratory Cox PH analyses yielded consistent evidence for more complex combined SNP effects and/or potential information in haplotype phase [43], conservative Bonferroni adjusted p -values for single interaction terms were somewhat equivocal (OLA

Tables 14 and 15). Nevertheless, HRs for genotype combinations estimated in a six-SNP model with three two-way interactions, and classified into protective, neutral and risk categories (OLA Table 16) correspond to a broad range of risks for severe retinopathy.

Family-based analysis

Family-based analyses were performed for 4 SNPs that were genotyped in available first-degree relatives (OLA Table 2). To adjust for covariates, Martingale residuals from the multivariate Cox PH model for SR without genotypes were treated as a quantitative trait. Excess transmission was observed for alleles C of rs699947 ($Z=2.19$, $P = 0.029$) and T of rs3025021 ($Z=2.49$, $P = 0.013$) (Table 4), confirming the significant association of these SNPs that we observed in multi-SNP regression analyses. Therefore, we also examined rs699947 and rs3025021 jointly ($df=2$, $p=0.0068$) and in haplotype analyses: haplotype C-T showed significant excess transmission ($P = 0.035$), whereas A-C was significantly less transmitted ($P = 0.040$). Association with the prevalence of SR was also examined in relatives with diabetes who were genotyped at the 4 SNPs (see above), but Fisher exact tests detected no significant association (OLA Table 17).

Discussion

Multivariate single- and multi-SNP analysis for SR

We examined association between VEGF SNPs and time to event for three retinal and two renal outcomes in the DCCT/EDIC cohort. SNPs were selected from HapMap, SeattleSNPs, and from the literature [28] to capture common

variation spanning ~ 22 kb encompassing VEGF.

Our approach, capturing associations due to the most common variations in VEGF, and taking into account factors known to be associated with the development of complications, is more comprehensive than previous studies of DR that examined a single or few variations in only the promoter region of VEGF. Our use of tagSNPs representative of bins with high LD, facilitates detection of association between polymorphic variation in and around the gene with relative efficiency in genotyping.

We note the importance of including known risk factors in analyses aiming to identify gene effects. Single-SNP analyses identified 8 SNPs that were significantly associated with SR in the multivariate analyses ($P < 0.05$), while only 3 of these were significant in the univariate analysis, underscoring the importance of inclusion of measured covariates.

As described in the OLA, a number of associations between DCCT baseline covariates and SNPs were observed (OLA Table 6), and we considered possible collinearity and/or confounding effects. The association of rs2146323 and rs3025010 with SR was sustained when either triglycerides or other lipids were excluded from the multivariate model (data not shown) arguing against mediating effects.

We attempted to reduce false positive results due to multiple testing by employing strict Bonferroni correction criteria both for global tests of all SNPs with the five outcomes as well as for the single- and multi-SNP analyses for SR. In spite of this conservative correction, which assumes independence among markers (Figure 1), the strongest

association signal observed for rs3025021 nevertheless retains significance at the overall 5% level.

In the backward selection of 15 SNPs including all covariates, 6 SNPs remained in the model (Table 3). Both rs3025007 and rs3025021 were selected in backward and forward selection models. The finding that multiple SNPs make independent contributions suggests two possible explanations: either one or both of these SNPs are in high LD with an as yet unidentified variant, or that allelic heterogeneity in VEGF predisposes to higher risk of developing SR. As pointed out by Clayton et al [43], functional studies are necessary to distinguish the indirect effect of an unobserved locus from the direct effect of two loci on the same chromosome. Due to the low LD between these two SNPs ($D' = 0.13$, $r^2 = 0.08$) it is likely that at least two etiologic variants exist. In this case different possible mechanisms need to be investigated concerning how these variants are involved in the pathological process of retinopathy that include effects on VEGF gene expression, splicing or other mechanisms.

Family-based single-SNP and haplotype analysis

This is the first study to perform family-based analysis of VEGF SNPs with SR in T1D. Using Martingale residuals from the multivariate Cox PH models as a quantitative trait, alleles at rs699947 and rs3025021 exhibited significant excess transmission with SR. rs3025021 was most strongly associated with SR in the individual-level single-SNP analysis, while rs699947 approached nominal 5% significance ($HR = 0.83$, $P = 0.045$). Family-based haplotype analysis of rs699947 and rs3025021 confirmed that the haplotype consisting of their risk

alleles (C-T) was transmitted more often to those with high risk of developing SR ($P = 0.035$), whereas the haplotype consisting of protective alleles (A-C) was transmitted less often ($P = 0.040$). Association of rs3025010 was detected in the individual-level single-marker analysis but not in the family-based analysis. However, this SNP was not retained in the multi-SNP selection model, suggesting that its association in the single-SNP analysis is an indirect result of its high LD ($r^2 = 0.93$) with rs2146323.

In summary, both rs699947 and rs3025021 met criteria for significance in individual-based single-SNP analysis, were retained in the backward selection of the multiSNP regression analysis, and showed excess allele and haplotype transmission with the Martingale residuals in family-based analysis. The consistency of these findings argues for a true positive finding that cannot be explained by population stratification.

Comparison with previous studies of VEGF and retinopathy

Three studies [26, 28-29] investigated rs2010963, a promoter variant, of which two studies were performed in T2D [28, 29] and one in both T1D and T2D [26]. Two studies [26, 29] found no association between this SNP and DR. While rs2010963 (C on the forward strand) approached nominal significance with time to SR in the DCCT/EDIC probands in our single marker analysis (HR = 1.21, 95%CI, 0.98-1.50, $P = 0.078$), it was not retained in backward or forward selection models. Rs2010963(C) and rs833070 have been reported to be associated with a severe neovascular/exudative form of age-related macular degeneration (AMD) [44] using family and haplotype-based analysis. Another study found a significant

association of rs1413711 C genotype with AMD [45]. This marker falls in the same bin as rs833070, which we found to be weakly associated with SR in our single-SNP analysis. We speculate there may be a real signal in the first block underlying the association with SR observed in our study as well as in the other studies of DR and AMD. Inconsistencies as to which marker is most strongly associated in each study may depend on which SNPs were genotyped from the first block (Figure 1). Our multi-SNP results, however, support rs699947, rs2146323 and rs3024987 being independently responsible for the signal seen in the first LD block, which contains the promoter and the 5' region. The global multi-SNP test results in DCCT/EDIC for the two other retinal outcomes (≥ 3 -step progression and macular edema) were non-significant: for ≥ 3 -step progression this was likely due to it measuring change from baseline; the effect size for macular edema may be smaller for which we have low power.

Functional relevance

In *Ex-vivo* transfection experiments using COS cells [46], vectors containing the entire 5' UTR in-frame with the cDNA encoding the VEGF¹⁸⁹ isoform produced more VEGF protein in the presence of the C allele of rs2010963 in comparison to the G allele. Rs2010963 C/C genotype was found to be associated with higher fasting VEGF levels in healthy subjects [28]. There is substantial evidence supporting the association between VEGF levels and development of DR. Transgenic non-diabetic mice expressing even minimally elevated levels of human VEGF protein in the eye were found to develop clinical and pathological changes consistent with those seen in non-PDR and very early stages of PDR [47]. There was also a positive correlation

between VEGF expression and severity of retinopathy [47]. We speculate that the production of higher VEGF levels through the variants significantly associated with SR (or variants that are in high LD with them) is one possible mechanism by which these variants mediate their effect on DR. Because several variants survived the multi-SNP association model with SR, yet are not in LD with known promoter variants that affect VEGF expression, other mechanisms are possible including mRNA stability or splice site variation. Rs3025021, which shows one of the strongest associations with SR is highly conserved in 12 species, arguing in favor of its functional relevance. To date, seven splice forms of VEGF have been identified, including VEGF165 which is missing exon 6 and appears to predominate quantitatively and functionally in most angiogenic states. A recently described family of VEGF isoforms, termed VEGFb [48], is formed by alternative splicing which results in the inclusion of an 18-bp fragment, called exon8b, in place of exon 8. This family produces proteins of the same length as other forms, but with a different C terminal amino acid sequence [49], including VEGF165b. VEGF165b lacks exon7b but despite the presence of the receptor-binding domain it does not stimulate angiogenesis. Further it inhibits the proliferative, migratory and vasodilator effects of VEGF165. VEGFb inhibits VEGF induced angiogenesis in a one to one stoichiometric manner. Decreased expression of VEGF165b has been observed in human eye tissues from patients with diabetes [50]. Interfering with the splice format could be one mechanism for the association, however, none of the associated SNPs are located in conserved splice sites.

Conclusion

Our study extends previous reports in several respects. The retinal phenotypes in DCCT/EDIC study have been measured 17 times over 15 years leading to a more sensitive measure of the risk of complications. A strength of prospective studies such as DCCT/EDIC is the reliability of outcome and covariates measured repeatedly over an extended period of time. In contrast to case-control studies, this allows inclusion of important covariates measured prior to development of the outcome. All known risk factors for retinopathy were measured and included in the multivariate time-to-event analysis. As expected longitudinal measures of HbA1c over the period at risk had a strong relationship to development of SR. This is in stark contrast to cross-sectional studies which typically only have a single measure at the time of observation. Moreover, unlike previous studies that focused only on variants in the promoter region, we investigated tagSNPs that cover the entire gene. Under the assumption of prior evidence for association with VEGF, the current study provides strong evidence that more than one SNP in VEGF is independently associated with the risk of developing severe diabetic retinopathy in patients with T1D. This suggests that allelic heterogeneity is operating through one or more pathological mechanisms or that an as yet unidentified variant is underlying the observed association. The generalizability of these findings must be interpreted with some caution given the extensive inclusion and exclusion criteria of the original DCCT study, specifically the exclusion of SR at DCCT baseline (31). These findings add to the growing literature concerning the important role that VEGF plays in this diabetic complication. Addressing the mechanisms by which VEGF variants exert effects on

diabetic retinopathy should be a priority for further research.

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Electronic Database Information:

HapMap: <http://www.hapmap.org>

SeattleSNPs:

<http://pga.gs.washington.edu/>

UCSC Genome Browser:

<http://genome.ucsc.edu>

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Table 1. Distribution of the retinal and renal outcomes and results of the likelihood ratio test (LRT) based on multivariate Cox proportional hazard global test including all 16 VEGF SNPs

	Number of events (%)	Sample size	LRT (df=16)	P value
Retinal outcomes†				
Progression to \geq 3-steps	724 (61%)	1181	12.43	0.71
Severe retinopathy	220 (19%)	1180	46.98	6.8×10^{-5}
Clinical significant macular edema	226 (19%)	1181	23.34	0.105
Renal outcomes*				
Persistent microalbuminuria	219 (20%)	1123	14.76	0.54
Severe Nephropathy	98(8%)	1181	18.21	0.31

Legend to table 1: †Retinal outcomes are from data up to EDIC year 10. *Renal outcomes include data up to EDIC year 8.

Table. 2 Covariate associations in multivariate Cox proportional hazards model for time to severe retinopathy

Covariate	HR	95% CI	P value
Secondary intervention vs. Primary prevention cohort	2.34	1.42-3.85	8.6 x 10 ⁻⁴
Intensive vs. Conventional treatment	0.82	0.42-1.59	0.56
Cohort by Treatment Interaction	0.93	0.45-1.91	0.85
Age at diagnosis (years)			0.25 *
Contrast between age of diagnosis at 30 vs. 20 years	1.18	0.86-1.63	0.30 §
Type 1 diabetes duration (years) at DCCT baseline			<10 ⁻⁴ *
Contrast between 3.5 and 1.5 years of diabetes duration	1.26	0.98-1.62	0.071 §
Contrast between 12 and 6 years of diabetes duration	3.08	2.34-4.05	<10 ⁻⁴ §
Female vs. Male gender	0.73	0.54-0.97	0.032
BMI: 10% increase (25.7 vs 23.4 kg/m ²) ¶	0.99	0.88-1.10	0.79
mean arterial blood pressure: 10% increase (95.1 vs 85.6 mmHg) ¶	1.00	1.00-1.00	0.66
Triglyceride: 10% increase (90.0 vs 81.8 mg/dl)¶	1.02	0.98-1.05	0.37
HDL cholesterol: 10% increase (55.4 vs 50.4 mg/dl)¶	1.03	0.96-1.10	0.36
Total cholesterol: 10% increase (194.9 vs 176.2 mg/dl)¶	0.99	0.91-1.08	0.82
HbA1c at DCCT Eligibility: 10% increase (9.94 vs 9.04%)	1.15	1.12-1.19	0.0025
Smoking at DCCT baseline (pack/yr)	0.97	0.94-0.99	0.0051
Updated mean HbA1c measured during DCCT/EDIC: 1 unit (8.0 vs 7.0 %) increase	2.63	2.22-3.11	<10 ⁻⁴
Updated hypertension indicator during DCCT/EDIC	1.75	1.26-2.43	8.9 x 10 ⁻⁴

Legend to Table 2: Sample size=1367, HR = hazard ratio, 95% CI = 95% confidence interval, ¶= 10% increase from the mean value at DCCT baseline, *=P value from 2df Wald test for joint contribution of linear and quadratic trend, § = p value from 1df Wald test derived from linear contrast of linear and quadratic trend.

Table 3. Single marker univariate and multivariate Cox proportional hazards analysis of VEGF SNPs with severe retinopathy.

Marker	Minor allele(%)	Strand	Sample Size	Univariate			Multivariate		
				HR	95%CI	P value	HR	95%CI	P value
rs1547651	T(16.4)	forward	1353	1.12	0.87-1.43	0.38	1.02	0.78-1.32	0.89
rs699947	A(49.5)	forward	1344	0.90	0.75-1.07	0.23	0.83	0.69-1.00	0.045
rs2010963	G(31.8)	reverse	1313	1.13	0.93-1.37	0.22	1.21	0.98-1.50	0.078
rs3024987	A(12.6)	reverse	1362	1.04	0.81-1.33	0.78	1.05	0.81-1.36	0.72
rs833068	A(32.4)	forward	1331	1.10	0.90-1.33	0.36	1.20	0.97-1.48	0.093
rs833070	A(49.7)	forward	1329	0.88	0.74-1.05	0.16	0.82	0.68-1.00	0.045
rs3024994	A(4.9)	reverse	1362	1.12	0.75-1.66	0.58	1.22	0.80-1.85	0.36
rs2146323	A(35.7)	forward	1362	0.89	0.74-1.08	0.23	0.77	0.63-0.94	0.011
rs3025007	A(44.0)	reverse	1358	0.80	0.67-0.96	0.017	0.80	0.66-0.96	0.017
rs3025010	C(37.8)	forward	1340	0.87	0.72-1.05	0.13	0.75	0.62-0.92	0.0052
rs3025020	T(32.0)	reverse	1361	0.82	0.67-0.99	0.039	0.75	0.61-0.92	0.0051
rs3025021	T(32.3)	forward	1341	1.19	0.98-1.43	0.073	1.37	1.13-1.66	0.0017
rs3025028	G(43.4)	reverse	1366	1.10	0.92-1.32	0.29	1.24	1.03-1.49	0.026
rs3025030	G(13.3)	reverse	1307	1.06	0.81-1.40	0.66	1.06	0.80-1.42	0.67
rs3025035	A(7.2)	reverse	1362	1.36	1.01-1.85	0.044	1.23	0.88-1.71	0.22
rs3025053	A(11.0)	forward	1362	1.05	0.79-1.39	0.76	1.06	0.79-1.44	0.69

Legend to Table 3: HR = hazard ratio, 95% CI = 95% confidence interval. The results are shown for the available genotype data set for each marker using an additive mode with the major allele frequency (i.e. common homozygote) used as the reference. Results of the same analysis for the common data set (1180 individuals) are provided in OLA Tables 9 and 12. The covariates included are listed in the OLA Supplementary Text (and Table 2 for the multivariate model).

Table 4. Family-based association analyses between VEGF markers and Martingale residuals from the Cox PH model of severe retinopathy via FBAT v1.7.3 software assuming an additive model of inheritance.

Marker(s)	Allele (Frequency)	Number of informative families	Z Statistic	P-value
Single marker analyses				
rs1547651	T (0.182)	291	0.66	0.51
rs699947	C (0.502)	441	2.19	0.029
rs3025010	T (0.614)	427	1.32	0.19
rs3025021	T (0.33)	548	2.49	0.013
Multi-maker analyses				
4-marker test of rs1547651, rs699947, rs3025010, and rs3025021: S_MM = 12.30; df = 4; P value = 0.015				
2-marker test of rs699947, and rs3025021: S_MM = 9.97; df = 2; P value = 0.0068				
Haplotype analyses of rs699947 and rs3025021				
1	A-C (0.372)	435	-2.052	0.040
2	C-C (0.321)	421	0.067	0.94
3	C-T (0.184)	328	2.098	0.035
4	A-T (0.123)	251	0.286	0.77

Figure 1

	rs1547651	rs699947	rs2010963	rs3024987	rs833068	rs833070	rs3024994	rs2146323	rs3025007	rs3025010	rs3025020	rs3025021	rs3025028	rs3025030	rs3025035	rs3025053
rs1547651		0.98	1.00	0.94	1.00	0.96	1.00	0.94	0.81	0.94	0.03	0.03	0.07	0.32	0.24	0.52
rs699947	0.44		0.98	0.99	0.98	0.98	1.00	0.98	0.42	0.94	0.26	0.20	0.09	0.00	0.18	0.18
rs2010963	0.30	0.66		1.00	0.99	0.99	1.00	0.99	0.97	0.99	0.02	0.14	0.02	0.13	0.01	0.86
rs3024987	0.16	0.37	0.26		1.00	1.00	1.00	1.00	0.94	0.94	0.69	0.18	0.04	0.28	0.09	0.59
rs833068	0.31	0.67	0.99	0.26		1.00	1.00	0.99	0.97	0.98	0.02	0.14	0.03	0.11	0.01	0.87
rs833070	0.43	0.16	0.67	0.38	0.69		1.00	0.99	0.43	0.95	0.25	0.20	0.08	0.00	0.16	0.17
rs3024994	0.10	0.16	0.16	0.09	0.16	0.22		1.00	1.00	1.00	0.66	0.71	0.64	0.74	0.03	0.65
rs2146323	0.56	0.74	0.51	0.28	0.51	0.75	0.17		0.09	0.98	0.28	0.35	0.43	0.24	0.01	0.45
rs3025007	0.32	0.38	0.59	0.40	0.60	0.38	0.20	0.08		0.12	0.02	0.13	0.03	0.04	0.39	0.14
rs3025010	0.54	0.75	0.53	0.28	0.53	0.76	0.18	0.93	0.10		0.30	0.35	0.37	0.23	0.05	0.40
rs3025020	0.02	0.18	0.01	0.18	0.01	0.17	0.10	0.26	0.01	0.26		0.99	0.93	0.67	0.97	0.70
rs3025021	0.02	0.14	0.14	0.10	0.14	0.14	0.11	0.18	0.08	0.19	0.47		0.97	0.96	0.94	1.00
rs3025028	0.03	0.08	0.01	0.02	0.01	0.07	0.17	0.28	0.02	0.26	0.56	0.77		0.99	0.95	0.78
rs3025030	0.06	0.00	0.07	0.04	0.06	0.00	0.07	0.12	0.02	0.11	0.18	0.26	0.34		1.00	1.00
rs3025035	0.15	0.05	0.00	0.06	0.005	0.04	0.00	0.00	0.10	0.02	0.19	0.18	0.23	0.11		0.52
rs3025053	0.80	0.06	0.21	0.08	0.21	0.06	0.42	0.12	0.06	0.11	0.17	0.24	0.31	0.14	0.06	