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## Challenges in elucidating the genetics of diabetic retinopathy

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### Abstract

**Importance**—In the past decade, significant progress in genomic medicine and technological advances have revolutionized our approach to common complex disorders in many areas of medicine, including ophthalmology. A major disorder that still needs major genetic progress is diabetic retinopathy (DR), one of the leading causes of blindness in adults.

**Objective**—To perform a literature review, present the current findings, and highlight some key challenges.

**Methods**—Thorough literature review of the genetic factors for DR, including heritability scores, twin studies, family studies, candidate gene studies, linkage studies, and genome-wide association studies (GWAS).

**Results**—While there is clear demonstration of a genetic contribution in the development and progression of DR, the identification of susceptibility loci through candidate gene approaches, linkage studies, and GWAS is still in its infancy. The greatest obstacles remain a lack of power due to small sample size of available studies and a lack of phenotype standardization. In this review, we also discuss novel technologies and novel approaches, such as intermediate phenotypes for biomarkers, proteomics, metabolomics, exome chips, and next-generation sequencing that may facilitate future studies of DR.

**Conclusions and Relevance**—The field of the genetics of DR is still in its infancy and is a challenge due to the complexity of the disease itself. This review outlines some strategies and lessons for future investigation to improve our understanding of this most complex of genetic disorders.

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## INTRODUCTION

Diabetic retinopathy (DR), an important microvascular complication of diabetes mellitus (DM), is a leading cause of visual impairment in adults 20 to 74 years of age<sup>1</sup>. Over 93 million people worldwide have DR, 17 million of whom have proliferative diabetic retinopathy (PDR) and 28 million of whom have vision-threatening DR<sup>2</sup>. This number will continue to escalate with an aging population, increasing obesity, and a rapidly progressing diabetes epidemic. More individuals, especially Hispanics, people of African descent, and Asians, will be vulnerable to blinding DR in coming years<sup>3,4</sup>. There is clearly a need to develop strategies to identify at-risk individuals for early interventions.

In comparison to other major causes of visual loss such as age-related macular degeneration (AMD)<sup>5</sup>, myopia<sup>6,7</sup> and glaucoma<sup>8,9</sup>, the search for genetic clues to DR has not progressed as rapidly. To date, few studies which have reported on possible susceptibility genes for DR have yielded inconsistent results. There is clearly a familial relationship in DR, as twin and family studies indicate a genetic basis<sup>10-17</sup>. Several candidate gene studies have reported promising genes<sup>18-21</sup> but few of them have been replicated, and the few positive findings show only weak genetic associations<sup>18-20,22</sup>. In genome-wide approaches, three linkage studies performed in Pima Indians and Mexican Americans have identified regions on chromosomes 1, 3, and 12 to be suggestive with DR<sup>13,23,24</sup>.

In contrast to AMD, myopia and glaucoma, very few genome-wide association studies (GWAS) have been conducted thus far on DR. The few GWAS are of modest sample sizes in Hispanics, Chinese, and Caucasian populations and have reported borderline associations with DR in either type 1 or type 2 diabetes<sup>25-28</sup>.

In this review, we will highlight these key genetic studies of DR with an emphasis on the most recent developments. We will also discuss issues and challenges with elucidating the genetics of DR and indicate approaches that will provide the opportunity to advance our knowledge of this complex genetic disorder.

## DEFINITION AND CLASSIFICATION OF DR

The diagnosis of DR is clinically defined by the presence of retinal microvascular lesions in subjects with diabetes; however these retinal lesions are not specific and may also be present in subjects without diabetes<sup>29,30</sup>. The classification of DR is graded by severity and divided into non-proliferative diabetic retinopathy (NPDR) and PDR. Key retinal changes in NPDR include microaneurysms, hard exudates, cotton wool spots, intraretinal microvascular abnormalities, and venous beading; these further subdivide NPDR into mild, moderate, and severe forms. Key retinal changes in PDR include neovascularization of optic disc or elsewhere, preretinal hemorrhage, or vitreous hemorrhage. On the other hand, clinically significant macular edema (CSME), which is graded as its own entity, can develop at any stage of the DR spectrum. Thus, the various classifications in DR grading, resulting in heterogeneity of DR phenotype, pose a significant challenge in any genetic study like DR. The assessment of DR via a standardized stereoscopic photograph has been proposed to overcome this issue and more researchers have utilized this approach by adopting and grading DR using the Early Treatment Diabetic Retinopathy Study (ETDRS) severity scale or a similar modification. Recently, the assessment of DR and diabetic macular edema (DME) via optical coherence tomography (OCT) has been proposed as an imaging modality to better visualize the intra-retinal morphological changes in subjects with diabetes<sup>31</sup>; however, the classification of DR and DME via OCT has not been clearly defined nor adopted for use.

## GENETICS OF DIABETES

DR occurs on the background of diabetes. That genetic factors play a major role in etiology of diabetes has long been appreciated due to ethnic differences in frequency, increased familial aggregation, and a dramatically higher concordance in monozygotic versus dizygotic twins. This is true for each of the major subforms of diabetes, type 1 and type 2.

To date, approximately 60 loci have been successfully identified for type 2 diabetes, of which only 3 were discovered prior to the GWAS era<sup>32, 33</sup>. Although most of these studies were carried out in individuals of European descent, more recent studies of Asians<sup>34</sup>, Hispanics<sup>35</sup>, and African-Americans<sup>35-37</sup> have also demonstrated some level of associations for these signals, supporting the hypothesis that these signals (or the causal variants that are in high linkage disequilibrium with these signals) are likely common alleles that are widely distributed in the human population and each contributing a small effect on disease risk<sup>32</sup>.

These important discoveries through large collaborative efforts by GWAS approaches have led to substantial progress in the understanding of genetics in type 2 diabetes, leading to the identification of novel pathways, demonstrating mechanistic associations, and supporting prior epidemiological studies<sup>38</sup>. These findings have illustrated some important key lessons that are useful in other genetics studies like DR. One, joining forces by international collaborative efforts is necessary to increase statistical power by increasing sample size<sup>38</sup>. Two, are the analysis methods. Both analysis by treating the phenotype as a dichotomous trait and analysis of a related, quantitative trait are useful<sup>38</sup>. Three, connection of genetic findings with more defined physiological parameters increases understanding<sup>38</sup>. Elucidating the genetic basis of type 2 diabetes offers an ideal model to approach the genetic study of DR. However, it should be apparent that the phenotype of type 2 diabetes does have several advantages, such as the ease of classification, and readily available large samples of subjects even without detailed assessment.

## ENVIRONMENTAL FACTORS FOR DR

The etiology of DR remains complex and poorly understood. Large epidemiological studies have consistently demonstrated that the duration of diabetes and adequacy of glycemic control are two of the major contributors to the development and progression of DR<sup>2, 39, 40</sup>. This was robustly documented in the Wisconsin Epidemiological Study of Diabetic Retinopathy (WESDR) study, which found that duration of diabetes was the strongest predictor for progression of DR, with prevalence of DR varied from 17% in type 1 DM to 29% in type 2 DM for subjects with diabetes for less than 5 years and the rate increasing dramatically to almost 100% for type 1 DM and 78% for type 2 DM in subjects with diabetes for more than 15 years<sup>41, 42</sup>. In other landmark studies, such as the Diabetes Control and Complications Trial (DCCT), UK Prospective Diabetes Study (UKPDS), the ACCORD Eye, and META-EYE study groups, intensive glycemic control was effective in reducing the rate of DR progression in both type 1 and type 2 diabetes<sup>2, 39, 40, 43</sup>. Other studies have also demonstrated that blood pressure control is another risk modifier<sup>2, 44, 45</sup>, although some studies did not support this finding<sup>43</sup>. Very recently, there is increasing evidence supporting an association between dyslipidemia and diabetic retinopathy<sup>46</sup>; the Fenofibrate Intervention and Event Lowering in Diabetes Study (FIELD)<sup>47</sup> as well as the ACCORD eye study<sup>43</sup> both demonstrated that reduction in lipids could also limit DR progression.

Despite strong evidence for DR susceptibility, these environmental risk modifiers by themselves do not account for the complete risk susceptibility. First, this is exemplified by clinical observations that some individuals develop DR despite good glycemic control and short duration of disease, while others do not develop DR, even with poor glycemic control

and longer duration of diabetes<sup>48</sup>. Second, the strongest environmental factors (duration of diabetes and glycosylated hemoglobin) only explained about 11% of the variation in retinopathy risk in the DCCT trial<sup>49, 50</sup>. Similarly, a combination of glycosylated hemoglobin, blood pressure, and total cholesterol only explained about 10% of the variation in retinopathy risk in the WESDR study<sup>51</sup>, suggesting that the remaining ~90% of the variation in retinopathy risk is presumably explained by other risk factors. Finally, population studies show that retinopathy signs such as microaneurysms was detectable in 7~13% of non-diabetics as well as in subjects where glycosylated hemoglobin level was well below 5%<sup>29</sup> and that SNPs associated with diabetes or hypertension were not associated with retinopathy in individuals without diabetes<sup>52</sup>, suggesting that other risk factors, independent of hyperglycemia and diabetes, contribute to the development and progression of retinopathy signs similar to DR.

## GENETIC FACTORS FOR DR

Attempts to identify gene or genes in the development of DR have been conducted over the past few decades. To date, these studies have been limited to twin studies<sup>10</sup>, family studies<sup>11–17</sup>, candidate gene studies<sup>18–22</sup>, linkage studies<sup>13, 23, 24</sup>, and small-scale GWAS with modest sample sizes<sup>25–28</sup>.

In support of a genetic hypothesis of DR, several studies have shown a discrepant rate of the prevalence of DR among US populations, with a significantly higher prevalence observed in Hispanics, African-Americans, and Chinese-Americans<sup>3, 4</sup>. In comparison to whites, other risk modifiers such as duration of diabetes, glycemic control, and blood pressure appear to account for the higher prevalence of DR observed in African-Americans, but these factors do not explain the higher prevalence seen in the Hispanics<sup>53–56</sup>, suggesting that other factors, including genetic factors, may influence susceptibility to DR.

### Twin and Family Studies

In twin studies, Leslie and Pyke found the same degree of severity in 95% (35 of 37) concordant type 2 diabetic twins, compared to only 68% (21 of 31) in concordant type 1 diabetic twins<sup>10</sup>. This early observation was extended by familial aggregation studies, with siblings and relatives of diabetics with DR having as high as a 3-fold increased risk for DR compared to siblings and relatives of diabetics without DR<sup>11–17</sup> (Table 1). This trend was seen in either type 1 or type 2 diabetes and also across different ethnicities. Furthermore, evidence for a familial aggregation is more consistently seen in the presence of more severe retinopathy and less in the presence of any retinopathy, with heritability scores ranging from 18–27% for any DR<sup>13, 17</sup> and 25–52% for PDR<sup>12, 17</sup> in either type 1 or type 2 diabetes (Figure 1). Thus, as previously mentioned, it is important to not only provide a standardized assessment of DR, but also to compare the “no DRs” with the “more severe stages of DR”. The Family Investigation of Nephropathy and Diabetes (FIND)-Eye study, where nearly half of their 2368 diabetic subjects are Mexican-Americans, demonstrated the heritability of any DR in this population of type 2 diabetes is as high as 24%<sup>17</sup>.

### Candidate gene studies

Most genetic research in DR have utilized the candidate gene approach. Several pathways and processes have been proposed to play an important role in the pathogenesis of DR. This led to the testing of a number of hypothesized candidate genes. Though a number of candidate genes and genetic variants have been proposed in the literature, few of them have been replicated, and the few positive findings only showed weak genetic associations with DR<sup>18, 19</sup>.

In this approach, the analysis compares the frequency of a particular genetic variant in subjects with (cases) or without DR (controls). Several pathways and processes have been proposed, including the renin-angiotensin system, glucose-induced pathways, vascular endothelial dysfunction, tissue matrix remodeling, and angiogenesis<sup>18, 19</sup>. Potential candidate genes involved in these pathways and processes include angiotensin-I converting enzyme (*ACE*), angiotensin II type 1 receptor (*AGTR1*), angiotensinogen (*AGT*), vascular endothelial growth factor (*VEGF*), aldose reductase (*AKR1B1*), receptor for advanced glycation endproducts (*RAGE*), glucose transporter 1 (*GLUT1*), apolipoprotein E (*APOE*), methylenetetrahydrofolate reductase (*MTHFR*), plasminogen activator inhibitor-1 (*PAI-1*),  $\alpha 2\beta 1$  Integrin (*ITGA2*), peroxisome proliferator-activated receptor gamma (*PPAR- $\gamma$* ), and nitric oxide synthases (*NOS3*). Their associations or lack thereof with DR have been extensively documented in prior reviews<sup>18–21</sup>. Here, we summarize the most important findings with a focus on aldose reductase (*AKR1B1*) and vascular endothelial growth factor (*VEGF*), because of their biological implications.

Aldose reductase (*AKR1B1*) is an enzyme which catalyzes the reduction of glucose to sorbitol during glucose metabolism. Increased activation of aldose reductase has been shown to induce metabolic and biochemical changes, leading to the development of early DR as well as PDR<sup>57, 58</sup>. For these reasons, *AKR1B1* was proposed as a highly suspect candidate for genetic association studies in DR. While a great deal of prior work have shown inconsistent results, a recent meta-analysis by Abhary et al. examining 20 candidate genes in DR found that variants in aldose reductase had the most significant association with DR.<sup>20</sup> In particular, the meta-analysis identified the z-2 microsatellite confers risk of DR (OR=2.33, 95% CI=1.49~3.64, p=0.0002) in either type 1 or type 2 diabetes. This trend was similar and significant in the subgroup analysis of NPDR (p=0.0075) and PDR (p=0.0023). On the other hand, the z+2 microsatellites conferred protection against overall DR (OR=0.58, 95% CI=0.36~0.93, p=0.02), but this association was only seen in subjects with type 1 diabetes, not type 2. It was independent of the studied ethnicity. In addition, a few studies examining the association of another *AKR1B1* polymorphism at the promoter (rs759853) found that the T allele confers protection for DR (OR=0.49, 95% CI=0.36~0.68, p<0.0001) in type 1 diabetes, but was not significant in type 2 diabetes<sup>20</sup>.

Vascular endothelial growth factor (*VEGF*), a key player involved in angiogenesis and a potent mediator of vascular permeability, is activated by microvascular changes associated with diabetes due to hypoxia. This activation of *VEGF* leads to breakdown of the blood-retinal barrier and retinal neovascularization<sup>59, 60</sup>. Conversely, anti-VEGF therapies, such as bevacizumab (Avastin, Genentech), ranibizumab (Lucentis, Genentech), and aflibercept (Eyelea, Bayer and Regeneron) have been shown to ameliorate these changes<sup>59, 61, 62</sup>. A number of polymorphisms (rs2010963<sup>20, 21, 63–71</sup>, rs25648<sup>20, 63, 68</sup>, rs1570360<sup>20, 63, 72</sup>, rs3095039<sup>20, 63, 70</sup>, rs35569394<sup>20, 73</sup>, rs699947<sup>20, 66, 74–77</sup>, rs13207351<sup>63, 72, 75</sup>, rs735286<sup>72</sup>, rs2146323<sup>72, 77</sup>, rs833061<sup>63, 68, 69, 75</sup>, rs3025021<sup>75, 76</sup>, rs10434<sup>76</sup>, rs833068<sup>76</sup> and rs833070<sup>77</sup>) in *VEGF* have been analyzed either with DR or severe DR. The only conclusive finding from these efforts is that the C allele of rs2010963 (-634C/G), though insignificantly associated with DR or PDR, does confer risk for NPDR (OR=1.61, 95% CI=1.23~2.10, p=0.0005) in the meta-analyses<sup>20, 21</sup>.

A number of other individual candidate genes have been examined with DR<sup>20, 65, 78–94</sup> and their findings are summarized (Table 2). However, it is difficult to draw any conclusions from these studies, since the sample sizes of individual studies were often quite small. The P-values obtained from these efforts are sometimes nominally significant, but cannot withstand corrections for multiple testing. In most cases, no replication has been attempted. Furthermore, there are also conflicting findings from multiple studies. Although meta-analysis techniques have been undertaken, findings remain largely inconclusive due to



problems with analysis in multiple and different ethnicities (direction of effect and allele frequencies may be different), publication bias, and lack of standardization for DR phenotype.

Thus, alternatively, two studies with a larger scale have examined candidate genes and DR using an approach that mimics a genome-wide approach. This methodology is useful when the effect sizes of individual variants, such as DR, are small and the study population is limited. The first study, the Candidate gene Association Resource (CARE) did not find genes previously associated with type 2 diabetes, diabetic nephropathy, and diabetic retinopathy to be associated with DR<sup>22</sup>. The most interesting finding from this study is that variants in the P-selectin (*SELP*), after adjusting for known DR risk factors, remained significantly associated with DR in the European Americans, but was not seen in the African-Americans, Hispanic Americans, or Asian Americans<sup>22</sup>. The second study, examining 193 candidate genes with DR of type 1 diabetic African-Americans, found nominal associations in 13 genes with progression of DR<sup>95</sup>. A number of these genes are involved in pathways related to glucose metabolism, inflammatory processes, angiogenesis/vascular permeability, insulin signaling, retinal development, or blood pressure regulation, highlighting not only the implications of these genes but suggesting that a number of biological pathways are simultaneously involved in DR. Even with these large-scale attempts of examining candidate genes in DR, no definite conclusion can be drawn at this time without replication efforts in larger cohorts.

### Linkage studies

A potential problem with the candidate gene approach is its basis depends on an *a priori* hypothesis implicating that a particular gene of interest plays a functional importance in the pathophysiology of DR. If the hypothesis is wrong, then the genetic association will be negative or inconsistent. This has led to hypothesis-free approaches (also known as agnostic approaches), first by linkage, and recently, by GWAS. In these two approaches, no initial biochemical or pathophysiological induction is proposed; the results are instead, driven by chromosomal location.

Linkage analysis is based on the principles of genetic recombination to map genomic regions by the observations seen in family members. It is based on the assumption of co-segregation of genetic marker with DR susceptibility loci within the family. If linkage is present, the marker is inherited together with the causal variant. If it is not present, the marker is inherited independently. As a result, the closer the physical distance of the marker to DR susceptibility loci, the stronger the evidence is for linkage.

Linkage analysis has been the mainstay approach for studying Mendelian disorders, and has succeeded for a handful of common complex disorders such as Crohn's disease; the latter success with the identification of *NOD2/CARD15* on chromosome 16<sup>96</sup>. However, certain presentations of DR pose significant challenges in family studies. For example, the late-onset of DR, especially for those with type 2 diabetes, suggests that the parents of the proband are often deceased, leaving only one generation of family members available to study. Thus, other study designs, such as sib-pair analysis, have been the dominant model used for linkage studies in DR.

Three linkage studies performed in Pima Indians and Mexican Americans have implicated regions on chromosomes 1, 3, and 12 for DR (Table 3)<sup>13, 23, 24</sup>. However, with the possible exception of 1p36, none of these regions reached genome wide statistical linkage significance of a LOD (logarithm of odds) score > 3.3. One study demonstrated a LOD of 3.01 for single-point and 2.58 for multiple point analysis at 1p36 in the Pima Indians, indicating suggestive evidence of linkage for DR in this population<sup>13</sup>. There are several

limitations with this approach. One, linkage studies only offer rough estimates of the genomic region as the mapping resolution is generally low, literally a test of millions of basepairs. Much more extensive efforts are required to pinpoint specific causal variants responsible for DR. Second, linkage studies often benefit from large families. Linkage studies on DR have thus far been conducted in Pima Indians and Mexican Americans, where large families are available for study. It has not been reported in other ethnicities. Third, the effect size (penetrance) of individual variants may be of sufficiently small magnitude that most study would be underpowered to detect genomic locations via co-segregation expected for complex multifactorial disorders like DR.

### Genome-Wide Association Studies (GWAS)

More recent technological advances have revolutionized the field toward the second hypothesis-free generating approach, GWAS, in which hundreds of thousand and even millions of single-nucleotide polymorphisms (SNPs) can be tested against traits such as DR. These developments include microarray-based technology with tag SNPs, utilizing the concept of linkage disequilibrium where adjacent or correlated SNPs co-segregate together in populations. Data from publicly available database such as the HapMap and the 1000 Genome Project have been instrumental in developing such arrays.

Since the first reported success of a well-designed GWAS just six years ago, more than 2000 loci have demonstrated significant and often replicated associations with one or more common complex disorders<sup>32</sup>. While this field has received a number of criticisms, the reality is that the use of GWAS has been the most successful approach in the genetics of common diseases to date. The utilization of this technology in the study of DR is relatively recent. Four small-scale GWAS with modest sample sizes conducted in Mexican-American, Chinese, and Caucasian populations have found borderline or weak associations with DR in either type 1 or type 2 diabetes<sup>25-28</sup>. One study, conducted in 103 cases (subjects with moderate-to-severe NPDR and PDR) versus 183 controls (subjects with normal to early NPDR) of Mexican-Americans, found borderline significance with DR at 6 loci<sup>25</sup> (Table 4). The second study conducted in 174 cases (subjects with NPDR and PDR) versus 675 controls (subjects who are diabetics with no DR and non-diabetics) in the Chinese found several SNPs that have appeared to attain genome-wide statistical significance with DR<sup>26</sup> (Table 4). The main problem in this latter study was the utilization of all 6 genetic models (genotype, allele, trend, additive, dominant, and recessive) simultaneously in their analysis to determine the most significant P-value. Had proper corrections for multiple testing been used, the stringent cut-off for P-value should have been multiplied by 6, due to 6 different genetic models run on each SNP. In this way, none of the SNP or loci reached typical genome-wide “statistical significance” of P-value  $10^{-8}$  after correction for multiple comparisons. The third study conducted in 973 cases (subjects with PDR and diabetic macular edema) versus 1856 controls (all others including NPDR) of Caucasian type 1 diabetics found borderline significance at several SNPs/loci with DR in a combined meta-analysis<sup>27</sup> (Table 4). However, a replication analysis conducted on the top signals in the WESDR type 1 diabetic did not confirm these associations<sup>97</sup>. The most recent DR GWAS study, conducted by the authors and colleagues, compared 1007 Chinese type 2 diabetic subjects with “extreme DR phenotype”, defined as 570 individuals with diabetes 8 years duration without DR (controls) versus 437 individuals with PDR (cases) (Table 4). Both groups had similar levels of HbA1c and duration of diabetes, two of the most important epidemiological confounders in the study of DR. Association analysis resulted in 3 top loci. Though borderline significant, the authors hypothesized that if the detected loci are true associations with DR, then subjects with the clinically intermediate eye phenotype (NPDR) would have intermediate frequencies of the risk allele. They then extended these top findings to 479 subjects with NPDR and did observe that the risk allele of the top 3 SNPs

had an intermediate frequency in the NPDR group, suggesting potential DR susceptibility genes in the Chinese that are independent of the level of HbA1c and diabetes duration<sup>28</sup>. To summarize the GWAS of DR to date, none of the regions reached genome-wide statistical significance. Some of the limitations in these studies include very modest sample size by GWAS standard, combining heterogeneous phenotypes (subjects with PDR, NPDR, diabetic macular edema) as cases, poor characterization of normal subjects (subjects with no DR) as these subjects are often only assessed one point in time, and poor DR standardization<sup>25–28</sup>.

## FUTURE DIRECTIONS

### Biomarkers, Proteomics, and Metabolomics

An interesting approach to find genetic susceptibility genes in DR is through the intermediate associations with biomarkers (also known as intermediate phenotypes), an approach analogous to that of the investigations seen in lipids with myocardial infarctions<sup>98</sup> and glucose related traits/obesity with type 2 diabetes<sup>99, 100</sup>. A number of systemic biomarkers have been a subject of investigation for association with DR. Many of these biomarkers are related to markers of systemic inflammation<sup>101–106</sup>, angiogenesis<sup>106</sup>, endothelial dysfunction<sup>102</sup>, insulin resistance<sup>101</sup>, hemostatic disturbance<sup>103</sup>, and homocysteinemia<sup>102, 103</sup>, suggesting that one or more of these processes are involved in the pathogenesis of DR. Analyzing the genetic associations of these biomarkers (genes for the quantitative assessments of biomarkers) might shed some important knowledge about the genetic interplays that are responsible for the development and progressions of DR.

Similarly, the investigation of proteomics and metabolomics and its relationship to the genome (also called functional genomics) will be another area of investigation in the study of DR. Proteomics is a large-scale study of the structure and function of proteins. A prior study examining the vitreous proteome in non-diabetic, diabetic without DR, and PDR subjects using label-free mass spectrometry-based spectral counting approaches found a number of proteins associated with key biological pathways in the kallikrein-kinin, coagulation, and complement systems to exhibit protein alterations in subjects with PDR compared to the other groups<sup>107</sup>. A review of key findings of proteomics in diabetic retinopathy of both animal and human studies concluded that multiple proteins such as apolipoprotein A-I and apolipoprotein H are more likely to contribute to retinal pathology than single proteins alone<sup>108</sup>.

Metabolomics is a global measurement of the immediate cellular state within a given biological system, taking into account the genetic profiles, altered enzymatic activities, environmental and lifestyle factors. A recent small study, examining the metabolomics in DR of 89 Chinese patients, found disturbances in fatty acid (stearic acid, linoleic acid, arachidonic acid), amino acids (aspartic acid) and glucose alterations to vary differently among diabetics without DR, NPDR, and PDR subjects<sup>109</sup>. Though the study of metabolomics in ophthalmology is rather new, its applications in other fields such as oncology has demonstrated successful clinical utility, ranging from quantitative assessment of metabolomic biomarkers for cancer diagnosis, optimization of therapeutic agents, evaluation of treatment efficacy and response, and prediction of treatment toxicity or resistance<sup>110</sup>. In the future, the application of proteomics and metabolomics to the study of DR may facilitate in the discovery, identification, or quantification of biomarkers to aid in early disease detection, diagnosis, and treatment response.

### Next-Generation Sequencing and Exome Chip studies

Massively parallel sequencing technology has been a breakthrough in the transformation of genomic medicine for Mendelian disorders. With high-throughput sequencing, scientists have been able to utilize large amounts of sequenced data with lower-cost reads to address a



range of biological diseases<sup>111–113</sup>, the origin of human protein-coding variants<sup>114</sup>, as well as determine population-specific whole-genome sequencing databases<sup>115</sup>. Although current exome sequencing studies are well powered to discover functional variants, current exome sequencing studies are not as well powered to establish an association. Thus, the exome chip was design to provide a cost effective way to examine large number of samples. The exome chip array was designed to test ~250,000 SNPs covering putative functional exonic variants (non-synonymous variants, splice variants, stop altering variants, etc.) from a range of diseases and populations<sup>116</sup>. This approach has been successfully applied to the identification of low-frequency and rare nonsynonymous variants that contribute to processes such as fasting insulin processing and secretion in non-diabetic subjects<sup>117</sup>. It is without a doubt that the future directions in the genetics of DR will encompass a number of these novel technologies.

## KEY POINTS & STRATEGIES

Thus, to approach the genetics of DR in a systematic way would require large collaborative efforts as well as several methodological improvements. First, the establishment of large scale consortia has been successful for a number of disorders, such as diabetes<sup>99, 118</sup>, lipids<sup>98</sup>, blood pressure and cardiovascular diseases<sup>119</sup>, and can be organized based on disease phenotypes or cohort<sup>120</sup>. Second, an important aim is the standardization of a DR phenotype, classified either with the ETDRS severity scale or a similar modification. A third aim is the standardization of associated phenotypes (diabetes duration, glycemic controls, blood pressure, lipid profiles, and medications), in order to minimize heterogeneity in the comparison. Fourth, the genetic effect of each variant on DR is likely to be modest and larger sample cohorts are required to find modest associations. Fifth, large meta-analyses between different cohorts and different ethnicities have proven difficult to conduct to date due to technical challenges. Standardization of study protocols between different studies could be improved upon to increase power. Sixth, novel statistical approaches such as utilizing a combination of GWA and genome-wide linkage studies to first prioritize the genome could be a more efficient means to identify candidate genes for DR<sup>121</sup>, though this approach would first require strong evidence of linkage peaks in families. Furthermore, studies of different ethnicities need to be conducted to find population-specific signals in DR, given the different prevalence rate observed in different populations. Lastly, novel approaches such as biomarkers as intermediate phenotypes, proteomics, metabolomics, exome array, and next-generation sequencing may integrate systematic information in the field of functional genomics or systems biology to better our understanding of the complexity of DR.

## CONCLUSION

DR remains as one of the most complex, heterogeneous, multifactorial disorders in any genetic studies. The identification of genetic susceptibility loci for DR through candidate gene approaches, linkage studies, and GWAS has not proven markedly successful to date, given the often conflicting and inconclusive results. It is clear that the study of the genetics of DR is still in its infancy and faces many challenges due to the complexity of the disease itself. A number of challenges and strategies are detailed in this review. Only when we achieve these important milestones may it be possible to understand the genetic contributions in DR, identify true genetic variants, and subsequently develop early screening assays for at-risk individuals and novel therapies to combat this common cause of blindness in adults.

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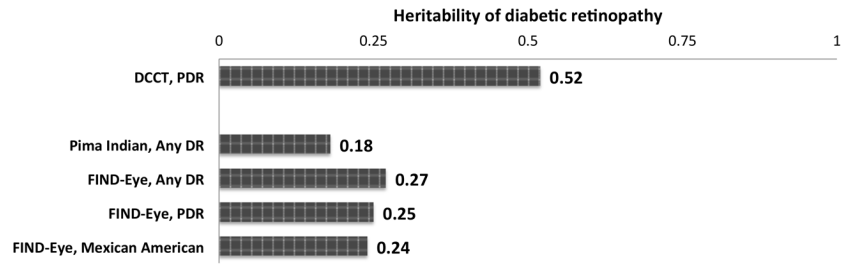
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**Figure 1.**

Table 1

Summary of familial aggregation studies in diabetic retinopathy

Ethnicity (Study)	Type of Diabetes	Heritability	Evidence of familial aggregation	References
Caucasians (DCCT)	1		Low evidence of increased risk for any DR. Strong evidence of familial clustering for severe DR; OR=3.1 in relatives of diabetics with and without DR.	11
Caucasians (FinnDiane)	1	PDR	0.52 Strong evidence of familial clustering in PDR.	12
Pima Indians	2	Any DR	0.18 Modest evidence of familial clustering.	13
South Indian	2		Strong evidence of familial clustering; OR=3.37 in sibling of diabetic with and without DR.	14
Chinese	2		Strong evidence of familial clustering in any DR. DR diagnosed in 7.1% of sibling without DR versus 29.7% of siblings with DR.	15
Mexican American	2		Strong evidence of familial clustering in severe DR (OR=1.72), but not any DR.	16
Multi-Ethnic* (FIND-Eye)	2	Any DR PDR Mexican-American	0.27 0.25 0.24 Strong evidence of familial clustering.	17

\* Mexican-American, European American, African Americans, and American Indians.



Table 2

Summary of individual candidate gene studies of diabetic retinopathy<sup>†</sup>

Gene	Polymorphism	Comments	References
<i>AKR1B1/ALR2</i>	(CA) <sub>n</sub> dinucleotide repeats	z-2 microsatellite confers risk in all DR, NPDR, and PDR of type 1 and type 2 DM *	20
		z+2 and z microsatellite protective against all DR in type 2 DM; no ethnic difference *	
	rs759853	T allele protective against DR in type 1 DM *	85
	rs35839483	not associated with DR	
<i>VEGF</i>	rs2010963 (-634 C/G)	insignificant finding for DR or PDR, but C allele confers risk for NPDR in meta-analysis *	20, 21, 63-71
	rs25648	T allele increases risk of DR but finding insignificant	20, 63, 68
	rs1570360 (-116A>G)	inconsistent and insignificant finding	20, 63, 72
	rs3095039	T allele increases risk, but finding inconsistent and insignificant	20, 63, 70
	rs35569394	-2549DEL increases risk but finding insignificant	20, 73
	rs699947 (-2578 A/C)	A allele increases risk but finding insignificant	20, 66, 74-77
	rs13207351 (-152A)	associated with PDR but insignificant in other studies	63, 72, 75
	rs735286 (+4618)	Haplotype-tagged SNP associated with severity of DR	72
	rs2146323 (+5092)	Haplotype-tagged SNP associated with severity of DR, associated with early progression of DR	72, 77
	rs833061 (-1498 C/T)	inconsistent and insignificant finding	63, 68, 69, 75
	rs3025021	inconclusive	7576
	rs10434	G allele associated with blinding DR.	76
	rs833068	G allele confers risk in DR	76
	rs833070	associated with early progression of DR but weak association	77
<i>ITGA2 (α2β1 integrin)</i>	rs2910964	A allele increases risk * but one study in Chinese was not significant	20, 78, 79
<i>AGTR1</i>	rs5186	C allele confers protection, but finding insignificant	20
<i>ACE</i>	INS/DEL at intron 16	inconsistent and insignificant finding	20
	rs4343	associated with DR in Chinese	122
<i>ADRB3</i>	rs4994	inconsistent and insignificant finding	20
<i>AGT</i>	rs4762	C allele confers protection *	20
<i>APOE</i>	E2/E3/E4	inconsistent and insignificant finding	20, 81, 82
<i>FGF2</i>	rs41456044	A allele increases risk, but finding insignificant	20
	rs308395	G allele increases risk, but finding insignificant	20
<i>NOS3</i>	rs1799983	G allele increases risk, but finding insignificant	20, 79
	rs41322052	inconsistent and insignificant finding	20, 83

Gene	Polymorphism	Comments	References
	rs3138808	inconsistent and insignificant finding	20
<i>SLC2A1</i>	rs841853	insignificant finding	20
<i>HLA</i>	DR1-8	insignificant finding	20
<i>SDH (sorbitol dehydrogenase)</i>	rs2055858 and rs3759890	Weak association	84
<i>ICAMI</i>	rs13306430	G allele confers protection *	20
	rs5498	not associated with DR	85
<i>MTHFR</i>	rs1801133	T allele increases risk but finding insignificant	20
<i>NPY</i>	rs16139	C allele increases risk but finding insignificant	20
<i>PAI-1</i>	rs1799768	4G/5G allele increases risk but finding insignificant	20, 86
<i>PON1</i>	rs662	inconsistent and insignificant finding	20
<i>PPARG</i>	rs1801282	G allele confers protection but finding insignificant	20
<i>AGER/RAGE</i>	rs1800624 (-374T/A)	inconsistent and insignificant finding	20, 85, 87, 88
	rs1800625 (-429T/C)	inconsistent and insignificant finding	20, 85, 89
	rs2070600	associated with DR of Indian ethnicity	85
<i>VDR</i>	rs10735810	T allele increases risk, but finding insignificant	20
<i>EDN1 (endothelin-1)</i>	rs5370 (Lys198Asn)	Reduced risk in Chinese	79
<i>ROCK2</i>	Thr431Asn and Arg83Lys	no association	90
<i>CPVL/CHN2</i>	rs39059	increases risk of DR, significant in meta-analysis *	91
<i>FRMD3</i>	rs10868025	significant in stage 1, but insignificant in stage 2.	91
<i>CARS</i>	rs451041	not significant	91
<i>IRS2</i>	rs1411766	not significant	91
<i>SOD2</i>	rs4880	C allele reduced risk	92
<i>MnSOD</i>	Ala16Val	Significant with DR	65
<i>CA (Carbonic anhydrase)</i>	rs2403104, rs17741410, rs1496533, rs17814594, rs12544332, rs1496529, rs725605, rs2645050, rs2645049, rs13278559	not associated with DR	93
<i>PEDF</i>	rs12150053, rs12948385, rs8697961, rs1136287	not associated with DR	85
<i>EPO</i>	rs1617640	not associated with DR	85
<i>HTRA1/ARMS2</i>	rs11200638, rs10490924	not associated with DR	85
<i>CFH</i>	rs1061170, rs3753394	not associated with DR	85
<i>PSMD9</i>	rs74421874, rs3825172, rs14259	associated with DR	94

† genome-wide approaches not included;

\* significant and consistent direction in more than 1 study

**Table 3**

Summary of linkage studies of diabetic retinopathy

Ethnicity	Type of Diabetes	Sample size	Definition of DR	Region	LOD score	Nearest markers	References
Pima Indians	2	103 sibpairs	Any DR	3q26	1.36	D3S3053, D3S2427	24
				9q21	1.46	D9S1120, D9S910	
Pima Indians	2	211 sibships	Retinopathy score in worst eye	1p36	3.01 and 2.58 *	DIS3669	13
Mexican Americans	2	282 sibpairs	Any DR	1p36	1.24	GGAT2A07	23
				3q12	2.41	GATA68D03	
				7p15	1.02	GATA41G07	
				12p13	2.47	GATA49D12	
				15q25	1.07	ATA28G05	
				15q26	1.16	GATA22F01	
				2q37	1.11	AFM112yd4	
				3p26	1.29	GATA22G12	
				3q12	1.40	GATA68D03	
				12q23	1.03	GATA85A04	
			Severe NPDR/PDR				

\* for single and multi-point analysis, respectively.

**Table 4**

Summary of Genome-wide association studies of diabetic retinopathy

Ethnicity	Type of Diabetes	Sample Size		Platform and Number of SNPs	Covariates	Top SNPs	Region	Risk Allele	Odds Ratio	P-value	Gene(s)	References							
		Cases (defined)	Controls (defined)																
Mexican-Americans	2	103 (moderate-to-severe NPDR and PDR)	183 (Normal to early NPDR)	Affymetrix GeneChip 100K; 421,010 SNPs (imputed)	age, gender, diabetes duration and HbA1c	rs6909083	6p12	NA	NA	$2 \times 10^{-5}$	<i>TINAG</i>	25							
						rs17083119	6q22	NA	NA	$3 \times 10^{-5}$	<i>C6orf170</i>								
						rs2300782	5q22	A	2.64	$6 \times 10^{-5}$	<i>CAMK4</i>								
						rs10519765	15q13	G	3.33	$6 \times 10^{-5}$	<i>FMN1</i>								
						rs899036	11p12	A	3.13	$3 \times 10^{-4}$	<i>API5</i>								
						rs10501943	11q22	C	3.04	$3 \times 10^{-4}$	<i>CNTN5</i>								
						rs17376456	5q15	A	3.63*	$3 \times 10^{-15}^\dagger$	<i>C5orf36</i>								
						rs2038823	13q32	C	2.33*	$5 \times 10^{-11}^\dagger$	<i>HS6ST3</i>								
						rs4838605	10q11	C	1.58*	$2 \times 10^{-9}^\dagger$	<i>ARHGAP22</i>								
						rs12219125	10p12	T	1.62*	$9 \times 10^{-9}^\dagger$	<i>PLXDC2-NEBL</i>								
Taiwanese (Chinese)	2	174 (NPDR and PDR)	575 diabetics (no DR) and 100 nondiabetics	Illumina HumanHap550; 550K SNPs	diabetes duration and HbA1c	rs4462262	10q21	C	1.54*	$9 \times 10^{-8}^\dagger$	<i>ZWINT-MRPS35P3</i>	26							
						rs2811893	1p32	T	1.50*	$3 \times 10^{-7}^\dagger$	<i>MYSM1</i>								
						rs1571942	10p12	C	1.67*	$3 \times 10^{-7}^\dagger$	<i>PLXDC2</i>								
						rs4470583	4q32	A	1.16*	$4 \times 10^{-7}^\dagger$	<i>RPS14P7-FSTL5</i>								
						rs476141	1q44	A	1.37	$1 \times 10^{-7}$	<i>AKT3</i> and <i>ZNF238</i>								
						rs4787008	16p13	G	1.47	$6 \times 10^{-7}$	<i>RBFOX1</i>								
						rs13064954	3q25	G	1.02	$7 \times 10^{-7}$	<i>LEKRI-CCNLI</i>								
						rs9866141	3q25	T	1.02	$9 \times 10^{-7}$	<i>KRT18P34-VEPPI</i>								
						Caucasians (GoKinD and EDIC)	1	973 (PDR and DME)	1856 (all others, including NPDR)	Affy 5.0 and Illumina HumanHap550; 2,543,887 SNPs (imputed)	no adjustments		rs13064954	3q25	G	1.02	$7 \times 10^{-7}$	<i>LEKRI-CCNLI</i>	27
													rs9866141	3q25	T	1.02	$9 \times 10^{-7}$	<i>KRT18P34-VEPPI</i>	

Ethnicity	Type of Diabetes	Sample Size		Platform and Number of SNPs	Covariates	Top SNPs	Region	Risk Allele	Odds Ratio	P-value	Gene(s)	References
		Cases (defined)	Controls (defined)									
						rs17404956	5q34	A	1.16	1 × 10 <sup>-6</sup>	RPLP0P9-ODZ2	
						rs10403021	19q12	C	1.01	2 × 10 <sup>-6</sup>	VSTM2B-POP4	
						rs10927101	1q44	A	1.33	2 × 10 <sup>-6</sup>	AKT3-ZNF238	
						rs2696835	16q24	C	2.27	3 × 10 <sup>-6</sup>	IRF8-FOXF1	
						rs1970671	18q21	G	1.37	3 × 10 <sup>-6</sup>	MAP1LC3P-TCF4	
						rs2115386	19p13	C	1.12	3 × 10 <sup>-6</sup>	INSR	
						rs10199521	2p25	T	1.46	3 × 10 <sup>-6</sup>	MYT1L-TSSC1	
						rs7772697	6q25	T	1.35	3 × 10 <sup>-6</sup>	UST-TAB2	
						rs6702784	1p34	C	1.08	4 × 10 <sup>-6</sup>	OSCP1	
						rs1342038	1q25	G	1.49	4 × 10 <sup>-6</sup>	TNFSF4-RPL26P11	
						rs3007729	1p36	T	1.35	5 × 10 <sup>-6</sup>	IGSF21-KLHDC7A	
						rs10910200	1q42	G	1.35	6 × 10 <sup>-6</sup>	KIAA1804-KCNKI	
						rs11867934	17p11	C	1.43	7 × 10 <sup>-6</sup>	TNFRSF13B-MPRIP	
						rs11765845	7p15	A	1.02	7 × 10 <sup>-6</sup>	CREB5	
						rs1073203	5q23	G	1.54	9 × 10 <sup>-6</sup>	RPSAP37-GRAMD3	
						rs9565164	13q22.2	C	1.7	1.3 × 10 <sup>-7</sup>	TBC1D4-COMMD6-UCHL3	
Taiwanese (Chinese)	2	437 (PDR)	570 diabetics; 8 years (no DR)	Illumina OmniExpress; 2,166,765 (imputed)	no adjustments; age and gender	rs1399634	2q31.1	A	1.5	2.0 × 10 <sup>-6</sup>	LRP2-BBS5	28
						rs2380261	2q37.2	T	1.5	2.1 × 10 <sup>-6</sup>	ARLAC-SH3BP4	

\* Dominant model;

† Determined by the most significant P-value from 6 genetic models (genotype, allele, trend, additive, dominant, and recessive)