Symposium - DR in India

Genetic components in diabetic retinopathy

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Diabetic retinopathy (DR) is a serious complication of diabetes, which is fast reaching epidemic proportions worldwide. While tight glycemic control remains the standard of care for preventing the progression of DR, better insights into DR etiology require understanding its genetic basis, which in turn may assist in the design of novel treatments. During the last decade, genomic medicine is increasingly being applied to common multifactorial diseases such as diabetes and age-related macular degeneration. The contribution of genetics to the initiation and progression of DR has been recognized for some time, but the involvement of specific genes and genetic variants remains elusive. Several investigations are currently underway for identifying DR susceptibility loci through linkage studies, candidate gene approaches, and genome-wide association studies. Advent of next generation sequencing and high throughput genomic technologies, development of novel bioinformatics tools and collaborations among research teams should facilitate such investigations. Here, we review the current state of genetic studies in DR and discuss reported findings in the context of biochemical, cell biological and therapeutic advances. We propose the development of a consortium in India for genetic studies with large cohorts of patients and controls from limited geographical areas to stratify the impact of the environment. Uniform guidelines should be established for clinical phenotyping and data collection. These studies would permit identification of genetic loci for DR susceptibility in the Indian population and should be valuable for better diagnosis and prognosis, and for clinical management of this blinding disease.

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Type 1 (formerly called juvenile onset or insulin-dependent diabetes) and Type 2 diabetes (formerly called adult-onset or noninsulin-dependent diabetes) result from the deficiency of insulin or impairment of its function at the cellular level, respectively. While diabetes becomes apparent generally during adulthood, Type 2 diabetes can develop at any age and accounts for 90–95% of diabetics. Type 1 diabetes, on the other hand, accounts only for 5–10% of diabetics. Although complications of diabetes develop gradually over a period, both forms of diabetes increase the risk of long-term complications, which include retinopathy, nephropathy, and neuropathy. In light of the predicted rise in the incidence of diabetes to 592 million worldwide by the year 2035, a significant proportion of diabetics would suffer from such complications. In 2013, the numbers of 20–79 years old diabetics in China, India, and the US, were estimated to be 98.4, 65.07 and 24.4 million, respectively.

Although genetic evidence primarily comes from twin[1] and family studies,[2] the involvement of genetic factors in diabetic retinopathy (DR) is undeniable. Numerous studies during the past two decades have identified genetic variants associated with DR; however, replication of such findings and defining their relevance to disease have been quite challenging.[3,4] It is noteworthy that DR is a complex trait, which is influenced by heritable factors[5-7] as well as the environment. Given the involvement of multiple genetic loci in the development of DR, the penetrance of individual variants may be too small to be detected by underpowered co-segregation studies. The success of genetic association studies in major complex diseases involving vision loss such as age-related macular degeneration (AMD),[8] glaucoma,[9] and myopia[10] can be attributed to the adequate statistical power of sample sizes.

The advent of genomic technologies has made it plausible to investigate complex disorders by genome-wide association studies (GWAS), which is a method of choice to analyze large numbers of individuals for their marker genotypes. The genetic resources made available by the International Human Genome Project, HapMap, and 1000 Genomes Project (http://www.1000genomes.org/) should be beneficial for DR genetics research.[1] Given the recent identification of all variants in the human genome globally[11] with precisely mapped regions in linkage disequilibrium, it is now feasible to perform large-scale association studies using tens of thousands of cases and controls from distinct ethnic populations. In this review, we have summarized the current literature related to etiology

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and genetics of DR and proposed an optimistic perspective for genetic analysis of DR in Indian population.

Retinal Complications of Diabetes

Phenotypes of diabetic retinopathy

The phenotypes of DR include changes in the neural retina as well as its associated microvascular network. The vascular leakage caused by chronic hyperglycemia-mediated microangiopathy in diabetic patients results in macular edema and capillary occlusion. The retinal ischemia associated with occluded capillaries induces the expression of vascular endothelial growth factor (VEGF), a key factor for the formation of new but abnormal blood vessels in the proliferative stage of DR. Thus, abnormal and leaky vessels constitute a distinguishing feature of DR pathology.

Classification and pathophysiology of diabetic retinopathy

Small blood vessels are especially vulnerable to poor glycemic control in diabetic patients, and accumulation of glucose in circulation damages these vessels in the retina. In the etiology of DR, this initial stage is called nonproliferative DR (NPDR). Typical changes seen during NPDR involve microaneurysms, retinal hemorrhages, and exudates. NPDR can progress to advanced or proliferative DR (PDR), which is accompanied by an excessive proliferation of blood vessels. As stated earlier, PDR is caused by enhanced levels of intraocular VEGF, which leads to neovascularization at the optic disk or in the vitreous. These newly formed vessels show leakage on fluorescein angiography and may cause vitreous hemorrhage and tractional retinal detachment. Retinal detachment results in separation of the neurosensory retina from the retinal pigment epithelium, and the detached sections of the retina exhibit scotoma and visual field defects.[12]

In addition to vascular pathology, pathways that modulate inflammation and autophagy, nerve growth factor, and epigenetic changes have been implicated in DR etiology.[12] As shown in Fig. 1, DR-induced biochemical alterations such as oxidative stress, activation of protein kinase C (PKC), and formation of advanced glycation end products (AGEs) have been detected as responses of the retina to hyperglycemia.[13] A detailed discussion of these pathways is beyond the scope of this review.

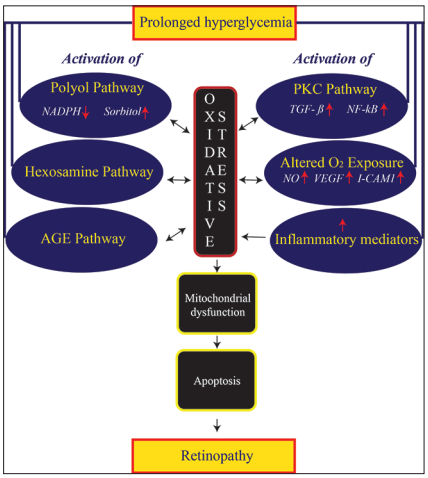

Figure 1: Metabolic pathways associated with the development of diabetic retinopathy

Retinal neurovascular damage in diabetes

Retinal dysfunction impacts distinct components of the retinal neurovascular unit in diabetic patients. The neurovascular unit is described as the physical and biochemical relationship among different cell types in the retina (neurons, glia, and specialized vasculature). The interdependency among these cells/tissues allows energy homeostasis and neurotransmitter regulation. Under the prolonged hyperglycemic condition, alterations of the blood-retinal barrier, neuronal damage, and inflammation appear to be the most relevant changes.[14] It is noteworthy that different components of the retinal neurovascular unit are affected at varying times during initial stages of the diabetic retinal disease. However, not all patients demonstrate a detectable alteration in blood-retinal barrier during the early stages of the disease,[15] and not all affected individuals present electrophysiological changes at the same stage of the disease.[16,17] Such complexity of interactions among retinal cell types indicates aberrant regulation of tissue-specific genes in DR. However, little is known about molecular pathological alterations in retinal neurons and glia in connection with DR.

Genetics of Diabetic Retinopathy

Ethnic differences in increased familial aggregation and higher concordance in monozygotic versus dizygotic twins in Type 1 and Type 2 diabetes illustrate the involvement of genetic factors in the etiology of diabetes. Genetic susceptibility and the association between DR and glycemic control are strongly supported by an old twin analysis study.[18] Furthermore, the role of genetic factors in DR has been corroborated by familial aggregation studies in patients with either Type 1 or Type 2 diabetes.[19] It is, therefore, plausible that a number of diabetes-induced genes may impact specific stages of DR, including NPDR, PDR, and macular edema, differentially in distinct genetic backgrounds.

The biochemical changes reported in diabetic patients have indicated a tentative set of candidate genes that may be associated with the development and progression of DR. These candidate genes regulate the pathways involved in the production of reactive oxygen species, sorbitol, and AGEs.[20] However, these may represent only a subset of candidates, and there definitely are unexplored or underexplored loci underlying DR genetic susceptibility. The approaches to identify additional genetic determinants of DR are illustrated in Fig. 2 and summarized in the following sections.

Linkage studies

Linkage studies are based on the principles of genetic recombination. In these studies, genomic regions are mapped by genotyping unaffected (control) and affected members of a pedigree, and by characterizing segregation of genetic markers with DR susceptibility. Based on the presence or absence of the linkage, a marker is either inherited together with the causal variant or inherited independently of the causal variant, respectively. As a result, the closer the physical distance of the marker to DR susceptibility loci, the stronger the evidence is for linkage. Linkage strategies are hypothesis-free and are likely to indicate linkage of the phenotype or disease with a specific region of the chromosome or chromosomal region. This strategy is driven by chromosomal location, and no biochemical or pathophysiological induction is proposed.

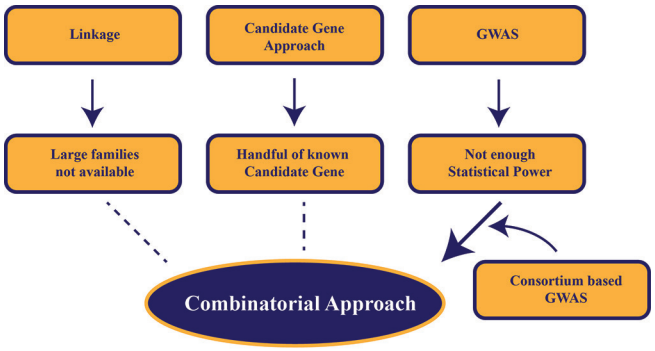

Figure 2: Crosstalk between different genetic approaches to study diabetic retinopathy

The members of multigenerational families are typed for genome-wide polymorphic markers to search for regions of the genome with more than the expected number of shared alleles among affected individuals within a family. A logarithm of the odds (LOD) score is calculated at a given recombinant fraction, and a LOD score of 3 or greater is generally considered significant for evidence of linkage.

In a dominant model of sib-pair linkage analysis for DR in Pima Indians with Type 2 diabetes, the modest evidence of linkage with LOD scores of 1.36 and 1.46 was found at chromosomes 3 and 9, respectively.[21] A subsequent genome-wide linkage analysis in this population found a stronger evidence for linkage on chromosome 1p with LOD scores of 2.58 and 3.1 for a single point and multipoint analyses, respectively.[22]

In principle, linkage analysis requires large sample sizes and extensive efforts to identify specific causal variants responsible for DR. Although these variants may account for a minority of DR cases, they could illuminate the etiology of the more common forms. Such linkage studies on DR have thus far been conducted in limited ethnicities (Pima Indians and Mexican Americans). Since multifactorial diseases are influenced by environmental risk factors and involve small individual effects contributed by multiple common genetic variants,[23] linkage studies on small DR families are likely to yield inconsistent results. However, large multigenerational pedigrees with multiple affected individuals, although suitable for linkage analysis, are not readily available. Therefore, population-based association approaches,[24] candidate gene studies and GWAS are favored for DR.

Candidate gene studies

Most genetic research in DR has utilized the candidate gene approach. Several biochemical pathways have been proposed to play an important role in the pathogenesis of DR [Fig. 1], leading to evaluation of a number of hypothesized candidate genes. Reported by several different groups, the candidate gene approach requires a good understanding of the pathogenic mechanisms underlying DR.[25-27] In this approach, the frequency of a particular genetic variant of a specific candidate gene in subjects with DR is compared to the subjects without DR. To accomplish this, genes of several pathways and processes have been proposed as DR candidates.[4,19] These genes include AGE, angiotensin-I converting enzyme, angiotensin II Type 1 receptor, angiotensinogen, plasminogen activator inhibitor-1, α 2β 1 integrin, peroxisome proliferator-activated receptor gamma, nitric oxide synthase (NOS) 3, VEGF, aldose reductase (ALR2), receptor for AGEs (RAGE), glucose transporter 1, transforming growth factor beta, among others. Their associations or lack thereof with DR have been extensively documented in prior reviews.[4,28,29] We have summarized below the most important findings with a focus on AGE, ALR2, and VEGF, because of their unique biological implications. However, the association of these gene variants with DR has not been successfully reproduced.[23] The lack of reproducibility has therefore questioned the reliability of these polymorphisms for risk assessments.

Advanced glycation end products

AGE is produced by prolonged exposure of proteins to aldoses, such as glucose, and is shown to accumulate in plasma and tissues at an accelerated rate in diabetics.[30] These products modulate signaling pathways by binding to the RAGE. The involvement of RAGE-mediated signaling pathways has also been reported in the development of DR.[31] The presence of RAGE on endothelial cells, mononuclear phagocytes, and vascular smooth muscle cells contributes to the enhanced adherence of red blood cells to the endothelium in diabetic patients. Furthermore, extensive contact of membrane-associated AGE in diabetic erythrocytes with RAGE on the vessel wall could be important in the development of vascular complications. In addition, AGE is involved in endothelial cell proliferation.[32] It is therefore not surprising that association of AGE-R1 gene polymorphisms has been observed with the severity of retinopathy.[33] Further investigations are warranted on the association of RAGE variants with DR.

Aldose reductase gene products

The *ALR2* gene product, a key regulator of the polyol pathway,[34] converts glucose to sorbitol in an NADPH-dependent reaction. The affinity of ALR2 to glucose is very low and, therefore, enough sorbitol is not produced under normal glycemic conditions. However, a significant amount of sorbitol accumulates during hyperglycemia, which causes the death of retinal pericytes and injury to endothelial cells, an early event in the development of DR.[35] Recently, Abhary *et al*. assembled meta-analyses of 85 studies, and demonstrated a number of genetic variants in the ALR2 gene to be significantly associated with DR.[28] Furthermore, several other studies have reported high levels of ALR in erythrocytes of both Type 1 and Type 2 diabetic patients with retinopathy.[36-38]

Vascular endothelial growth factor

In humans, angiogenesis is accompanied by modulation of microvascular hyperpermeability and selective degradation of the basement membrane. The hyper-permeability of vasculature and induction of new vessel formation are mediated by VEGF, a 46 kD dimeric heparin-binding protein that specifically acts on endothelial cells.[39] The significance of VEGF and new blood vessel formation is supported by observations indicating elevated concentrations of VEGF in the vitreous and aqueous fluid of patients with PDR, retinal ischemia- and hypoxia-induced increase in VEGF production, and inhibition of retinal vessel proliferation by intraocular injection of anti-VEGF drugs.[40,41] The expression of retinal VEGF early in DR suggests that VEGF may also act as a permeability-inducing factor leading to progression of the early stages of retinopathy.[42]

However, a recent meta-analysis of French and Danish Type 2 diabetic patients failed to demonstrate significant effects of VEGF variants on risk of DR.[43]

Sobrin *et al*.[44] investigated a large sample of Type 2 diabetics ($n = 2691$) for the association of 2000 candidate genes identified for Type 2 diabetes, DR and diabetic nephropathy with DR in the Candidate gene Association Resource. These studies revealed the tentative association of three single nucleotide polymorphisms (SNPs) in P-selectin gene and several variants of α-L-iduronidase with DR. Many other associations observed in these studies could not be replicated in independent cohorts, thereby indicating an inconsistent association of previously identified candidate genes with DR. Although there are potential novel candidates of interest as discussed above, new studies with additional cohorts are required with a goal to differentiate patients on the basis of their glycemic profiles. In this regard, information about the details of the disease (duration and biochemical parameters) should be made available.

A major concern with the candidate gene association described above relates to the size of samples. While some of these studies were conducted with a sample size of 50, others with larger sample size were restricted to unique/rare populations.[45,46] Furthermore, most studies failed to take into account the role of haplotype diversity at the candidate gene locus, which made the exclusion of a gene from consideration difficult.[47]

Genome-wide association studies

The completion of the International HapMap Project in 2005[48] has enabled better elucidation of the genetic components of common multifactorial diseases. GWAS is preferred over candidate gene studies, as the latter is based on an *a priori* hypothesis. The first GWAS success story of the discovery of the association CFH with AMD, a landmark study of the most common form of blindness in the Western world, was independently described in three different cohorts.[8,49,50] A coding variant Y402H in exon 9 of CFH was significantly associated with AMD. The landmark GWAS in AMD has paved the way to understand the genetics of complex diseases. GWAS have also been successful in revealing the involvement of specific pathways in Type 2 diabetes.[51] But there have been only a few such studies for DR performed in different populations of small sample sizes.[52] The two largest GWAS of DR in Type 1[53] and Type 2[54] DM investigated 973 and 749 cases, respectively. The strongest association with severe DR was observed in a meta-analysis of results from the Kidney in Diabetes and Epidemiology of Diabetes Intervention and Control Trial studies,[53] which reported associations of SNP rs476141 ($P = 1.2 \times 10^{-7}$) located between AKT3 and ZNF238 on chromosome 1, and SNP rs227455 ($P = 1.6 \times 10^{-7}$) on chromosome 6. Other studies in Mexican-Americans and Taiwanese Chinese in sample sizes of NPDR/PDR varying from 103 to 437 revealed the association of borderline significance with a few SNPs.[37,54-56] However, all these associations need to be replicated to draw any meaningful biological conclusion.

The lack of definitive association of genomic markers to DR can be attributed to the power of sample size. Unlike other diseases where analysis of several thousand cases and controls by GWAS has been successful,[51,53] determination of genetic effects in DR have been performed in relatively small sample sizes. Furthermore, a broad spectrum of DR manifestation, inaccurate diagnosis, misclassification of patients, and pooling of suboptimal datasets are likely to compromise the results. Thus, large sample sizes, accurate diagnosis of patients and classification of control subjects recruited in the study are important factors for determining the reliable association. To overcome the issues described above, ongoing study protocols are aimed at recruiting more cases and controls, combining datasets from different studies, and incorporating newer algorithms for aggregating the results. It is expected that the subject recruitment protocols employed by various groups would eliminate the heterogeneity in subjects and allow better replication of results. The multicenter studies are also including collaborative replication of findings to distinguish true findings from the false positives. Future studies would illuminate genetic architecture of DR and separate it from the genetics of diabetes.

Suggestions for Genetic Studies of Diabetic Retinopathy in India

Diabetic retinopathy incidence

A recent report estimates that at least 387 million people (8.3% of the adult population with equal rates in both women and men) have diabetes worldwide, and Type 2 diabetics constitute about 90% of these patients.[57] International Diabetes Federation predicts the number of adults with diabetes in the world to be 552 million by 2030, and the largest increase is expected to occur in Asia and Africa.[58] During the period spanning 1989–2003, the incidence of diabetes in rural India increased by 3-fold (2.2–6.3%).[59] Alarming as these figures are, it is also of concern that DR affects 3–5% of the population, and these numbers rise to 16.6–20.9% for demographics representing the age group of 50 years and higher.[60,61] Over the last 20 years, DR has become one of the most common causes of low vision and blindness in India and currently DR is the 6th cause of blindness.[62] A recent study suggests DR prevalence of 33.9% in Type 2 diabetics of western India,[63] which included 25.5% of NPDR and 8.33% of PDR. These statistics are further compounded by the lack of access to eye health care as illustrated by the ophthalmologists to population ratio of 1:9000 nationwide and 1:608,000 for some regions in India.[64]

To manage the DR phenotype in India, collaborative efforts as well as several methodological improvements, as suggested in Fig. 3, are needed. The organization of DR phenotype-based consortia standardized phenotype criteria, and patient profile with diabetes duration, glycemic control, blood pressure, lipids, and medications will greatly help minimize heterogeneity.

Genetics and statistics of diabetic retinopathy

Some of the candidate gene association studies specific to Indian population are described below. SNP analysis was carried out on nine candidate genes (RAGE, pigment epithelium derived factor [PEDF], AKR1B1, EPO, HTRA1, intercellular adhesion molecule [ICAM], HFE, CFH, and ARMS2) that play a major role in DR pathology. Of 15 SNPs studied only 1 SNP rs2070600 (G > A) in exon 3 of RAGE, displayed statistically significant ($P = 0.016$) association with DR in this study population.[65]

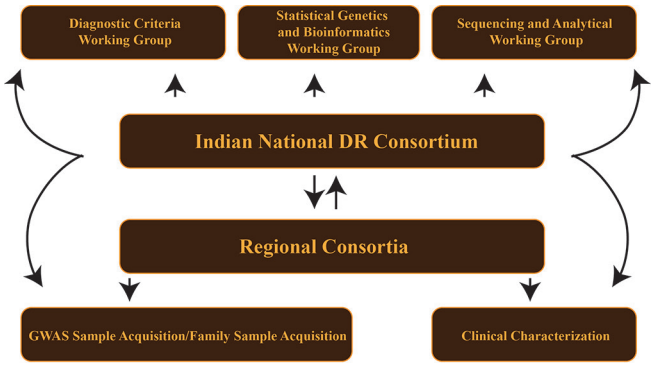

Figure 3: Strategic approach to analyze diabetic retinopathy in Indian population

The weak association of VEGF, insulin-like growth factor (IGF-1), PEDF, endothelial NOS (eNOS), PKC beta (PKC-β), ALR2, inducible NOS (iNOS), RAGE, and ICAM-1 with DR has been documented in Indian population.[64,65] These data indicated variants Z-2 of ALR2, $(CA)_{18}$ of IGF-1 and AA genotype of ICAM-1 as high-risk alleles for DR.[50-52] Genotyping of the specific patient population has also revealed variants $(GT)_9$ of TNFβ and $(CCTTT)_{15}$ of iNOS genes as low-risk alleles for DR.[66,67] However, these studies also indicated the lack of association with promoter variants of PKC-β, 3′UTR variants of VEGF and the 27 bp variable number tandem repeat in eNOS intron 4 in Type 2 DR patients.

The effect of an individual (rare or common) genetic variant on DR is likely to be modest, and larger sample cohorts in Indian patient population are required to find such associations. In light of rising incidence of DR in India, we believe the available genetic resources must be leveraged for identifying relevant DR susceptibility loci. The success, however, would depend on the establishment of national and/or regional consortia [Fig. 3], standardization of clinical phenotyping, identification of families with DR, meta-analyses of genetic data from multiple centers, and combined GWA and genome-wide association studies. Access to high throughput genotyping and next generation sequencing would greatly advance elucidation of genetic susceptibility variants associated with DR in India. Such studies can then be combined with proteomics and metabolomics to develop systems-level understanding of DR.

Conclusion

The genetics of DR is relatively complex, and a majority of the reported genetic studies have not been replicated or reproduced in different ethnic populations. Although a susceptible genetic background is necessary for the development of overt diabetes, it is only fully sufficient in rare Mendelian forms of diabetes; such susceptibility loci have not been confirmed for DR.

Based on the evidence for genetic heritability of DR, it is predicted that some strong genetic association with candidate genes would emerge in the near future. The success of genetic approaches for establishing the association and/or linkage with Type 1 or Type 2 diabetes has largely been due to concerted efforts of numerous research groups, availability of large families, and recruitment of case and control subjects. The current paucity of validated genetic associations in DR can be attributed in part to the inadequate power of sample sizes to identify gene variants of genome-wide significance. We believe that these limitations can be overcome by establishing consortia for high throughput genotyping analysis, sharing of data and resources, and incorporating powerful new statistical genetics tools. Given the increase in the incidence of DR and the presence of multiple research centers, time is ripe for taking advantage of this rich genetic resource by establishing uniform guidelines for clinical data collection and genetic analysis as part of larger collaborations within India and with institutions worldwide.

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Conflicts of interest

There are no conflicts of interest.

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