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CLINICAL STUDY

Molecular genetics of microvascular disease in diabetic retinopathy

KM Warpeha and U Chakravarthy

Abstract

Diabetic retinopathy is a sight-threatening complication of the retinal microvasculature. While important environmental factors have been clearly identified as influencing its development, increasing evidence suggests that diabetic retinopathy has a genetic component. A variety of studies have explored associations between candidate genes and frequency and severity of retinopathy. Overall, this review has found that the majority of candidate genes studied exhibit weak or no association with retinopathy status, and where associations have been detected these results have not been replicated in multiple populations. This may reflect inaccurate case definition, small subject numbers and possibly inadequate markers for genetic studies

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Genetic susceptibility to diabetic retinopathy

It is recognised that polymorphic variability in the genetic make-up of an individual can profoundly influence the expression of a gene and its response to environmental factors.[1,2] Useful clinical markers for genetic susceptibility to a disease are either familial aggregation or a variation in disease frequency, which are not explained by environmental, biochemical, or biological risk factors. Diabetic retinopathy (DR) displays these characteristics as clinical studies on human subjects with diabetes reveal substantial variation in the onset and severity of retinopathy that are not fully explained by the known risk factors such as duration of diabetes, level of glycaemic control, or concomitant vascular disease.[3,4] The risk of severe DR in the siblings of affected individuals is substantially increased,[5] and the Diabetes Control & Complications Trial has shown that retinopathy tends to cluster in families.[4] Furthermore, differences in the frequency of disease in ethnic populations[6] also suggest that genetic influences are operating in DR. With the complex metabolic environment of the retina, many risk factors have been proposed in the past.[7,8]

The search for genetic factors in multifactorial, complex late-onset human diseases is characterised by two approaches: genome-wide scans for markers linked to disease and candidate genes studied individually based on the putative function of the gene product. In terms of DR, few studies have employed the former method. Recently, Imperatore *et al*[9] undertook a genome-wide scan for susceptibility genes to diabetic retinopathy (and nephropathy) in families using affected sib-pair linkage analysis. There were indications that elements on chromosomes 3 and 9 influenced both nephropathy and retinopathy, but no clear genomic region was designated for retinopathy alone.[9]

Candidate genes for retinopathy

Association between genetic variability and retinopathy may be because of increased frequency or increased severity of retinopathy within the population of interest. A large number of candidate genes have been examined in subjects with diabetes, but few groups have identified a strong association between a gene and the frequency or severity of retinopathy. Most published studies have been based on small patient samples, and case definitions were not based on prospectively agreed and standardised criteria. Moreover, selection of genes for association studies in DR poses particular difficulties, as many of the obvious candidate genes are involved with the normal function and regulation of the microvasculature in the retina. In this review, we focus on groups

Ophthalmology and Vision Science
Queen's University and Royal Hospitals
Institute of Clinical Science
Belfast, UK

Correspondence:
U Chakravarthy
Ophthalmology and Vision Science
Queen's University and Royal Hospitals
Institute of Clinical Science
Grosvenor Road
Belfast BT12 6BA, UK
Tel: +44 2890 330744
Fax: +44 2890 240503

E-mail: u.Chakravarthy@qub.ac.uk

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Table 1 Summary of studies on associations between genetic markers and diabetic retinopathy

Gene	*Product*	*Marker*	*Association with*	*Ethnicity*	*Type of Diabetes*	*Reference*	*Sample size*
AR2	Aldose reductase	Substitution[a]	Retinopathy	Asian	2	Kao *et al*[15]	NR=97; AR=67 (adolescents)
		Microsatellite	No retinopathy	Multiethnic	2	Olmos *et al*[16]	AR=27
			No retinopathy	Asian	2	Ko *et al*[17]	NR=22; AR=22
			Retinopathy	Asian	2	Fujisawa *et al*[18]	NR=83; PPR=87
			Retinopathy	Asian	2	Ichikawa *et al*[19]	NR=30; SR=30; PPR=27
			Retinopathy	Asian	2	Ikegishi *et al*[20]	NR=34; PPR=27
			Retinopathy	Caucasian	1	Demaine *et al*[22]	NR=70; PPR=159
			None	Multiethnic	1	Chistyakov *et al*[21]	NR=31; PPR=19
			None	Caucasian	1	Heesom *et al*[13]	(Neph study) stratified for DR
HLA region genes	Region of chromosome 6p	Various	Retinopathy	African	1	Cisse *et al*[23]	Not known
			Retinopathy	Caucasian	1	Falck *et al*[24]	NR=52; AR=51 (adolescents)
			Retinopathy	Caucasian	1	Agardh *et al*[25]	NR or SR=28; PPR=28
			None	Caucasian	1	Stewart *et al*[26]	NR=39; AR=20 (adolescents)
			None	Asian	2	Hawrami *et al*[28]	NR=45; PPR=40
			None	Caucasian	1	Serrano-Rios *et al*[27]	AR=32
					2	Serrano-Rios *et al*[27]	AR=35
IgG	Immunoglobulin subclass heavy chains	Allotypes	Retinopathy	Caucasian	1	Stewart *et al*[26]	NR=58; AR=44 (adolescents)
IgM Sμ	Immunoglobulin M heavy chain switch region	Substitution	Retinopathy	Asian	2	Hawrami *et al*[28]	NR=45; PPR=40
GLUT1	Glucose transporter 1	Nucleotide substitution	None	Caucasian	2	Guitterez *et al*[29]	NR=92; AR=68
			None	Caucasian	1	Hodgkinson *et al*[30]	NR=44; AR=30 (neph study)
			None	Asian	2	Liu *et al*[31]	AR=45 (neph study)
PAI	Plasminogen activator inhibitor	Insertion/deletion and nucleotide substitution	None	Indian	2	Nagi *et al*[32]	NR=101; AR=70
			None	Caucasian	1	Tarnow *et al*[33]	(Neph study) stratified for DR
APOE	Apolipoprotein E	Allelic variation	None	Caucasian	1	Tarnow *et al*[33]	(Neph study) stratified for DR
TNF	Tumour necrosis factor in MHC	Microsatellite	Retinopathy	Asian	2	Hawrami *et al*[34]	NR=46; PPR=53
β3-AR	β-3 Adrenoreceptor	Substitution	Retinopathy	Asian	2	Sakane *et al*[35]	NR=121; NP=48; PR=46
			None	Caucasian	1	Vendrell *et al*[36]	NP=56; PR=12
PON1	Paraoxonase 1	Substitution	Retinopathy	Multiethnic	2	Kao *et al*[37]	NR=119; AR=80
			None	Multiethnic	2	Mackness *et al*[39]	NR=93; AR=101
α2β1I	α2β1 Integrin (platelet collagen receptor)	Various (silent base) changes and nucleotide transition	Retinopathy	Asian	2	Matsubara *et al*[39]	NR=108; AR=119
Collagen IV α1	Basement membrane protein	Substitution	None	Caucasian	2	Alcolado *et al*[40]	PR=43
			None	Caucasian	1	Chen *et al*[41]	(Neph study) stratified for DR
Gβ3	β-Subunit of heterotrimeric G-protein	Substitution	None	Caucasian	1	Shcherbak and Schwartz[42]	NR=96; AR=76
NPY	Neuropeptide Y	Substitution	Retinopathy	Caucasian	2	Niskanen *et al*[43]	NR=46; AR=40
ACE	Angiotensin-converting enzyme	Insertion/deletion	None	Caucasian	2	Guitterez *et al*[48]	NR=92; AR=68
			None	Asian	2	Fujisawa *et al*[49]	Meta-analysis (18 studies)
			None	Caucasian	1	Nagi *et al*[50]	NP=92; PR=94
			Retinopathy	Asian	2	Nagi *et al*[50]	NP=186; PR=177

Table 1 (*continued*)

Gene	*Product*	*Marker*	*Association with*	*Ethnicity*	*Type of Diabetes*	*Reference*	*Sample size*
					2	Fujisawa *et al*[51]	AR=267 (MI study)
			Retinopathy	Asian	1	Hanyu *et al*[52]	PPR=45 (neph study)
			Retinopathy	Caucasian	2	Rabensteiner *et al*[53]	NP=81; PPR=94
			Retinopathy	Asian	2	Matsumoto *et al*[54]	NR=90; SR=60; PPR=60
AGTII-IR	Angiotensin-II Type 1 receptor	Substitution	None	Caucasian	1	Tarnow *et al*[55]	NR=67; PPR=155
			None	Caucasian	1	Tarnow *et al*[56]	(Neph study)
AGT	Angiotensinogen	Substitution	None	Caucasian	2	Guiterrez *et al*[48]	NR=92; AR=68
NOS2A	Inducible nitric oxide synthase	Microsatellite	No retinopathy	Caucasian	1 and 2	Warpeha *et al*[62]	NR=107; PPR=93
NOS3	Constitutive nitric oxide synthase	Microsatellite	None	Caucasian	1 and 2	Warpeha *et al*[63]	NR=97; PPR=78
EDN1	Endothelin-1	Microsatelite	None	Caucasian	1 and 2	Warpeha *et al*[63]	NR=86; PPR=70

[a]Substitution indicates silent change or resulting in amino acid substitution.
Symbols: NE, Northern European; MI, myocardial infarction; DR, diabetic retinopathy; NA, North American. Retinopathy codes: NR, no retinopathy (<5 microaneurysms); SR, simple retinopathy, ie microaneurysms only with occasional small haemorrhage; PPR, severe preproliferative and/or proliferative; AR, any retinopathy (mixed grades of retinopathy), or classed as NP, nonproliferative, PR, proliferative.

of genes involved in distinct metabolic and functional pathways known to be affected in diabetes. In Table 1, we provide a summary of the studies undertaken to date on the role of variability in candidate genes that have been examined for association with retinopathy.

Aldose reductase pathway The aldose reductase pathway has been studied in a number of population samples, and variation in the genes expressed in this pathway may influence microvascular susceptibility. Aldose reductase is encoded by the aldose reductase (AR2) gene and is involved in the conversion of glucose to sorbitol, acting as the rate-limiting enzyme of the polyol pathway. The protein is strongly expressed in retinal pericytes and is also found in the vascular endothelium.[10] It has been postulated that 7q35 is a susceptibility region for diabetic retinopathy and nephropathy by virtue of the AR2 gene and nearby genes.[11] It is still uncertain if the AR2 gene itself is directly causative in pre-disposition to retinopathy or protection from its development.[12] Heesom *et al*[13] investigated the role of a polymorphism consisting of a $[CA]_n$ repeat in the 5′ region of this gene with severity of retinopathy. No obvious genetic association was detected in subjects segregated by retinopathy status, but a decrease in prevalence of allele $Z+2$ was associated with nephropathy. The same group also reported an increased frequency of the $Z-2$ allele in subjects with neuropathy.[14] Fujisawa *et al*[15] postulated that the length of the polymorphism and not the actual repeat itself was important, concluding that shorter alleles were associated with retinopathy. They compared their findings with other studies on AR2 and retinopathy and concluded that the data were consistent. Most studies of the genetics of the AR2 gene pathway have been conducted in subjects[16–21] of Asian descent, but recently Demaine *et al*[22] reported a significant association of a particular haplotype with the development of severe retinopathy in Caucasian subjects.

MHC and immunity markers In the case of Type 1 diabetes, the strongest genetic risk component is localised within the major histocompatibility complex (MHC) and is designated IDDM I. This locus contains the HLA-DQ genes and other MHC-encoding genes that contribute to risk of Type 1 diabetes depending on the ethnic population investigated. This HLA region, that is located on 6p21, has also been implicated as a genomic region of interest for susceptibility to retinopathy in both Type 1[23–27] and Type 2[27,28] diabetes. However, the results appear to vary based on retinopathy classification (see Table 1) and ethnicity, and could be clarified by replication in large samples of adult patients.

Glucose transporters Another potential candidate gene is the glucose transporter 1 (GLUT1), which encodes a

protein that facilitates transport of glucose into cells. It has been implicated as a susceptibility factor for Type 2 diabetes itself and in the development of microvascular complications. In the three studies listed in Table 1, no association between polymorphisms in the GLUT1 gene and retinopathy status was found.[29–31]

Cell communication and the extracellular matrix A number of other candidate genes involved in cellular communication and the extracellular matrix have also been investigated for genetic association with retinopathy including plasminogen-activating factor (PAI-1),[32,33] APOE,[33] tumour necrosis factor alpha (TNF-α),[34] β-3 adrenergic receptor gene (β-3AR),[35,36] Paraoxonase 1 (PON1),[37,38] α2β1 integrin,[39] collagen IV α1,[40,41] G-protein β-3 subunit[42] and neuropeptide Y.[43] However, in some cases, there were no consistent associations with frequency or severity of retinopathy, and, in others, significant associations have not been replicated in additional patient groups.

Endothelins and nitric oxide synthases There is incontrovertible evidence that endothelium-mediated vasoregulation is defective in diabetes mellitus and is a precursor to pathological alterations in retinal blood flow.[44–46] The complex interactions between vasodilator and vasoconstrictor modulation of blood flow in the retinal microcirculation are unclear, and the longitudinal influence of diabetes on this interplay is even less well understood. What is clear, however, is that over a period of time, in diabetes, the retinal vasculature undergoes a series of irreversible pathological changes culminating in a severe retinopathy.[47]

A variety of molecules with potent vasoactive functions are produced and released by the vascular endothelium. These include the nitric oxide synthases (NOS) that mediate vasodilation and the endothelins (ETs) that are vasoconstrictors. Angiotensin-converting enzyme (ACE), which converts angiotensinogen to angiotensin, is another important mediator of vasoconstriction and homeostasis; however, studies to date on genetic markers of members[48–56] of this signalling pathway have not shown definitive evidence of direct genetic risk (see Table 1).

NOS catalyse the formation of the potent vasodilator gaseous molecule NO from the substrate L-arginine.[57] There are three isoforms of NOS, each of which is encoded by a distinct gene. NOS1 is constitutively expressed in the brain and thus termed neuronal NOS. The NOS3 gene is expressed constitutively in the endothelium of blood vessels (endothelial NOS) and is responsible for the normal dilator tone. NOS2A is not expressed in any tissue under normal conditions. However, upregulation of NOS2A in a variety of tissues by cytokines can result in a sudden burst of NO synthesis leading to severe vasodilation and circulatory collapse.

The ETs are a class of long-acting vasoconstrictor peptides with marked similarity to snake venom, sarafotoxin. There are three different endothelin peptides, ET-1, ET-2, and ET-3, encoded by the EDN1, EDN2, and EDN3 genes, respectively, which are located on different chromosomes. All three EDN genes translate a large precursor, which is cleaved by an endothelin-converting enzyme (ECE) to the active peptide. The most potent vasoconstrictor peptide produced and released from the endothelium is ET-1 where ECE1 cleaves the inactive precursor peptide, big ET-1, to the active ET-1 peptide,[58] and is critical in controlling the production of ET-1.[59,60] The ETs are vasoconstrictors and are mitogens for vascular smooth muscle.[61] Expression of ECE1 is widespread in human tissues, but particularly high in vascular endothelial cells. NOS and ET are counterregulatory and the NO/ET pathway is crucial to the state or tone of the vasculature, which is delicately controlled by the balance in their expression. A number of studies have identified perturbations of the NO/ET pathway in most vascular beds including that of the retina in the early stages of diabetes. There has been substantial interest in the polymorphic variability of NOS and ET genes as potential markers for vascular disease.

In studies from our laboratory, we identified microsatellite polymorphic markers in members of the ET and NOS families as well as those of the ECE1 gene. Segregation of the alleles was assessed for diabetic retinopathy in two separate populations of patients recruited from two distinct geographical locations (Northern Ireland and Liverpool, England).[62] Subjects with no retinopathy despite 15 years or more of diabetes (controls) and any subject with severe retinopathy regardless of duration (ETDRS level 50 or worse) were prospectively recruited into these studies. None of the polymorphisms studied in the NOS1 (unpublished data) or NOS3[63] genes was significantly associated with cases or controls. However, studies on the NOS2A gene showed that a 14-repeat allele of a pentanucleotide polymorphism in the 5′UTR of the NOS2A gene was protective (OR = 0.21) against developing diabetic retinopathy in both patient populations.[62] Further *in vitro* studies using constructs of the NOS2A gene showed that this polymorphism influenced cytokine-induced NOS2A transcription.[62] Increasing allele size resulted in better transcription, with peak transcription occurring in constructs containing the 14-repeat allele. Further increases in allele size (15, 16, and 17 repeats) did not improve transcription.[62] When similar experiments were conducted in the presence of high concentrations of glucose mimicking a diabetic state, there was inhibition of NOS2A transcription, but this inhibition effect was

minimal in constructs containing the 14-repeat allele.[62] These findings suggest a plausible mechanism for the protective effects seen with the 14-repeat allele, and this is in agreement with published studies on the expression of NOS3 and NOS2A in high glucose. The level of expression of NOS3 is reduced in the retinal vascular endothelial cells *in vivo*[64] and *in vitro*[65] in a diabetic milieu. There is evidence to suggest that when NOS3 expression is low, induction of NOS2A may occur in an attempt to achieve homeostasis.[66] Inducibility of NOS2A may be crucial in preventing or delaying pathological alterations in the microcirculation in diabetes. It is noteworthy that specific alleles of the NOS2A were associated with the absence of retinopathy suggesting that vascular damage is the inevitable consequence of a prolonged high glucose environment *unless* genetic variation confers protection.

A recent review has highlighted the importance of the ET system and the impact of perturbations in this system on vascular complications[67] in diabetes. Evidence from EDN1 genetic mutants shows that ET and its converting enzyme are necessary for correct vascular development in the embryo.[68–70] Huang *et al*[71] reported that the EDN1 gene was directly involved in hypertension, and polymorphisms in the gene encoding ET receptor-A have been shown to be associated with essential hypertension testifying to the necessity of balance within the system for normal functioning in vascular tissues.[72]

While we have reported on the importance of ET-1 expression in retinal microvasculature in high glucose,[73] there appears to be a lack of association between a polymorphism in the EDN1 gene and diabetic retinopathy after correction.[63] There is also a similar lack of association between the ECE1 gene and retinopathy status after correction (data in preparation).

Conclusions

The loci summarised in Table 1 have been logical choices for candidate genes to investigate potential genetic contribution to diabetic retinopathy. A number of these loci showed modest associations with either lack of retinopathy or severe retinopathy, indicating the presence of genetic determinants for resistance or susceptibility to vascular complications. However, there appears to be an inability to replicate findings of either positive or negative associations (ie association or lack thereof) in multiple population groups. These inconsistencies may reflect variation in (a) case definition, (b) standardisation of grading of severity of retinopathy, (c) delineation of duration of disease, and (d) accurate recording of other associated risk factors. In addition, there is a tendency for the studies to be relatively small, and often they were undertaken in specific ethnic groups. Thus the role of 'genetic influences' in diabetic retinopathy has been difficult to define.

It is increasingly obvious that there are distinct morphological manifestations in diabetic retinopathy with some subjects showing exudative changes only in the macula (maculopathy) and others showing much more extensive retinal vascular disease. To date, the factors that determine the evolution of the clinical picture of retinopathy have not been identified and it is unclear whether the different morphologies represent distinct pathogenetic mechanisms. Future studies should undertake a more accurate and defined phenotype with respect to retinopathy status, to ensure that subsequent recategorisation can be undertaken if necessary. Agreed international standards for data collection, particularly agreement on a minimum data set for the phenotyping of retinopathy in subjects with diabetes, would permit the pooling of data from the many studies with enhanced power to detect associations. Among the various pathways that have been explored in the association studies thus far, variations in the genes involved in the NOS/ET pathway and the aldose reductase (AR2) pathway represent a fruitful area for further study.

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