DIABETIC STUDY

Dinucleotide repeat polymorphisms in *EDN1* and *NOS3* are not associated with severe diabetic retinopathy in type 1 or type 2 diabetes

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Abstract

Purpose **Endothelin-1, and constitutive endothelial nitric oxide synthase, have been implicated in the pathogenesis of diabetic retinopathy. We therefore screened polymorphisms within the genes encoding these two vasoactive agents in a sample of individuals with 15 years of diabetes and no retinopathy (ETDRS level 10 or better) and those with severe retinopathy (ETDRS level 50 or worse).**

Methods **PCR primers for highly polymorphic sites within the *EDN1* and *NOS3* genes were used to genotype individuals with type 1 or type 2 diabetes with severe or no retinopathy. Allele frequencies were compared between groups using chi-squared analysis and adjusting for multiple comparisons.**

Results **No significant differences were observed in allele frequencies for these two markers between the patients who had retinopathy and the patients who did not.**

Conclusion **Polymorphic variability in the *EDN1* and *NOS3* genes does not appear to have a major impact on determining susceptibility or resistance to diabetic retinopathy.**

Key words Diabetic retinopathy, *EDN1*, Genetic susceptibility, *NOS3*

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Diabetes mellitus is a common condition affecting 2–3% of the population of the UK. The earliest detectable abnormality in the retinal circulation is an increase in blood flow. Pathological changes soon follow that include selective loss of retinal pericytes, basement membrane thickening, and subsequent endothelial cell loss and closure of the small capillaries.[1] These events lead to failure of the blood–retinal barrier with leakage of plasma constituents into the extravascular compartments. Vascular closure causes retinal ischaemia and upregulation of growth factors, inducing retinal neovascularisation. Eventual visual loss is the result.

There is strong evidence that the earliest pathological changes in diabetic vascular disease are involved with vascular endothelial dysfunction.[2–4] A variety of molecules with potent vasoactive functions are produced and released by the vascular endothelium. The two major classes of agents with potent effects on the vasculature include the nitric oxide synthases, which mediate vasodilation, and the endothelins, which are vasoconstrictors.[5] In clinical and experimental diabetes both vasodilator and vasoconstrictor responses are aberrant early in the disease, suggesting that changes in blood flow may contribute to diabetic microangiopathy.[6,7] The retinal circulation is devoid of any extrinsic enervation, and is dependent entirely on endothelium-mediated autoregulation;[8] thus endothelial dysfunction in diabetes is likely to have a major impact on the circulation within the retina.

At the cellular level the factors that initiate and promote the development of diabetic retinopathy include duration of diabetes, poor glycaemic control and the concomitant presence of hypertension and hyperlipidaemia.[9] Clinical trials[10] and family studies indicate that in addition to environmental influences, genetic factors may also affect the onset or severity of diabetic retinopathy.[11,12] The published evidence indicates that specific genes may contribute to susceptibility or resistance to diabetic retinopathy.

Exposure of retinal vascular endothelial cells to high ambient glucose causes a downregulation of constitutive endothelial nitric oxide synthase (*NOS3*) expression.[13] Targeted disruption of the endothelin-1 (*EDN1*)[14] and *NOS3*[15] genes in mice confer aberrant cardiovascular phenotypes. Huang *et al.*[16] also used genetic experiments to show that *NOS3* appeared to be directly involved with

hypertension. Evidence thus suggests that *EDN1* and *NOS3* genes may be directly involved in microvascular disorders including diabetic retinopathy. We chose to study the polymorphic variability in the genes encoding the proteins ET-1 (*EDN1*) and cNOS (*NOS3*) in two groups of diabetic patients, one with severe retinopathy, the other with no retinopathy.

Materials and methods

Clinical assessment

Institutional ethical approval was obtained at both study centres (Northern Ireland and Liverpool area) for this project. Individuals with diabetes mellitus were recruited after giving informed consent. Patients recruited into the study fell into two categories. The first category consisted of individuals with no retinopathy in either eye (5 microaneurysms or fewer) and, if type 1 diabetics, the duration of disease had to be 15 years or more from diagnosis. The second category consisted of individuals with severe retinopathy (level 50 or worse) in either eye. Fundi were examined using slit-lamp biomicroscopy and graded for retinopathy by experienced ophthalmologists based on the ETDRS (Early Treatment Diabetic Retinopathy Study) modification of the Airlie House classification.[17] Patients with intermediate grades of retinopathy (levels 20–50) and/or maculopathy alone were excluded to maximise separation of the two groups selected for study.

The following clinical data were collected: age and sex, type, duration and family history of diabetes, presence of vascular disease (angina, hypertension, peripheral vascular disease), renal function (dipstick microalbuminuria, albumin/creatinine ratio) and glycaemic control (biannual HbA_{1c} readings were obtained over the preceding 3 years for 85% of patients). Type 1 diabetes was defined as diabetes diagnosed before 30 years of age or evidence of absolute insulin dependence (e.g. an episode of ketoacidosis). All other cases were deemed to be type 2 diabetes. The data presented herein represent 211 patients from Northern Ireland and 127 patients from Liverpool.

DNA extraction and PCR

Standard methods were used for blood collection, DNA extraction and the polymerase chain reaction (PCR). PCR primers and the conditions for amplification have been described previously for both markers: *EDN1*[18] and *NOS3*.[19] The *EDN1* marker is a dinucleotide repeat located in the 5'-untranslated region of the endothelin-1 gene with 11 reported alleles (197–217 bp; we designated the largest fragment A1) and a heterozygosity of 0.78. The *NOS3* marker is a dinucleotide repeat located within intron 13 and 23 reported alleles (134–190 bp; the 134 bp fragment is 'A1') with heterozygosity of 0.92. Published allele frequencies for *EDN1*[18] and *NOS3*[19] markers are available for Northern European Caucasians. We have typed 91 and 66 random anonymous adults for these markers, respectively, in Northern Ireland in order to compare frequencies in the general population with those in diabetes patients, as shown in Table 1.

Statistical methods

It was planned to recruit 75 patients with retinopathy and 75 patients without retinopathy for the Northern Ireland study. This was sufficient to give the study in excess of 80% power to detect as statistically significant ($p < 0.05$ with Bonferroni correction for up to 20 comparisons) a difference in the frequency of an allele between groups of 10% versus 30%. A similar number of samples were collected from Liverpool. Allele

Table 1. *Incidence of EDN1 repeat in Northern Ireland patients with diabetic retinopathy or no retinopathy and frequencies of control samples for comparison*

		A. Retinopathy		B. No Retinopathy		C. Northern Ireland	D. Northern European[18]
Allele	bp	No. of alleles	Frequency	No. of alleles	Frequency	Frequency	Frequency
Al	221	0	(0.000)	0	(0.000)	(0.000)	(0.00)
A2	219	0	(0.000)	0	(0.000)	(0.011)	(0.00)
A3	217	1	(0.007)	1	(0.006)	(0.011)	(0.02)
A4	215	1	(0.007)	0	(0.000)	(0.016)	(0.02)
A5	213	0	(0.000)	4	(0.023)	(0.005)	(0.01)
A6	211	14	(0.100)	11	(0.064)	(0.082)	(0.02)
A7	209	0	(0.000)	9	(0.052)	(0.044)	(0.02)
A8	207	21	(0.150)	25	(0.145)	(0.121)	(0.11)
A9	205	24	(0.171)	18	(0.105)	(0.104)	(0.12)
A10	203	15	(0.107)	24	(0.140)	(0.115)	(0.21)
A11	201	52	(0.371)	56	(0.326)	(0.352)	(0.37)
A12	199	8	(0.057)	20	(0.116)	(0.115)	(0.08)
A13	197	3	(0.021)	4	(0.023)	(0.022)	(0.01)
A14	195	0	(0.000)	0	(0.000)	(0.000)	(0.00)
A15	193	0	(0.000)	0	(0.000)	(0.000)	(0.00)
A16	191	0	(0.000)	0	(0.000)	(0.000)	(0.00)
Total alleles		140		172		182	160

Columns A and B are Northern Ireland patients with diabetes with retinopathy or no retinopathy. If we compare frequencies of columns A and B: $\chi^2 = 15.46$, d.f. 8, $p = 0.051$; p critical = 0.025 (combining A1–A3, A14).
Columns C and D are frequencies of Northern Ireland controls and Northern European controls. If we compare frequencies of columns C and D: $\chi^2 = 15.4$, d.f. = 10, $p = 0.12$ (combining A2 and A5).

frequencies were compared between various subgroups of patients and controls using Pearson's chi-squared test. Rare alleles were pooled for analysis to ensure that expected cell values in the chi-squared test calculation all exceeded 2. The p values for comparison between diabetes subgroups were adjusted for the number of comparisons by the Bonferroni correction. Tests of allele distribution were corrected for two comparisons ($p < 0.025$ critical) while tests of the frequency of individual alleles were corrected for 20 potential comparisons ($p < 0.0025$). The 5% level of significance was used after correction for the number of comparisons.

Results

Demographic and clinical details

We examined two markers in two different genes, *EDN1* and *NOS3*, which have been implicated in the early pathogenesis of diabetic retinopathy. The individuals genotyped included patients with diabetes, with or without retinopathy, from Northern Ireland and Liverpool. In Northern Ireland, the Retinopathy and No Retinopathy groups were well matched for age and duration of diabetes and the majority of the patients sampled had type 1 diabetes. The Retinopathy group had more than twice as many renal complications as the No Retinopathy group, and a higher incidence of hypertension. HbA_{1c} was also significantly higher in the Retinopathy group compared with the No Retinopathy group. When patients from Liverpool were similarly subdivided into Retinopathy and No Retinopathy groups, the differences in renal complications and hypertension were less significant when compared with the Northern Ireland sample, possibly due to the preponderance of patients with type 2 diabetes in the Liverpool sample.

Comparisons of allele frequencies of controls from Northern Ireland and Northern Europe

There were no significant differences between Northern Ireland and Northern European control frequencies for the *EDN1* marker (Table 1). The observed difference in distribution of alleles of the *NOS3* marker could be partly attributed to difference in sample size and representation of the rarer alleles.

Specific alleles of EDN1 *are not significantly associated with Retinopathy or No Retinopathy in Northern Ireland*

Although the data for the EDN1 marker revealed differences in allele frequencies between the Retinopathy group and No Retinopathy group in Northern Ireland, upon correction to take into account multiple comparisons, the p value did not reach significance (critical $p = 0.025$, Table 1). The differences observed between Retinopathy and No Retinopathy patients could be in part attributed to the incidence of the A7 allele. Carriage rate for A7 in the Retinopathy group compared with the No Retinopathy group was 0/140 (0.000) compared with 9/172 (0.052), respectively. This is interesting; however, upon correction of Fisher's exact test to account for multiple comparisons, the result is not significant (critical $p = 0.0025$). It is possible that this allele may be important in influencing retinopathy for a small proportion of individuals affected with diabetes.

There is no association between any allele of EDN1 *and Retinopathy or No Retinopathy in Liverpool*

The data for patients recruited from Liverpool are shown in Table 2. Retinopathy and No Retinopathy patients were not significantly different from each other at any allele observed. The A5 allele had the same trend of higher incidence in the No Retinopathy patients; however, it was not statistically significant ($\chi^2 = 3.00$, d.f. = 1, $p = 0.08$). A pooled analysis of the results from both Northern Ireland and Liverpool stratified by centre

Table 2. *Incidence of EDN1 dinucleotide repeat in Liverpool*

		A. Retinopathy		B. No Retinopathy		C. Northern European[18]	
Allele	bp	No. of alleles	Frequency	No. of alleles	Frequency		Frequency
A1	221	0	(0.000)	1	(0.009)		(0.000)
A2	219	1	(0.009)	0	(0.000)		(0.000)
A3	217	1	(0.009)	0	(0.000)		(0.019)
A4	215	0	(0.000)	0	(0.000)		(0.019)
A5	213	2	(0.017)	1	(0.009)		(0.013)
A6	211	12	(0.120)	4	(0.038)		(0.019)
A7	209	3	(0.025)	8	(0.076)		(0.019)
A8	207	12	(0.102)	14	(0.132)		(0.113)
A9	205	17	(0.144)	16	(0.151)		(0.120)
A10	203	13	(0.110)	8	(0.076)		(0.214)
A11	201	40	(0.339)	36	(0.340)		(0.371)
A12	199	15	(0.127)	14	(0.132)		(0.082)
A13	197	1	(0.009)	4	(0.038)		(0.013)
A14	195	0	(0.000)	0	(0.000)		(0.000)
A15	193	1	(0.009)	0	(0.000)		(0.000)
A16	191	0	(0.000)	0	(0.000)	(0.000)	
Total alleles		118		106		160	

Comparison of column A with B showed $\chi^2 = 13.42$, d.f. = 12, $p = 0.339$.

Table 3. *Incidence of NOS3 alleles in patients from Northern Ireland with retinopathy or no retinopathy, and in control samples*

		A. Retinopathy		B. No Retinopathy		C. Northern Ireland	D. Northern European[18]
Alleles	bp	No. of alleles	Frequency	No. of alleles	Frequency	Frequency	Frequency
A1	190	0	(0.000)	0	(0.00)	(0.000)	(0.001)
A2	186	0	(0.000)	1	(0.005)	(0.000)	(0.007)
A3	184	1	(0.006)	3	(0.015)	(0.008)	(0.010)
A4	182	6	(0.039)	3	(0.015)	(0.008)	(0.015)
A5	180	9	(0.058)	7	(0.036)	(0.000)	(0.039)
A6	178	16	(0.103)	10	(0.052)	(0.023)	(0.064)
A7	176	23	(0.147)	31	(0.160)	(0.038)	(0.103)
A8	174	15	(0.096)	17	(0.088)	(0.098)	(0.105)
A9	172	13	(0.083)	15	(0.077)	(0.136)	(0.099)
A10	170	15	(0.096)	24	(0.124)	(0.106)	(0.091)
A11	168	11	(0.071)	19	(0.098)	(0.159)	(0.100)
A12	166	15	(0.096)	17	(0.088)	(0.068)	(0.075)
A13	164	6	(0.039)	10	(0.052)	(0.076)	(0.079)
A14	162	7	(0.045)	15	(0.077)	(0.114)	(0.092)
A15	160	3	(0.019)	2	(0.010)	(0.046)	(0.018)
A16	158	4	(0.026)	3	(0.016)	(0.046)	(0.019)
A17	156	8	(0.051)	4	(0.021)	(0.046)	(0.039)
A18	154	3	(0.019)	3	(0.016)	(0.030)	(0.011)
A19	152	1	(0.006)	7	(0.036)	(0.000)	(0.025)
A20	150	0	(0.000)	0	(0.000)	(0.000)	(0.002)
A21	148	0	(0.000)	3	(0.016)	(0.000)	(0.002)
A22	142	0	(0.000)	0	(0.000)	(0.000)	(0.001)
A23	134	0	(0.000)	0	(0.000)	(0.000)	(0.001)
Total alleles		156		194		132	596

Columns A and B are Northern Ireland patients with diabetes with retinopathy (A) or no retinopathy (B). Comparison of allele frequencies in columns A and B reveals: $\chi^2 = 19.1$, d.f. = 16, p = 0.26; p critical = 0.025 (combining alleles A1–3 and A21–23). Columns C and D are frequencies of Northern Ireland controls (C) and Northern European controls (D). Comparison of allele frequencies reveals: $\chi^2 = 33.5$, d.f. = 15, $p = 0.004$ (combining alleles A1–4 and A20–23).

for A7 are not statistically significant after correction for multiple comparisons ($\chi^2 = 8.38$, d.f. =1, $p = 0.0038$, critical $p = 0.0025$).

NOS3 *had no associations with Retinopathy or No Retinopathy*

The distribution of alleles of the *NOS3* marker did not differ significantly between the Retinopathy and No Retinopathy patients in Northern Ireland (Table 3). When the data were compared with the population frequencies reported for Northern Europe there were also no significant differences. There were also no significant differences in the distribution of alleles in Retinopathy and No Retinopathy patients in the Liverpool population ($p = 0.25$; data not shown).

Discussion

Nitric oxide (NO) and nitric oxide synthases (NOS) signal transduction pathways have been implicated in the pathogenesis of diabetic retinopathy. In targeted disruption of the *NOS3* gene, a critical role for endogenous NO in the vascular system in general was shown.[15] NO is a negative regulator of vascular smooth muscle proliferation and can promote aberrant remodelling of the vasculature.[15] Thus the gene encoding constitutively expressed endothelial NOS was considered an important candidate gene in assessing genetic susceptibility to diabetic retinopathy. However, the present study showed that variability in the *NOS3* gene was not strongly associated with retinopathy or the absence of retinopathy. Bonnardeaux *et al.*[20] found a similar lack of association between the molecular variants of *NOS3* and essential hypertension in a population study. Nonetheless, in recent studies we have demonstrated that an allele of the *NOS2* gene is associated with absence of diabetic retinopathy.[21] Other NOS signal transduction components cannot be ruled out in the pathology of retinopathy.

Huang *et al.*[16] reported that the *EDN1* gene appeared to be directly involved in hypertension. Polymorphisms in *EDN1* and the gene encoding endothelin receptor-A have already been associated with essential hypertension.[22] It is interesting to note that the endothelins are implicated in a number of vascular diseases including the pathology of retinopathy,[23] so perhaps it was disappointing not to see a more direct involvement of a marker of the *EDN1* gene. Where mutant mice have been studied, modification in the endothelin components has had critical effects on the development of the cardiovascular system and other tissues.[14,24,25] In the present study the distribution of the *EDN1* alleles was not statistically significant between groups of diabetic patients with and without retinopathy in both Northern Ireland and Liverpool. However, one of the rarer alleles was less commonly encountered in patients with retinopathy in both sample populations. It is possible that minor contributions from this gene may influence an individual's susceptibility to retinopathy, but the study had insufficient power to detect this association. As other genes of the endothelin and nitric

oxide synthase signal transduction pathway may be relevant to the pathogenesis of diabetic retinopathy these studies have not ruled out genetic contributions from these families of genes to this disease.

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