

A VARIANT OF PARAOXONASE (PON1) GENE IS ASSOCIATED WITH DIABETIC RETINOPATHY IN IDDM

YAN-LIN KAO^{1,2}, KIM DONAGHUE^{1,3}, ALBERT CHAN¹, JOHN KNIGHT², MARTIN SILINK^{1,3}

¹Ray Williams Institute of Paediatric Endocrinology, Diabetes and Metabolism, and ²Centre for Kidney Research, The Royal Alexandra Hospital for Children, Westmead 2145. ³University of Sydney 2006, Australia

ABSTRACT Serum paraoxonase is a glycoprotein which binds to high-density lipoproteins (HDL) and may prevent oxidation of LDL by hydrolyzing lipid peroxides. Two polymorphisms identified in the paraoxonase gene (Met-Leu 54 and Gln-Arg 192) have been associated with cardiovascular disease. Oxidative low-density lipoprotein (LDL) is also toxic to retinal capillary endothelial cells and pericytes, so that mildly modified LDL may contribute to the development of diabetic retinopathy. To investigate the potential significance of these polymorphisms in the pathogenesis of diabetic retinopathy in IDDM, 80 patients with diabetic retinopathy and 119 controls without diabetic retinopathy were investigated in the current project. The allelic frequency of leucine 54 (L) was significantly higher in the group with retinopathy than without retinopathy (73% vs. 57%, $p < 0.001$). The genotype L/L was strongly associated with the development of diabetic retinopathy ($p < 0.001$), but a similar association was not found with Gln-Arg 192. Leucine 54 is a risk factor for diabetic retinopathy.

Oxidative low-density lipoprotein (LDL) cholesterol is toxic to the endothelial cells and pericytes in retinal capillaries (1). An important mechanism contributing to diabetic retinopathy may be the oxidation of LDL with consequent injury to retinal endothelia and pericytes (1). Oxidation of LDL plays an important role in the development and progression of cardiovascular disease, with high-density lipoprotein (HDL) cholesterol potentially protecting LDL from oxidation. Recent data shows that the protective effect of HDL may be due to paraoxonase (PON1) (2,3), a glycoprotein which binds HDL.

Two variants of the PON1 gene have been associated with an increased risk of cardiovascular disease: Gln-Arg 192 and more recently Met-Leu 54 (4,5,6). The current study was designed to investigate whether the two variants are associated with diabetic retinopathy.

Materials and Method

Study population

One hundred and ninety-nine adolescents with IDDM were classified with or without retinopathy

according to results of stereoscopic fundal photography of seven standard fields. The presence of background retinopathy was defined as any microaneurysm or hemorrhage. Patient characteristics are shown in Table 1. Blood was taken for genotyping. Informed consent was given by all participants and the study was approved by the Ethics Committee of the Royal Alexandra Hospital for Children.

Molecular Analysis

DNA was extracted from peripheral leukocytes by a conventional phenol-chloroform method. PCR primers were prepared as described by Humbert et al (7). An aliquot of 500ng DNA was denatured at 94C for 5 min and then amplified for 35 cycles. Each cycle consisted of denaturation at 94C for 30 sec, annealing at 61C for 30 sec and extension at 72C for 30 sec with a final extension time of 7 min. The PCR products, 170bp and 99bp, corresponded to the polymorphisms affecting positions 54 and 192, and were digested with restriction enzymes Hsp92 II and Alw I respectively. The digested products were separated by electrophoresis on 3.5% agarose gel and identified by ethidium

Table 1. Clinical characteristics of the patients with IDDM

	No Retinopathy	Retinopathy	p-value
Gender	60 M, 59 F	34 M, 46 F	NS
Age (years)	13.9 [12.3 - 15.6]	15.4 [13.4 - 17.4]	0.0008
Duration (years)	5.7 [4.1 - 8.3]	7.9 [6.1 - 11.2]	0.0001
HbA _{1c} (%)	8.7 [7.9 - 9.7]	8.6 [7.7 - 9.6]	NS
Mean AER (µg/min)	4.1 [3.0 - 6.1]	6.3 [4.3 - 10.8]	0.0003
Cholesterol (mmol/L)	4.3 [3.8 - 4.8]	4.4 [3.8 - 5.0]	NS
Systolic BP (mmHg)	110 [105 - 120]	110 [110 - 120]	NS
Diastolic BP (mmHg)	70 [60 - 70]	70 [65 - 70]	NS

Results above are given as median [interquartile range]

bromide staining. At position 54, allele L (leucine) corresponds to the presence of a non-digested fragment of 170bp, while allele M (methionine) corresponds to 2 digested fragments of 126bp and 44bp. At position 192, a single substitution of A to G creates a unique Alw I site. Allele A (glutamine) corresponds to the non-digested 99bp product and allele B (arginine) corresponds to 69 and 30bp products.

Statistical analysis

The software package of SAS (Version 6.11, Cary NC, SAS Institute, 1995) was used to analyse the data. For each genotype and allele, the Z-test was used to compare the difference in proportions between those with and without retinopathy. The Kruskal-Wallis test was used for comparing continuous variables between the retinopathy groups.

Results

Table 2 shows the frequencies of alleles and genotypes at the two loci for the diabetic subgroups with and without retinopathy and for a group of 156 nondiabetic Australians.

For Met-Leu 54, the frequencies of allele L and L/L genotype were significantly higher ($p < 0.001$) in those with retinopathy than without retinopathy (73% vs. 57%, and 50% vs. 27% respectively). The frequencies of allele and genotype in this

diabetic and in the nondiabetic groups were similar to those in a Caucasian group (5).

For Gln-Arg 192, neither genotype nor allele frequencies differed significantly between the two diabetic groups. The genotype A/A was the most common and the B/B was rarest, as found in two other populations (5,6).

The odds ratios for age and duration were 1.14 (CI 1.01, 1.28) and 1.15 (CI 1.04, 1.27) respectively. After allowing for the confounding effects of age and duration, the odds ratio for retinopathy for L/L was 2.55 (CI 1.34, 4.88, $p = 0.0046$) and for M/M was 0.24 (CI 0.06, 0.90, $p = 0.035$).

Discussion

The oxidation pathway may play a role in the early development of retinopathy. A case-control study of early retinopathy found that higher HDL-cholesterol was protective against diabetic retinopathy (8). The paraoxonase genes are good candidates for an increased vascular risk. The enzyme is entirely bounded to HDL-cholesterol which could explain its anti-oxidant capacity (2,3), and there is a 10-40-fold difference in serum PON1 activity between individuals which is stable over time (7). In the current study the difference in risk for retinopathy between the L and M homozygote is a factor of ten.

Table 2. Frequency of PON1 allele and genotype in patients with and without retinopathy

			Diabetic group		Nondiabetic group	p-value *
			No retinopathy	Retinopathy		
Met-Leu54	Haplotype	L	135 (57%)	117 (73%)	60%	0.00087
		M	103 (43%)	43 (27%)	40%	
	Genotype	L/L	32 (27%)	40 (50%)	34%	0.00088
		M/L	71 (60%)	37 (46%)	53%	NS
		M/M	16 (13%)	3 (4%)	13%	0.023
Gln-Arg 192	Haplotype	A	159 (67%)	96 (60%)	63%	NS
		B	79 (33%)	64 (40%)	37%	
	Genotype	A/A	60 (50%)	35 (44%)	48%	NS
		A/B	39 (33%)	26 (32%)	30%	NS
		B/B	20 (17%)	19 (24%)	22%	NS

* The p-values indicate the significance of comparison between those with and without retinopathy in the diabetic group

Whilst two polymorphisms of the PON1 gene have been identified, the early reports suggested that only Gln-Arg 192 was associated with PON1 activity (7,9). Considerable attention has focused on this variant, which has been strongly associated with cardiovascular disease in some groups (4,6,10,11) but not in others (12,13). Met-Leu 54 was not considered to be involved in determination of the allozyme type (7). Subsequently the Met-Leu 54 polymorphism was found associated with modified serum concentration of PON1, and contributing to the PON1 activity (5,14,15). In NIDDM the L/L genotype doubled the risk of heart disease after allowing for other risk factors (5).

In vitro, the effect of PON1 polymorphism 192 varies for different substrates. While the Arg 192 isoform hydrolyses paraoxon more rapidly, both Gln 192 and Arg 192 hydrolyse chlorpyrifos oxon and phenylacetate at approximately the same rate (16). As yet no physiological substrate has been identified. The association between polymorphisms and enzyme activity remains to be studied in vivo. Toxicity of mildly modified LDL to cultured retinal capillary endothelial cells and pericytes has been observed (1). In vitro, inactivation of paraoxonase by pretreating HDL with heat or EDTA reduces the ability of HDL to

inhibit LDL modification (2). Leucine 54 may contribute to the development of diabetic retinopathy by attenuating HDL's protection of LDL against oxidation.

Our results show that the PON1 variant, Met-Leu 54, is a significant genetic risk factor for diabetic retinopathy. PON1 gene is one member of a multigene family (17). Recently two other members of the PON family, designated PON2 and PON3, have been identified, and they are all located on chromosome 7. The Cys-Ser 311 variant of PON2 gene was associated with coronary heart disease in Indians (18). Other reports showed that PON2 might be involved in insulin resistance (19,20). The family of paraoxonase genes has potential significance for understanding diabetes and its complications.

Acknowledgment: Yan-Lin Kao was supported by a generous donation from the Florence Theresa Pitt Estate.

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