Polymorphisms of the Aldose Reductase Gene and Susceptibility to Retinopathy in Type 1 Diabetes Mellitus

Andrew Demaine, Deborah Cross, and Ann Millward

Purpose. Aldose reductase (ALR2) is the first and rate-limiting enzyme of the polyol pathway and is involved in the pathogenesis of diabetic retinopathy. Polymorphisms of the ALR2 gene are associated with susceptibility to diabetic retinopathy in Chinese and Japanese patients with type 2 diabetes. There are no reports investigating these polymorphisms in white patients with type 1 diabetes from either Western Europe or North America. A CA dinucleotide repeat polymorphism (5'ALR2; located at −2100 bp) as well as a novel C(106)T polymorphism was investigated in 229 white patients with type 1 diabetes, with or without retinopathy.

Methods. The DNA was typed for these polymorphisms using conventional polymerase chain reaction techniques.

Results. There was a highly significant increase in the frequency of the Z′−2 5'ALR2 allele and Z′−2/X (where X is not Z′+2) genotype in patients with diabetic retinopathy (n = 159) compared with those without who had diabetes of 20 years' duration (uncomplicated, n = 70; χ² = 17.0, P < 0.0001). There was a similar decrease in the Z′+2/Y genotype (where Y is not Z′−2; χ² = 30.1, P < 0.00001) in the patients with retinopathy compared with the uncomplicated diabetes group. The C′/Z′−2 C(106)T/5' ALR2 haplotype was found in 33.3% of the patients with retinopathy and 8.7% of the patients with uncomplicated diabetes.

Conclusions. These results confirm previous studies in other populations and in type 2 diabetes showing that polymorphisms in the promoter region of the ALR2 gene are associated with susceptibility to diabetic retinopathy. (Invest Ophthalmol Vis Sci. 2000;41:4064–4068)

Retinopathy is a leading cause of visual impairment in patients with type 1 diabetes mellitus.1 It is well known that long-term exposure to hyperglycemia is a major cause of diabetic retinopathy, and strict glycemic control may help to prevent or delay the onset of this complication of diabetes.2–4 It is becoming clear that genetic factors may also play a role in the pathogenesis of diabetic retinopathy. At present, little is known about these genetics factors, although recent studies have implicated the gene encoding aldose reductase (ALR2).5–13 Aldose reductase is the first and rate-limiting enzyme of the polyol pathway and converts glucose to sorbitol in an NADPH-dependent reaction. Sorbitol is subsequently converted to fructose by the enzyme sorbitol dehydrogenase (SORD) with the cofactor nicotinamide adenine dinucleotide phosphate (NADPH)–dependent reaction. Under hyperglycemic conditions there is increased flux through the polyol pathway, and this in turn leads to a number of metabolic and vascular abnormalities that may ultimately cause tissue ischemia.

It has recently been shown that a polymorphism (5′ALR2) consisting of a (CA)n repeat located 2.1 kb upstream of the initiation site of ALR2 is associated with diabetic nephropathy and neuropathy.17–19 Chinese and Japanese patients with type 2 diabetes and retinopathy have been found to have an increased frequency of the Z′−2 5′ALR2 allele consisting of 23 CA repeats.5,12 Similar findings have been reported for young white adolescents from Australia with type 1 diabetes and an early onset of retinopathy.9 However, to date there are no studies investigating a population of patients with type 1 diabetes from either Western Europe or North America. Therefore, the purpose of this study was to investigate the 5′ALR2 locus in a large population of patients with type 1 diabetes with established retinopathy.

Methods

Patients and Normal Control Subjects

DNA samples from 229 white British patients with type 1 diabetes were randomly taken from the freezer for analysis. A total of 114 DNA samples from normal healthy white British subjects were randomly taken from the freezer to obtain control frequencies. The normal control subjects consisted of DNA from cord blood samples collected sequentially after normal obstetric delivery from the Obstetric Department, Derriford Hospital, Plymouth (UK). Local ethics committee approval was
obtained. The study protocol adhered to the tenets of the Declaration of Helsinki. The patients were classified according to their microvascular complications, as previously described.15,16 These are summarized as follows.

Patients with Uncomplicated Diabetes
These patients (n = 70) had had type 1 diabetes for at least 20 years but remained free of retinopathy (fewer than five dots or blots per fundus) and proteinuria (negative results in urine Albustix testing [Bayer, West Haven, CT] on three consecutive occasions over 12 months).

Patients with Retinopathy
These patients (n = 159) had retinopathy defined as more than five dots or blots per eye; hard or soft exudates, new vessels, or fluorescein angiographic evidence of maculopathy or previous laser treatment for preproliferative or proliferative retinopathy; and maculopathy or vitreous hemorrhage. Ninety of these patients also had proteinuria. Fundoscopy was performed by both a diabetologist and ophthalmologist. The clinical features of the patients are shown in Table 1.

Preparation of DNA and Analysis of ALR2 Promoter Region Polymorphisms
For the 5′ALR2 locus, DNA samples were taken at random from the freezer, and an aliquot (50–100 ng) was used for amplification of the DNA. Briefly, a pair of amplimers was constructed that flanks a 138-bp region that contains the dinucleotide (CA)n repeat.8 The antisense primer was labeled with 32P-deoxyadenosine triphosphate (dATP) by T4 polynucleotide kinase. A 50-μl reaction mix was prepared using the end labeled 5′ALR2 antisense and the sense amplimer, dNTPs and Taq polymerase. The samples were subjected to 30 cycles of amplification, which consisted of denaturing for 1 minute at 95°C, annealing for 1 minute at 61°C, and extension for 1 minute at 72°C. An aliquot of the amplified genomic DNA was electrophoresed through a 5% formamide-urea gel at 90 W for 3 to 4 hours and dried, and autoradiography was performed using radiographic film (X-Omat; Eastman Kodak, Rochester, NY) with intensifying screens at ~85°C overnight.

A C(-106)T polymorphism upstream of the ALR2 gene was typed by taking 50 to 100 ng of genomic DNA together with amplimers that span the polymorphic site to amplify the region using previously published sequences.9 The following conditions were used: The samples were denatured for 2 minutes at 96°C and then amplified for 35 cycles at 94°C for 30 seconds and 70°C annealing-extension for 2 minutes. The amplification products were then purified using a commercial system (Wizard PCR Prep DNA purification system; Promega, Madison, WI) and a laboratory vacuum manifold (Vac-Man; Promega).

The C(-106)T substitution creates a new BfaI restriction endonuclease site. To detect the C(-106)T polymorphic site, 20 μl of purified product was digested to completion using 10 U of BfaI and incubated in the appropriate buffer at 37°C for 2 hours. Digested products were run out on a 3.5% agarose gel containing ethidium bromide at 150 V against a 50-bp DNA molecular weight marker and visualized under a UV illuminator.

Statistical Analysis
The number of 5′ALR2 and C(-106)T alleles was obtained using gene counting. The frequency of 5′ALR2 and C(-106)T alleles and genotypes in the patient and normal control groups were compared using the χ² test and contingency tables. The probability was corrected for the number of comparisons made (Pc) using the Bonferroni inequality method.20 C(-106)T/5′ALR2 haplotype frequencies were obtained by the gene-counting method using the DNA of those subjects who were homozygous for either one or both loci.

RESULTS
The frequency of the 5′ALR genotypes in the patients and normal control subjects are shown in Table 2. The frequencies of the 5′ALR2 and C(-106)T genotypes in the patient subgroups as well as the normal control subjects were found to be in Hardy–Weinberg equilibrium. Nine alleles were detected; Z+8, Z+6, Z+4, Z+2, Z, Z−2, Z−4, Z−6, and Z−8 where Z is the most common allele and consists of 24 CA repeats. Of the 159 patients with retinopathy, 49.1% had the Z−2/X 5′ALR2 genotype (where X is not Z) compared with only 20.0% in the uncomplicated diabetes group (P < 0.0001). In contrast, the Z+2/Y genotype (where Y is not Z) was found in only 16.4% of the patients with retinopathy but in 51.4% of the patients with uncomplicated diabetes (P < 0.0001). The normal control subjects had intermediate frequencies of these genotypes. There were no other differences in the frequency of the 5′ALR2 genotypes between the patient subgroups.

Table 1. Clinical Features of the Patient Groups and Normal Control Subjects

<table>
<thead>
<tr>
<th></th>
<th>Uncomplicated (n = 70)</th>
<th>Retinopathy (n = 159)</th>
<th>Normal (n = 114)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male:Female</td>
<td>26:44</td>
<td>77:82</td>
<td>50:64</td>
</tr>
<tr>
<td>Age at onset of diabetes (yr)</td>
<td>17.2 (10–36)</td>
<td>20.3 (1–58)</td>
<td></td>
</tr>
<tr>
<td>Duration of diabetes (yr)</td>
<td>33.4 (20–59)</td>
<td>24.2 (12–53)</td>
<td></td>
</tr>
</tbody>
</table>

Figures in parentheses refer to the range in years. All the patients had type 1 diabetes. The uncomplicated group had no evidence of retinopathy or proteinuria and had a duration of diabetes of more than 20 years.

Table 2. Frequency of 5′ ALR2 Genotypes in Patients with Type 1 Diabetes Mellitus and Diabetic Retinopathy

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Uncomplicated (n = 70)</th>
<th>Retinopathy (n = 159)</th>
<th>Normal (n = 95)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z−2/X</td>
<td>20.0 (14)</td>
<td>49.1 (78)†</td>
<td>29.0 (27)</td>
</tr>
<tr>
<td>Z+2/Y</td>
<td>51.4 (36)</td>
<td>16.4 (26)†</td>
<td>28.0 (26)</td>
</tr>
<tr>
<td>Z−2/Z+2</td>
<td>4.3 (3)</td>
<td>8.2 (13)</td>
<td>5.4 (5)</td>
</tr>
<tr>
<td>X/Y</td>
<td>24.3 (17)</td>
<td>26.4 (42)</td>
<td>37.6 (35)</td>
</tr>
</tbody>
</table>

Figures in parentheses are number of subjects. X is any allele other than Z+2 and Y is any allele other than Z−2. A total of 9 alleles were detected designated Z+8 to Z−8 where Z = 24 CA repeats.† Frequency compared with uncomplicated χ² = 17.0, P < 0.0001.
The frequency of the 5' ALR2 alleles is shown in Table 3. The patients with retinopathy had an increased frequency of the Z−2 allele compared with the patients with uncomplicated diabetes (33.6% and 14.3%, respectively; $\chi^2 = 18.1, P < 0.0001$). Conversely, the Z+2 allele was decreased in the patients with retinopathy compared with the patients with uncomplicated diabetes (13.2% and 33.6%, respectively; $\chi^2 = 39.8, P < 0.0001$). There were no other significant differences, although it is interesting that there was a small increase in the frequency of the Z−4 allele in the patients with retinopathy compared with the patients with uncomplicated diabetes (6.6% and 2.9%, respectively). The normal control subjects had intermediate Z−2 and Z+2 allelic frequencies compared with those for the uncomplicated diabetes and retinopathy groups.

Table 4 shows the frequency of the C/C(-106)ALR2 alleles and genotypes in the patients with type 1 diabetes and normal control subjects. The retinopathy group had an increased frequency of the C/C(-106) ALR2 genotype compared with the uncomplicated diabetes group (46.7% and 25.0%, respectively; $\chi^2 = 5.18, P < 0.025, P_c = 0.05$). Consequently, the frequency of the C(-106) allele was increased in the retinopathy group compared with the uncomplicated diabetes group (71.9% versus 55.6%, respectively; $\chi^2 = 6.7, P < 0.01$).

It is possible to deduce the 5' ALR2/C(-106)T haplotypes in those subjects who were homozygous for at least one of these loci. In the retinopathy group, it was possible to determine 155 of the 318 haplotypes; similarly, 46 of the 140 haplotypes in the uncomplicated diabetes group were identified. The frequency of the 5’ ALR2/C(-106)T haplotypes is shown in Table 5. The most common haplotype in the retinopathy group was the Z−2/C, which was present in 31.0% compared with only 8.7% of the uncomplicated diabetes group ($\chi^2 = 9.2, P < 0.001, P_c = 0.005$) and with a frequency of 14.3% in the normal control subjects. In contrast, the Z+2/C as well as the Z+2/T haplotypes occurred with a reduced frequency in the retinopathy group compared with the uncomplicated group (9.0% and 41.3%, respectively, $\chi^2 = 26.6, P < 0.0001, P_c = 0.0005$). The frequency in the normal control subjects was 18.5%.

### Discussion

We have recently shown that the Z−2 5' ALR2 allele is strongly associated with the susceptibility to diabetic nephropathy and neuropathy.17,18 However, in our previous report, although the frequency of the Z−2 allele was increased in patients with retinopathy, the change did not attain significance. In this study we have presented data on a greatly expanded patient population and find a highly significant increase in the frequency of the Z−2 allele in those patients with retinopathy compared with patients with long-term uncomplicated diabetes. To our knowledge, this is the first report of a study investigating these genetic markers in a white patient population with retinopathy from Western Europe or North America.

The results presented here confirm the recent reports studying young adolescents with type 1 diabetes and retinopathy from Australia as well as studies in Chinese and Japanese patients with type 2 diabetes. However, in contrast to these studies we also found a protective effect of the Z+2 allele in our population. Indeed, the decreased frequency of this allele in our patients with retinopathy are more significant than the increase in the frequency of the Z−2 allele. The discrepancy between findings in the studies may reflect the allelic frequencies between the different ethnic groups. For instance, the Z+2 allele is relatively uncommon in Japanese populations. An alternative explanation is duration of diabetes in the uncomplicated diabetes groups used in these studies. The duration of disease for the uncomplicated diabetes group in our study was

#### Table 3. Frequency of 5’ ALR2 Alleles in Patients with Type 1 Diabetes Mellitus and Diabetic Retinopathy

<table>
<thead>
<tr>
<th>5’ ALR2 Allele</th>
<th>Uncomplicated (n = 140)</th>
<th>Retinopathy (n = 318)</th>
<th>Normal (n = 186)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z−2</td>
<td>14.3 (20)</td>
<td>33.6 (107)*</td>
<td>21.5 (40)</td>
</tr>
<tr>
<td>Z+2</td>
<td>33.6 (47)</td>
<td>13.2 (42)†</td>
<td>22.0 (41)</td>
</tr>
<tr>
<td>Z</td>
<td>40.7 (57)</td>
<td>40.3 (128)</td>
<td>45.6 (85)</td>
</tr>
<tr>
<td>Z−4</td>
<td>2.9 (4)</td>
<td>6.6 (21)</td>
<td>3.8 (7)</td>
</tr>
<tr>
<td>Z+8</td>
<td>0.7 (1)</td>
<td>0.3 (1)</td>
<td>0.0</td>
</tr>
<tr>
<td>Z+6</td>
<td>1.4 (2)</td>
<td>0.5 (1)</td>
<td>2.7 (5)</td>
</tr>
<tr>
<td>Z+4</td>
<td>6.4 (9)</td>
<td>4.1 (13)</td>
<td>2.7 (5)</td>
</tr>
<tr>
<td>Z−6</td>
<td>0.0</td>
<td>1.5 (4)</td>
<td>1.6 (5)</td>
</tr>
<tr>
<td>Z−8</td>
<td>0.0</td>
<td>0.3 (1)</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Figures in parentheses are number of subjects.

* Frequency compared with uncomplicated $\chi^2 = 18.1, P < 0.0001$.
† Frequency compared with uncomplicated $\chi^2 = 39.8, P < 0.000001$.

#### Table 4. Frequency of C/C(-106)ALR2 Alleles and Genotypes in Patients with Type 1 Diabetes Mellitus and Diabetic Retinopathy

<table>
<thead>
<tr>
<th>C-C(-106)T</th>
<th>Uncomplicated (n = 36)</th>
<th>Retinopathy (n = 105)</th>
<th>Normal (n = 114)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C/T</td>
<td>61.1 (22)</td>
<td>50.5 (55)</td>
<td>56.1 (64)</td>
</tr>
<tr>
<td>C/C</td>
<td>25.0 (9)</td>
<td>46.7 (49)*</td>
<td>29.8 (34)</td>
</tr>
<tr>
<td>T/T</td>
<td>13.8 (5)</td>
<td>2.8 (3)</td>
<td>14.1 (16)</td>
</tr>
</tbody>
</table>

#### Table 5. Frequency of 5’ ALR2/C(-106)T Haplotypes in Patients with Type 1 Diabetes Mellitus and Diabetic Retinopathy

<table>
<thead>
<tr>
<th>Haplotype 5’ ALR2/C(-106)T</th>
<th>Uncomplicated (n = 46)</th>
<th>Retinopathy (n = 155)</th>
<th>Normal (n = 140)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z−2/C</td>
<td>8.7 (4)</td>
<td>31.0 (48)*</td>
<td>14.3 (20)</td>
</tr>
<tr>
<td>Z+2/C</td>
<td>23.9 (11)</td>
<td>7.1 (11)†</td>
<td>11.4 (16)</td>
</tr>
<tr>
<td>Z−2/T</td>
<td>6.5 (3)</td>
<td>2.6 (4)</td>
<td>7.9 (11)</td>
</tr>
<tr>
<td>Z+2/T</td>
<td>17.4 (8)</td>
<td>1.9 (3)†</td>
<td>7.1 (10)</td>
</tr>
<tr>
<td>X/C</td>
<td>28.3 (13)</td>
<td>29.0 (65)</td>
<td>35.0 (49)</td>
</tr>
<tr>
<td>X/T</td>
<td>15.2 (7)</td>
<td>15.5 (24)</td>
<td>24.3 (34)</td>
</tr>
</tbody>
</table>

X, neither Z−2 or Z+2 allele. The haplotype frequencies were obtained by using the gene-counting method in those subjects who were homozygous for either the 5’ ALR2 or the C/C(-106)T locus or both.

* Compared with uncomplicated $\chi^2 = 9.2, P < 0.001, P_c = 0.005$.
† Compared with uncomplicated $\chi^2 = 26.6, P < 0.00001, P_c = 0.00005$. 

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at least 20 years. Although this does not exclude the possibility that retinopathy will develop in some of these patients in the future, the risk is clearly much lower than in those individuals who have had diabetes for only 5 to 10 years, which is the cutoff point that was used in many other studies.

In the diabetic patients with retinopathy the C(-106) allele was associated with the Z−2 5′ ALR2 allele. Conversely, in the uncomplicated group, both the C and the T-allele was associated with the Z+2 5′ ALR2 allele. This suggests that the 5′ ALR2, together with the C(-106)/T loci is close to the region that confers susceptibility to retinopathy. These results are consistent with those of Kao et al.11 who studied a young adolescent population of patients with type 1 diabetes and retinopathy in Australia. However, Moczulski et al.28 recently showed that in an North American population of patients with type 1 diabetes and nephropathy the T(-106) allele was associated the Z−2 allele, and this haplotype was found more often in this group of patients than in those without proteinuria (but not necessarily retinopathy). None of these studies included any normal control frequencies. In our normal control population the C/Z−2 haplotype was relatively common, suggesting that these two markers may display linkage. However, family studies are needed to confirm this.

It has recently been shown that the Z−2 5′ ALR2 allele is associated with the increased mRNA expression in patients with nephropathy compared with patients without the allele.19 Therefore, the C/Z−2 haplotype may identify individuals who have enhanced levels of ALR2 mRNA, and the presence of this haplotype may lead to increased flux through the polyol pathway. In contrast, the C/Z+2 and T/Z+2 haplotypes may be associated with reduced gene expression. Ultimately, increased gene expression would result in excessive production of sorbitol and fructose, metabolic and vascular abnormalities, and oxidative stress in the cell.

The polymorphisms in the current study are in a region of the ALR2 gene that is known to contain a number of osmotic response elements (OREs).12–21 We have recently described the sequencing of those patients with or without nephropathy as well as sequencing of the region using a cosmid clone.21–24 We did not find any sequence differences between those patients with microvascular disease and those without. These observations together with those of Shah et al.19 and Ikigishi et al.10 suggest that the 5′ ALR2 may be directly involved in modulating the expression of the ALR2 gene. It is possible that the length of the CA repeat may influence the accessibility of the binding sites for the transcription factors. The length of the microsatellites has been shown to modify the expression of other genes including interferon-γ.20 Further studies are now required to explore the functional relevance of these polymorphisms in the susceptibility to diabetic retinopathy. Further, it is still possible that the association with this region is due to linkage with an adjacent susceptibility gene. In this respect, it is interesting that the endothelial nitric oxide synthase (eNOS) gene has also been localized to chromosome 7q35, the same region as ALR2.27 Recent studies have shown that polymorphisms of eNOS are associated with diabetic microvascular disease.28

In conclusion, we have investigated two polymorphic regions in the promoter region of the ALR2 gene and confirm the role of the gene in the susceptibility to and possibly the protection from diabetic retinopathy. Individuals with retinopathy have a significantly increased frequency of the Z−2/C haplotype compared with those with no retinopathy after diabetes of 20 years’ duration.

Acknowledgment

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References


