# Brief Genetics Report

# A Novel Polymorphism in the Aldose Reductase Gene Promoter Region Is Strongly Associated With Diabetic Retinopathy in Adolescents With Type 1 Diabetes

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A $(CA)_n$ dinucleotide repeat sequence –2.1 kb upstream of the transcription start site of the aldose reductase gene has been found to be strongly associated with human diabetic nephropathy and retinopathy (1,2) and linked to increased expression of the aldose reductase gene (3). The current project was designed to detect potential variants in the putative promoter and encoding regions and to determine their potential association with early diabetic retinopathy in adolescents with type 1 diabetes. A novel polymorphism in the promoter region of the aldose reductase gene was identified, which was located at C(–106)T. The frequencies of homozygote CC and C alleles were significantly higher in those with retinopathy compared with those without retinopathy ($P$ = 0.0035 and 0.005, respectively). We also confirmed that the Z-2 allele (2) was strongly associated with retinopathy among our patients ($P$ < 0.0005). The two polymorphisms are in strong linkage disequilibrium ($\chi^2$ test, $P$ = 0.001). The C allele and the Z-2 allele in the promoter region of the aldose reductase gene increase the risk of diabetic retinopathy in type 1 diabetic adolescents.

Aldose reductase ($AR_2$), the first enzyme of the polyol pathway, has been thought to play an important role in the pathogenesis of the long-term complications of diabetes (4–6). In vivo, both increased enzyme activity and expression of the $AR_2$ gene have been reported in patients with diabetic microvascular complications (7–9).

Variants in the encoding or promoter regions of the human $AR_2$ gene may contribute to diabetes complications. The current project was designed to detect potential variants and their potential association with early diabetic retinopathy in type 1 diabetes. Informed consent was given by adolescents and their families, and the project was approved by the hospital's ethics committee.

No common polymorphisms were found in the encoding region of the $AR_2$ gene in 20 diabetic adolescents. The conservation of the encoding region of $AR_2$ contrasts with that of another enzyme (sorbitol dehydrogenase) of the sorbitol pathway, for which at least 10 polymorphic sites have been described (10). Two 1-bp deletions were identified close to the osmotic response element (the deletions of T and C located at –3624 and –3760), compared with the previously reported sequence (11). However, all 20 adolescents were homozygous for these deletions, making them unlikely to be contributors to the general pathogenesis of diabetes complications. It is possible that the published sequences may be in error or are very rare polymorphisms.

A novel polymorphism was identified in the basal promoter region: a single substitution C(–106)T. For homozygote CC subjects, the 263-bp polymerase chain reaction (PCR) product contained only a single *Bfa*I site, at which the product was cleaved into 206- and 57-bp fragments. The C(–106)T substitution created another *Bfa*I restriction site. For homozygote TT subjects, the 206-bp fragment was further cleaved into 147- and 59-bp fragments. Heterozygotes (CT genotypes) therefore showed 206-, 147-, 59-, and 57-bp fragments. The frequency of the T allele was 35% in 76 nondiabetic control subjects.

The novel polymorphism was assessed in 164 diabetic adolescents with and without retinopathy, defined as any microaneurysm or hemorrhage assessed by seven fields of stereoscopic fundal photography. Because longer duration is a significant risk factor for diabetic retinopathy, only those with retinopathy and diabetes duration <13 years were used in this analysis. The frequencies of both allele C and homozygote CC were significantly higher in those with retinopathy than in those without retinopathy ($P$ = 0.0035 and 0.005, respectively; Table 1). Logistic regression showed an increased risk of retinopathy for the homozygous genotype CC (odds ratio 2.47 [95% CI 1.30–4.67], $P$ = 0.006) compared with the other two genotypes (CT and TT). Diabetes duration and $HbA_{1c}$ were not significantly associated with retinopathy in this group.

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$AR_2$, aldose reductase enzyme; nt, nucleotide; PCR, polymerase chain reaction; RT, reverse transcription.

TABLE 1
Distribution of demographic variables, C(–106)T and Z-2 polymorphisms for patients with and without diabetic retinopathy

| | Type 1 diabetic adolescents | | |
|---|---|---|---|
| | No retinopathy | Retinopathy | *P* value |
| *n* | 97 | 67 | |
| Age (years) | 14.3 (12.1–15.6) | 14.8 (12.7–16.3) | NS |
| Diabetes duration (years) | 6.0 (5.1–8.8) | 7.2 (5.8–9.9) | NS |
| $HbA_{1c}$ (%) | 8.7 (8.0–9.7) | 8.6 (7.7–9.6) | NS |
| Aldose reductase promoter | | | |
| Haplotype | | | |
| C | 105 (54) | 94 (70) | 0.0035 |
| T | 89 (46) | 40 (30) | |
| Genotype | | | |
| CC | 35 (36) | 39 (55) | 0.005 |
| CT | 35 (36) | 16 (26) | NS |
| TT | 27 (28) | 12 (19) | NS |
| Microsatellite | | | |
| Haplotype | | | |
| Z-2 | 59 (31) | 95 (71) | <0.0005 |
| No | 133 (69) | 39 (29) | |
| Genotype (Z-2) | | | |
| Homo | 19 (20) | 41 (62) | <0.0005 |
| Hetero | 21 (22) | 13 (19) | NS |
| No | 56 (58) | 13 (19) | < 0.0005 |

Data are medians (interquartile range) or *n* (%). *P* values indicate the significance of comparison between patients with and without retinopathy.

Also examined in this population were the potential associations between the Z-2 allele and the novel polymorphism. We confirm that the frequency of the Z-2 allele is significantly higher in those patients with retinopathy than those without retinopathy ($P < 0.0005$; Table 1). We also observed that Z-2 was rarely associated with the T allele and strongly associated with the C allele ($P = 0.001$; Table 2). This suggests that these two polymorphisms are in linkage disequilibrium.

In this study, the retinopathy detected was at a very early stage. Increased expression of the $AR_2$ gene may only be pathogenic in the early stages of complications, which would explain why $AR_2$ inhibitors have not been effective in slowing progression of more advanced disease (12). No common mutation was found in the encoding region of the $AR_2$ gene. The current results do not support the hypothesis that a mutation in the encoding region of the $AR_2$ gene modulates retinopathy development by more efficient conversion of glucose to sorbitol (2). Increased $AR_2$ expression has been linked with the Z-2 allele (3). The novel C(–106)T polymorphism and the identified $(CA)_n$ repeats are in strong linkage disequilibrium. Either or both variants in the promoter region of the $AR_2$ gene may directly contribute to early diabetic retinopathy by increasing expression of the $AR_2$ gene. Another possibility is that a gene near this locus contributes to the early onset of diabetic retinopathy rather than $AR_2$ gene itself. These polymorphisms will provide useful markers for genetic studies in the role of $AR_2$ in and searching for potential candidate genes in diabetes complications.

## RESEARCH DESIGN AND METHODS

For the encoding region, 10 exons were assayed by reverse transcription (RT)-PCR (total RNA from peripheral leukocytes) and direct DNA sequencing. Two sets of primers were used to amplify from exon 1 to exon 5 and from exon 6 to exon 10 (13).

To amplify the promoter region, three pairs of primers were designed according to published sequences of the human $AR_2$ gene (11,14). PCR products included the basal promoter region (from –609 nucleotide [nt] to +31) and the osmotic response element (from –3850 nt to –3551). The primers, conditions for RT-PCR, PCR, or PCR digestion are available by request.

The primers for the basal promoter region were used to amplify the 263-bp fragment that included the novel C(–106)T polymorphic site. The upstream primer was 5′-CCTTTCTGCCACGCGGGGCGCGGG-3′ (nt –222 to –199), and the downstream primer was 5′-CATGGCTGCTGCGCTCCCCAG-3′, which extended from nt +21 to the translation start ATG codon. PCR was conducted in a volume of 50 µl containing 50 ng of template DNA, 200 µmol/l of each dNTP, 50 pmol each of the primers, and 1 U of Taq polymerase in the buffer supplied by the manufacturer (Boehringer Mannheim, Sandhofester, Mannheim, Germany). After initial denaturation at 95°C for 2 min, 35 cycles were run for 1 min each at 95, 66, and 72 and an additional 7 min at 72°C after the last cycle. The PCR products were purified by Wizard PCR Preps DNA purification system (Promega, Madison, WI), and directly sequenced on an Applied Biosystems automated DNA sequencer (Bio-Rad, Hercules, CA) using standard technique.

The restriction enzyme *Bfa*I was used to determine a novel polymorphism. There were 8 U of *Bfa*I (New England Biolabs, Beverly, MA) and 10 µl of PCR products incubated at 37°C for 2 h in the manufacturer's buffer. Genotypes were determined by electrophoresis in 3.5% agarose gel and ethidium bromide staining.

The genotyping of the $(CA)_n$ repeat was analyzed by automated fluorescent gene scanning. PCR primers and condition were as described by Ko et al. (2). The sense primer was labeled at the 5′-end with the fluorescent dye ROX (red fluorescent, Genosys Biotechnologies, the Woodlands, TX). The PCR product was combined with an aliquot of the internal lane standard labeled with fluorescent dye TAMRA (yellow fluorescent; Perkin-Elmer, Norwalk, CT). The mixture was then

TABLE 2
Association between Z-2 and C(–106)T polymorphisms

| | Z-2 microsatellite | | | |
|---|---|---|---|---|
| | Homo | Hetero | No | Total |
| CC | 57 | 15 | 1 | 73 |
| CT | 2 | 15 | 34 | 51 |
| TT | 1 | 4 | 34 | 39 |
| Total | 60 | 34 | 69 | 163 |

$P = 0.001$, $\chi^2$ test.

resolved by electrophoresis on 6% urea-PAGE denaturing gel using ABI s73 DNA sequencer (Perkin-Elmer, Norwalk, CT). The internal lane standard was used to create a calibration curve of peak arrival time, which in turn was used to calculate the length of unknown PCR product automatically by ABI GENESCAN 672 software (Perkin-Elmer).

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