JKAU: Med. Sci., **Vol. 15** No. 4, pp: 19-29 (2008 A.D. / 1429 A.H.)

Genotype at the Aldose Reductase Locus Affects Retinopathy-Free Survival in Type I Diabetes

Abdulmoein E. Al-Agha, FRCP, Abdulmohsin M.S. Ahmadjee, MBBS, Bruce H. Bennetts[1], MD, Albert K. Chan[2], MD, and **Kim F. Donaghue[2], MD**

Department of Pediatrics, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia
[1]Department of Molecular Genetics, Children's Hospital at Westmead and [2]Ray Williams Institute of Pediatric Endocrinology and Diabetes, Sydney, Australia
abdulmoein@dr-agha.com

Abstract. The aim of this study is to examine whether the retinopathy-free survival in diabetics is linked to the genotype at the Adose Reductase Locus**.** Two hundred and fifty-six Australian adolescents with type 1 diabetes were attending for complication, screenings were genotyped. One microsatellite at the aldose reductase was amplified by Polymerase chain reaction to produce fragments of 126-152 base pair (Z = 138). Kaplan-Meier survival analysis was used to determine the duration free of retinopathy according to genotypes Z-2/Z-2, Z-2/non Z-2 and non Z-2/non Z-2. A stereoscopic fundal photography was performed; blinded to the patients' genotype. The presence of a background retinopathy was defined as any microaneurysm or hemorrhage. The results of our study show that, Median survival free of retinopathy was significantly shorter in patients having the Z-2 allele (p = 0.014). When survival analysis was performed on combinations of both Z-2 and Z+2, the only significant difference was between Z+2/non Z+2 and non Z+2/non Z+2 (p = 0.04). We concluded that survival free of retinopathy was significantly reduced in Australian patients with a Z-2 allele with less evidence for other alleles. This may represent that some sort of protective mechanism of retinopathy in patients with absent Z-2 allele.

Keywords: Aldose reductase, Diabetes, Microangiopathic complications, Genetic polymorphism, Dinucleotide repeat.

Correspondence & reprint request to: Dr. Abdulmoien E. Al-Agha
P.O. Box 80215, Jeddah 21589, Saudi Arabia
Accepted for publication: 30 November 2008. Received: 12 July 2008.

Introduction

Diabetes Control and Complications Trial (DCCT)[1] showed that the incidence of microvascular complications were significantly influenced by glycaemic control. In addition, familial clustering of retinopathy as well as nephropathy was identified when first degree relatives were also investigated[2].

The aldose reductase gene (AR2) is a good candidate for this familial aggregation. A substantial evidence points to a key role for increased glucose metabolism by cytosolic enzyme, aldose reductase enzyme through the polyol pathway[3]. Polyol pathway is activated by hyperglycaemia. An elevated tissue level of AR2 were associated with diabetes complications[4-7]. The discovery of the AR2 (AC)n dinucleotide repeats sequence at 2.1 kb upstream of the transcription start site was followed by the initial demonstration, that the Z-2 allele marker was associated with susceptibility for retinopathy in type 2 diabetes[8]. Subsequently, this allele was also linked to an increased risk of nephropathy in type 1 diabetes[9]. A more recent study has suggested that the repeat length, rather than a specific allele was important for retinopathy in type 2 diabetes[10]. Specifically in the Japanese population the Z-4 allele showed an association with retinopathy[11]. A protective effect of the Z+2 allele has been proposed[12-13].

Most individuals given sufficient diabetes duration, eventually developed retinopathy. A survival analysis was used in the current study to investigate whether the retinopathy-free survival period was linked to AR2 (AC)n dinucleotide a repeated sequence polymorphism.

Materials and Methods

Recruitment of Adolescents with Type 1 Diabetes with/without Microvascular Complications

Two hundred and fifty-six Australian adolescents with type 1 diabetes were sequentially genotyped when they attended for their annual complication screening. Stereoscopic fundal photography of seven standard fields was performed and a trained ophthalmologist, blinded to the patient's genotype, graded the photographs. Patients' characteristics are shown in Table 1 and 93% of this population is Caucasian. These measurements were those from the last annual assessment or those from

the first assessment when retinopathy was detected. All participants gave informed consent, and the Hospital's Ethics Committee approved the study.

Table 1. Patient clinical characteristics.

	Retinopathy	No retinopathy	p Value
Gender	55 M, 67 F	59 M, 75 F	0.87
Age (years)	15.2 (13.7 - 17.5)	15.1 (13.3 - 16.8)	0.28
Duration (years)	8.3 (5.6 - 11.3)	5.8 (4.2 - 9.1)	0.0001
BMI (kg/m^2)	21.6 (20.1 - 24.7)	22.2 (19.6 - 25.3)	0.67
HbA_{1c} (%)	8.4 (7.7 - 9.4)	8.7 (8.0 - 9.7)	0.03
Median HbA_{1c} (from diagnosis) (%)	8.6 (7.9 - 9.3)	8.7 (7.9 - 9.4)	0.30
Cholesterol (mmol/L)	4.4 (3.8 - 4.9)	4.3 (3.8 - 4.8)	0.23
Systolic bp (mmHg)	115 (110 - 125)	115 (110 - 120)	0.43
Diastolic bp (mmHg)	70 (65 - 75)	70 (60 - 70)	0.052

Summary statistics shown as median (interquartile range)

Genotyping for 5′ AR2 (AC)n Dinucleotide Repeats Polymorphism

Genotyping of patients was performed using genomic DNA prepared from white blood cells. The 5' AR2 (AC)n polymorphism was amplified by PCR using a FAM labeled oligonucleotide to produce fragments of 126-152 bp (Z = 138 bp). The primers used to amplify the region of interest were ARpr1 (5'-FAM-TGGTCAGGCCTGGCCCTCCT-3') and ARpr2 (5'-GATACCTCTCGGTGTGGCTGA-3')[8]. PCRs were performed in 20 µl reactions with 100 ng genomic DNA as a template, 5 pmol of each primer, 200 µM dNTPs, 25 mM $MgCl_2$ and 1 U Taq DNA polymerase (Boehringer Mannheim). Samples were subjected to 35 cycles of amplification using a touchdown protocol. Denaturation for 2 min at 95°C, annealing temperature was reduced by 2°C/cycle from 66 to 54°C for 1 min with the final 25 cycles at 54°C and extension at 72°C for 30 sec. PCR fragments were sized on an automated DNA sequencer (ABI 373) using a standard techniques. The size of the (AC)n allele was determined by comparison with previously genotyped specimens[13].

Statistical Analysis

The software packages of SAS (Version 6.12, Cary NC, SAS Institute, 1996) and SPSS were used to analyze the data. Wilcoxon rank-

sum test was used to compare continuous variables between two groups. A Chi-square test was used to assess the association between gender and the stratified variable.

Kaplan-Meier survival analysis was used to determine retinopathy-free survival for the three microsatellite alleles: Z-2, Z+2 and Z+4, as well as allele length shorter or longer than Z-2. Difference in survival times was tested by log rank test. P-values of less than 0.05 were considered statistically significant.

Results

The distribution of alleles of the AR2 polymorphism in our cohort is summarized in Table 2. A total of fourteen alleles were observed (Z+14, Z+12, Z+10, Z+8, Z+6, Z+4, Z+2, Z, Z-2, Z-4, Z-6, Z-8, Z-10, Z-12). The three major alleles detected were Z-2, Z, and Z+2. There were no significant differences in allele distribution for those with and without retinopathy, when no correction was made for the significantly longer duration of those with retinopathy.

Table 2. Frequency of allele distribution in the 256-patient cohort.

Allele	Retinopathy (N = 122)	No retinopathy (N = 134)	Total
Z-12	1 (0.4%)	2 (0.8%)	3
Z-10	0	3 (1%)	3
Z-8	1 (0.4%)	3 (1%)	4
Z-6	2 (1%)	7 (3%)	9
Z-4	17 (7%)	24 (9%)	41
Z-2	86 (35%)	82 (31%)	168
Z	79 (32%)	77 (29%)	156
Z+2	29 (12%)	41 (15%)	70
Z+4	17 (7%)	19 (7%)	36
Z+6	5 (2%)	4 (1%)	9
Z+8	1 (0.4%)	1 (0.4%)	2
Z+10	0	1 (0.4%)	1
Z+12	3 (1%)	1 (0.4%)	4
Z+14	3 (1%)	3 (1%)	6
Total	244	268	512

Median retinopathy-free survival was significantly shorter in patients having the Z-2 allele (p = 0.014) (Fig. 1 and Table 3). There was a significant difference in the retinopathy-free survival between those having any allele shorter than or equal to Z-2 allele and those with both alleles longer than Z+2 (p = 0.049). There was no significant difference in the survival time free of retinopathy according to the presence or absence of the Z+2 or the Z+4 allele (Table 3).

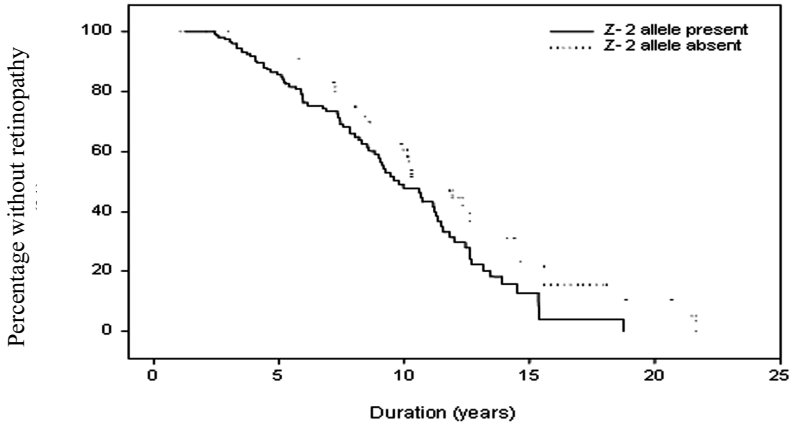

Fig. 1. Kaplan-Meier curve for survival free of retinopathy for presence and absence of Z-2 allele. The difference in survival free of retinopathy was significant by logrank test (p = 0.014).

Table 3. Survival analysis for duration until retinopathy in relation to various microsatellite alleles.

Microsatellite allele	Number	Median survival time (Years) (Interquartile range)
Z-2 absent	102	11.48 (8.01 - 14.64)
Z-2 present	154	9.76 (6.75 - 12.62)
		p = 0.014
Z+2 absent	190	10.12 (6.87 - 13.33)
Z+2 present	66	10.29 (8.01 - 12.68)
		p = 0.20
Z-4 absent	217	10.24 (7.42 - 13.15)
Z-4 present	39	12.45 (7.38 - 14.64)
		p = 0.66
Both Z or longer	81	10.29 (8.09 - 15.56)
Any Z-2 or shorter	175	10.58 (7.23 - 13.15)
		p = 0.049

When survival analysis was performed on combinations of both Z-2 and Z+2, the only significant difference was between Z+2/non Z+2 and non Z+2/non Z+2 ($p = 0.04$).

HbA1c was surprisingly significantly lower in those with retinopathy on the day of assessment, but was not different when median of all measurements from diagnosis for an individual were analyzed (Table 1).

Discussion

In the current study, survival free of retinopathy was significantly reduced in the group with the Z-2 allele of the AR2 microsatellite marker. This data does not support a susceptibility role for the Z+4 allele, nor a protective effect of the Z+2 allele.

The increased risk of retinopathy associated with the Z-2 allele is in agreement with the findings described in Chinese patients with type 2 diabetes[8] and in Caucasian patients with type 1 diabetes[12,13]. However, there was no role for alleles shorter than Z-2 was found. Indeed, when the shorter alleles were included with Z-2, this actually negated the susceptibility risk. This contrasts with two Japanese studies of type 2 diabetes in which proliferative retinopathy was associated with alleles equal to or shorter than Z-2[10] and with the Z-4 allele[11].

Recent studies support a role for the upstream (AC)n repeat sequences, in modulating the expression level of AR2. In American and Italian patients with type 1 diabetes, AR2 mRNA levels were found higher in association with the Z-2 allele, but without diabetes, the Z-2 allele did not influence AR2 mRNA levels[14]. In type 2 Japanese patients, higher AR2 protein levels were found with the Z-4 allele[11]. Furthermore, in a retinal epithelial cell line, transfect with the Japanese Z-4 AR2 gene promoter region in a luciferase vector, gene transcription was up-regulated compared to constructs with other (AC)n repeat sequences, including the Z-2 construct.

It is tempting to speculate that the association of the Z-2 allele with diabetes complications seen in this study is equivalent to that of the Z-4 allele in Japanese patients. The Z-4 allele in Japanese patients and the Z-2 allele in other populations may both act epistatically with another susceptibility polymorphism in the AR2 gene or in other genetic loci. This discrepancy may reflect differences between type 1 and type 2

diabetes plus other genetic and environmental factors unique to the two populations.

The advantage of survival analysis in a longitudinal study over chi-squared analysis in a case-control study is that the interaction between the study effect (AR2 allele) and time (diabetes duration) can be identified. Survival analysis also allows for different lengths of patient follow-up. The majority of the patients with diabetes developed diabetic retinopathy if followed for long enough: 98% after duration of 15 years or more[15]. In the current study the frequency of Z-2 allele was not significantly higher in the group with retinopathy who had slightly longer diabetes duration (Table 1), but its presence was associated with shorter survival free of retinopathy.

Survival analysis is a sensitive method to investigate the population significance of a single candidate gene, which may increase or decrease the risk of early onset of an outcome. Selection of patients based on the outcomes of a long duration absence or a short duration presence of a complication. In case control studies, does not provide clear evidence of whether susceptible or protective polymorphisms are sufficient in them or need to work in synergy with other genes to produce the effect. Indeed, the protective effect of the Z+2 allele has only been detected in the study of English type 1 diabetic subjects who survived 20 or more years without retinopathy[12]. The lack to identify the protective effect in the current study may be because of some other factor(s) which is also needed, and that only a small proportion of individuals with Z+2 are protected.

Environmental factors, presumably interact with genetic determinants to regulate gene expression and the risk for complications. However, hyperglycemia as measured by HbA_{1c} was surprisingly not higher in those with diabetic retinopathy in the current study. Similarly there was no effect seen for blood pressure.

Aldose reductase inhibition represents an attractive strategy for prevention of diabetic complications[16]. The beneficial effects of aldose reductase inhibitors (ARIs) in preventing or substantially delaying the onset of diabetic complications have largely been demonstrated in experimental models, in which the inhibition was initiated at onset of diabetes[17,18]. If more is understood about susceptibility alleles in the

aldose reductase gene, then specific individuals with such alleles may be targeted for intervention with aldose reductase inhibitors.

The current study supports an important role for the aldose reductase gene (AC)n dinucleotide to repeat a sequence of polymorphism in the early development of diabetic retinopathy.

Acknowledgments

The authors wish to thank Dr. Jan Fairchild for assisting with patient recruitment; Ms. Janine Cusumano and Mandy Crocker for retinal photography; Dr. Stephen Hing for retinal grading; Mr. Craig Kennedy and Ms. Julianne Jackson for assistance with the molecular typing; and Dr. Ray and Mrs. Rita Williams for their encouragement and financial support.

References

[1] **[No authors listed].** The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. The Diabetes Control and Complications Trial Research Group, *N Engl J Med*, 1993; **329**(14): 977-986.

[2] **[No authors listed].** Clustering of long-term complications in families with diabetes in the diabetes control and complications trial. The Diabetes Control and Complications Trial Research Group, *Diabetes*, 1997; **46**(11): 1829-1839.

[3] **Hamada Y, Nishimura C, Koh N, Sakakibara F, Nakamura J, Tanimoto T, Hotta N,** Influence of interindividual variability of aldose reductase protein content on polyol-pathway metabolites and redox state in erythrocytes in diabetic patients, *Diabetes Care*, 1998; **21**(6): 1014-1018.

[4] **Dunlop M,** Aldose reductase and the role of the polyol pathway in diabetic nephropathy, *Kidney Int Suppl*, 2000; **77**: S3-12.

[5] **Hamada Y, Kitoh R, Raskin P**, Association of erythrocyte aldose reductase activity with diabetic complications in type 1 diabetes mellitus, *Diabet Med*, 1993; **10**(1): 33-38.

[6] **Nishimura C, Saito T, Ito T, Omori Y, Tanimoto T,** High levels of erythrocyte aldose reductase and diabetic retinopathy in NIDDM patients, *Diabetologia*, 1994; **37**(3): 328-330.

[7] **Williamson JR, Chang K, Frangos M, Hasan KS, Ido Y, Kawamura T, Nyengaard JR, van den Enden M, Kilo C, Tilton RG,** Hyperglycemic pseudohypoxia and diabetic complications, *Diabetes*, 1993; **42**(6): 801-813.

[8] **Ko BC, Lam KS, Wat NM, Chung SS,** An (A-C)n dinucleotide repeat polymorphic marker at the 5' end of the aldose reductase gene is associated with early-onset diabetic retinopathy in NIDDM patients, *Diabetes*, 1995; **44**(7): 727-732.

[9] **Heesom AE, Hibberd ML, Millward A, Demaine AG**, Polymorphism in the 5'-end of the aldose reductase gene is strongly associated with the development of diabetic nephropathy in type I diabetes, *Diabetes*, 1997; **46**(2): 287-291.

[10] **Fujisawa T, Ikegami H, Kawaguchi Y, Yamato E, Nakagawa Y, Shen GQ, Fukuda M, Ogihara T,** Length rather than a specific allele of dinucleotide repeat in the 5' upstream

region of the aldose reductase gene is associated with diabetic retinopathy, *Diabet Med*, 1999; **16**(12): 1044-1047.

[11] **Ikegishi Y, Tawata M, Aida K, Onaya T,** Z-4 allele upstream of the aldose reductase gene is associated with proliferative retinopathy in Japanese patients with NIDDM, and elevated luciferase gene transcription *in vitro*, *Life Sci*, 1999; **65**(20): 2061-2070.

[12] **Demaine A, Cross D, Millward A,** Polymorphisms of the aldose reductase gene and susceptibility to retinopathy in type1 diabetes mellitus, *Invest Ophthalmol Vis Sci*, 2000; **41**(13): 4064-4068.

[13] **Kao YL, Donaghue K, Chan A, Knight J, Silink M,** A novel polymorphism in the aldose reductase gene promoter region is strongly associated with diabetic retinopathy in in adolescents with type 1 diabetes, *Diabetes*, 1999; **48**(6): 1338-1340.

[14] **Shah VO, Scavini M, Nikolic J, Sun Y, Vai S, Griffith JK, Dorin RI, Stidley C, Yacoub M, Vander Jagt DL, Eaton RP, Zager PG,** Z-2 microsatellite allele is linked to increased expression of the aldose reductase gene in diabetic nephropathy, *J Clin Endocrinol Metab*, 1998; **83**(8): 2886-2891.

[15] **Klein R, Klein BE, Moss SE, Davis MD, DeMets DL,** The Wisconsin epidemiologic study of diabetic retinopathy. II. Prevalence and risk of diabetic retinopathy when age at diagnosis is less than 30 years, *Arch Ophthalmol*, 1984; **102**(4): 520-526.

[16] **Krans HM**, Recent clinical experience with aldose reductase inhibitors, *Diabet Med*, 1993; **10**(Suppl 2): 44S-48S.

[17] **Oates PJ, Mylari BL,** Aldose reductase inhibitors: therapeutic implications for diabetic complications, *Expert Opin Investig Drugs*, 1999; **8**(12): 2095-2119.

[18] **Pfeifer MA, Schumer MP, Gelber DA,** Aldose reductase inhibitors: the end of an era or the need for different trial design? *Diabetes*, 1997; **46**(Suppl 2): S82-S89.

اعتلال الشبكية لمرضى السكري اليافعين تختلف على حسب الأَلائِلِ السَّائِلَةِ المِكْرَوِيَّةِ لِمُخْتَزِلَةِ الأَلْدُوز

عبدالمعين عيد الأغا، وعبدالمحسن محمد أحمدجي، وبروز اتش بانيتز[١]، وألبيرت كي شان[٢]، و كيم اف دونجهو[٢]

قسم الأطفال، كلية طب، جامعة الملك عبدالعزيز

جدة – المملكة العربية السعودية

[١] كلية طب الأطفال علم الوراثة الجزيئي

[٢] ومعهد وليامز لطب الأطفال والغدد الصماء والسكري

سيدني – أستراليا.

المستخلص. الهدف من هذا البَحْثُ فحص ما إذا كان اِعْتِلال الشَّبَكِيَّةِ مُرْتَبِطًا بالأَلائِلِ السَّائِلَةِ المِكْرَوِيَّةِ لِمُخْتَزِلَةِ الأَلْدُوز. تَمَّ التَّنْمِيطُ الجِينِيُّ لمائتين وستِّين يَافِعًا أُسْتُرالِيًّا مُصابًا بالسُّكَّرِيِّ من النَّمَطِ الأَوَّل كانوا يُراجِعُون من أجلِ التَّحَرِّي عن المُضاعَفاتِ. تَمَّ تَضْخِيمُ النَّاحِيَةِ السَّائِلَةِ المِكْرَوِيَّةِ لِمُخْتَزِلَةِ الأَلْدُوز بِواسطَةِ تَفاعُلِ سِلْسِلَةِ البُولِيميراز لإنتاجِ شُدَفٍ مُؤَلَّفَةٍ من ١٢٦ إلى ١٥٢ زَوْجَ قَواعِدَ (Z= ١٣٨). استخدمت طريقة كابلان–ماير لتحديدِ مُدَّةِ البُقْيا الخَالِيَــةِ من اعْتِــلالِ الشَّبَكِيَّــةِ بِحَسَبِ الأَنْماطِ الجِينِيَّةِ Z-2/Z-2، وَ non Z-2/Z-2 وَ non Z-2/non Z-2.:كذلك تمّ إجراء التَّصْوِيرُ الضَّوْئِيُّ التَّجْسِيمِيُّ لِقاعِ العَيْن لجميع المرضى مع معرفةِ النَّمَطِ الجِينِيِّ لكلِّ مريض. (تَمَّ تعريف وُجُود اعْتِلالِ شَبَكِيَّةٍ خَلْفِيٍّ بأنه أَيُّ أُمٍّ دَمٍ مِجْهَرِيَّةٍ أو نَزْفٍ). كان متوسط مُدَّةِ البُقْيَا الخَالِيَةِ من اعْتِلالِ الشَّبَكِيَّةِ عِنْدَ المَرْضَى ذَوِي النَّمَطِ الجِينِيِّ Z-2/Z-2 (٨,٨ سنة) أو النَّمَطِ الجِينِيِّ non Z-2/non Z-2 (٦. سنة) أَقْصَرَ بشكلٍ مُعْتَدٍّ

بالمُقارَنَةِ مع ذلك الذي عِنْدَ المَرْضَى ذَوِي النَّمَطِ الجِينِيِّ Z-2/Z-2 (٨,١١ سنة). كانتْ مُدَّةُ البُقْيَا الخَالِيَةُ من اعْتِلالِ الشَّبَكِيَّةِ عِنْدَ المَرْضَى ذَوِي الأَلِيلِ Z-2 قَصِيرَةً، ولم تَكُن كذلك عِنْدَ الذين يَمْتَلِكُون أَلائِلَ أُخْرى. وهذا يُمْكِنُ أن يُمَثِّلَ الآلِيَّةَ الوَظِيفِيَّةَ لِقِصَرِ مُـدَّةِ البُقْيا الخَالِيَـةِ من اعْتِلالِ الشَّبَكِيَّةِ عِنْدَ المَرْضَى ذَوِي الأَلِيلِ Z-2.