ORIGINAL ARTICLE

The relationship between aldose reductase gene C106T polymorphism and the severity of retinopathy in Type 2 diabetic patients: A case–control study

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Background: Hyperglycemia over-activates glucose reduction to sorbitol by aldose reductase (ALR) leading to osmoregulation disruption and cellular damage that cause diabetic complications. We investigated the association of C106T polymorphism of *ALR2* gene with the severity of diabetic retinopathy (DR) in Jordanian Type 2 diabetic patients in this case-control study at the Ophthalmology clinic of the National Centre of Diabetes, Endocrinology, and Genetics. **Materials and Methods:** A total of 277 subjects participated in the study (100 diabetics without retinopathy, 82 diabetics with retinopathy, and 95 controls). Blood samples were withdrawn followed by DNA extraction. C106T polymorphism was examined by polymerase chain reaction followed by restriction fragment length polymorphism and gel electrophoresis. Statistical analysis was performed by SPSS software using analysis of variance, multiple logistic regression or Chi-square test. **Results:** The CT and TT genotypes were significantly more prevalent in DR patients than those without DR (CT 50% vs. 38%, TT 16.7% vs. 8%, $P = 0.02$ and 0.01, respectively). DR patients had T allele more frequently than those without it (41.7% vs. 27%, $P = 0.007$). Diabetics without retinopathy showed similar genotype and allele frequency to those of nondiabetic controls. No correlation between CT/TT genotypes and the severity of DR in affected subjects was found (χ^2: 3.049, $P = 0.550$). **Conclusion:** C106T polymorphism increased the risk to develop retinopathy in Jordanian Type 2 diabetic patients. T allele of *ALR2* was associated with DR. The severity of DR did not show an association with this polymorphism.

Key words: Complications, diabetes, polymorphism, polyol, sorbitol

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INTRODUCTION

Hyperglycemia and disease duration are known factors for developing diabetic microvascular complications, such as retinopathy.[1,2] This diabetic complication is a common finding even though it does not develop in all diabetic patients.[1,2] Poor glycemic control affects both the development and progression of diabetic retinopathy (DR).[3] Family history of genetic polymorphisms in several genes including aldose reductase gene *(ALR2)* increases diabetic complications risk suggesting that genetic factors may also play a role in the pathogenesis of DR.[4-7]

The increase in ALR enzymatic activity is one of the biochemical mechanisms underlying the structural and functional abnormalities associated with overexposure of the vascular tissues to hyperglycemia. ALR is the first and rate-limiting enzyme in the polyol pathway that catalyzes glucose reduction to sorbitol. Excessive glucose, as detected in hyperglycemic diabetic patients,

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over-activates this pathway resulting in the accumulation of sorbitol intracellularly and alterations in intracellular signaling.[8-10] The high intracellular levels of sorbitol disturb osmoregulation in ocular and neural cells and damage them. To date, several studies have investigated whether ALR2 C106T polymorphism is correlated with the risk of DR. However, the contribution of this polymorphism to DR is still controversial. For instance, Kaur *et al*. have shown that *ALR2* C106T polymorphism was associated with DR, whereas Deng *et al*. have reported the opposite result.[11,12] In a recent meta-analysis that involved 23 studies, a significant association between the *ALR2* gene C106T polymorphism and DR susceptibility was shown. In addition, they found a significantly higher risk for DR in Type 1 diabetes mellitus (DM) East Asian populations, and Middle Eastern populations.[13]

In our study, we investigated the relationship between *ALR2*-C106T polymorphism and DR severity in Jordanian Type 2 diabetic patients. To the best of our knowledge, there are no studies investigating the influence of *ALR2* C106T promoter polymorphism on Jordanian or even Arab diabetic patients to date. Building databases about Jordanian population as a part of the global populations may assist in planning preventive measures and reducing treatment costs of the complications of a common disease like diabetes.

MATERIALS AND METHODS

Patient selection

In this case-control study, 277 subjects were recruited at the ophthalmology clinic of the National Centre of Diabetes, Endocrinology and Genetics (NCDEG), 82 were diabetics with DR, 100 were diabetics free of DR, and 95 were nondiabetic controls. Subjects attending ophthalmology clinic at NCDEG who matched the following selection criteria were recruited in this study. Patient selection criteria included age range between 40 and 65 years, Type 2 diabetes diagnosed by standard means defined by the American Diabetes Association, diabetes duration of < 15 years, and no retinal problems prior to the diagnosis of diabetes. The American Diabetes Association standards for diagnosing diabetes include using one of four tests to establish a firm diagnosis of diabetes: (i) fasting plasma glucose (FPG) >6.9 mmol/L (>125 mg/dL), the most commonly used test; (ii) random plasma glucose ≥ 11.1 mmol/L (≥200 mg/dL) with diabetes symptoms such as polyuria, polydipsia, fatigue, or weight loss; (iii) 2-h postload glucose ≥ 11.1 mmol/L (≥200 mg/dL) on a 75 g oral glucose tolerance test; (iv) or HbA1c ≥ 48 mmol/mol (≥6.5%). All these tests require confirmation with a second test, which may be the same test or a different test. A written informed consent was obtained from all participants included in the study before a blood sample was withdrawn. Each subject was given a coding number to preserve privacy rights. The Deanship of Scientific Research at the University of Jordan and the Institutional Review Board at the NCDEG approved the study. This study was performed in accordance with the ethical standards of the Helsinki Declaration of 1975, as revised in 2000. HbA1c test was done for diabetic patients but not for controls. We referred to medical history record to exclude diabetes in control group. Personal data including age and gender were collected. Medical information including height and weight, in addition to history of diabetes, hypertension, ischemic heart disease, and dyslipidemia were obtained. Consistent gender distribution in each group was also considered. Retinopathy was defined by the presence of characteristic changes, including hemorrhages, exudates, new vessels, and fibrous proliferation, detected by slit lamp biomicroscopy through dilated pupils by an experienced ophthalmologist. DR severity was determined based on the evidence-based International Clinical DR Disease Severity Scale that is agreed on by the American Academy of Ophthalmology in 2001 and the International Council of Ophthalmology in 2002. This scale classifies DR severity as follows; no apparent retinopathy by the absence of ophthalmoscopic abnormalities, mild nonproliferative DR (NPDR) by the presence of microaneurysms (MA) only, moderate NPDR by the presence of more than just MA but less than the severe form, severe NPDR by the presence of more than 20 intraretinal (IR) hemorrhages in each of the 4 quadrants, definite venous beading in 2 or more quadrants, or prominent IRMA in one or more quadrants, and the absence of proliferative retinopathy signs; and finally, proliferative DR by the presence of neovascularization and/or vitreous/preretinal hemorrhage. Blood samples were collected for all subjects in EDTA tubes for DNA extraction.

Genotyping

Genomic DNA was extracted from whole blood using QIAGEN Puregene Blood Core Kit B (QIAGEN Sciences, Maryland, USA) according to the manufacturer's instructions. Amplification of genomic DNA samples by polymerase chain reaction (PCR) analysis was carried out using the forward primer CAGATACAGCAGCTGAGGAAC and the reverse primer GCCTTCTGATTGGTTGCACT yielding 159-bp band. The PCR conditions were: 5 min of initial denaturation at 94°C, followed by 35 cycles of 94ºC for 60 s, 60°C for 60 s, and 72°C for 60 s, with a final extension at 72°C for 7 m (Bio-Rad, S1000 Thermal cycler™, USA). PCR products were detected on a 2.5% agarose gel. The presence of the C allele was indicated by 138 and 21-bp fragments and T allele was indicated by a 159-bp fragment after overnight digestion with *BsrI* at 37°C. The detected fragment sizes were: 138 and 21 bp for CC; 159, 138 and 21 bp band for CT and 159 bp band for TT, [Figure 1]. PCR findings were validated by: (1) running a negative control that contains all PCR components except the DNA template in every

PCR run; (2) repeating around 13% of all samples by other laboratory personnel to confirm findings of the PCR.

Statistical analysis

The statistical analysis was performed using SPSS version 16 (Chicago, Illinois, USA). All values represent mean ± standard deviation, or counts (%). The correlation between variables in each group was compared by analysis of variance or multiple logistic regression depending whether the variable is quantitative or categorical, respectively. The association of the *ALR2* polymorphism with DR and its severity was determined using multiple logistic regression. Genotype and allele frequencies were analyzed by Chi-square test. A $P < 0.05$ was considered statistically significant. Genotype and allele frequencies were analyzed for concordance to the Hardy–Weinberg equilibrium.

RESULTS

Clinical characteristics of the three groups are shown in Table 1. A higher percentage of diabetic patients had hypertension, dyslipidemia, and ischemic heart disease compared to nondiabetic controls confirming the frequent combination of diabetes with these diseases and implying a low probability that normal controls might have an undiagnosed diabetes. Body mass index (BMI) was higher in diabetic patients than in controls. Clinical characteristics did not show a significant difference between diabetic patients who developed the complication and those who did not except in the duration of diabetes.

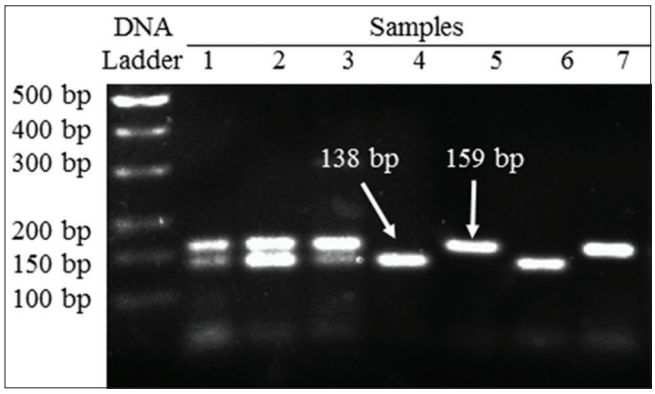

Figure 1: Agarose gel electrophoresis (2.5%) of polymerase chain reaction products, after digestion with *BsrI* endonuclease, and genotyping of the *ALR2* gene C106T polymorphism. Lanes 1-3, 159, 138 and 21 bp bands indicating CT genotype; lane 4 and 6, 138 and 21 bp bands indicating CC genotype; lane 5 and 7, 159 bp band indicating TT genotype

Table 2 represents the distribution of *ALR2* genotypes and allele frequencies among diabetic patients, including subjects with or without DR, and controls. We were able to obtain genotypes of 178 out of 182 subjects due to sample depletion, failure of PCR, or failure of endonuclease digestion. No significant differences in allele or genotype frequency between diabetic patients and controls were noticed. When comparing diabetic patients without retinopathy as a complication to their counterparts with retinopathy, CT and TT genotypes were significantly common among patients with retinopathy [Table 3]. In addition, the substituting allele was significantly more common in patients with retinopathy than diabetic patients without DR as shown in Table 3.

The allelic distribution of the C106T polymorphism was in Hardy–Weinberg equilibrium (C106T: DR (χ^2: 0.10, $P = 0.58$); DM (χ^2: 0.14, $P = 0.67$); controls: (χ^2: 0.68, $P = 0.98$)).

As shown in Table 4, we show the clinical characteristics of the diabetic patients with DR according to *ALR2* genotypes. There were no significant differences in age ($P = 0.573$), BMI ($P = 0.438$), HbA1C ($P = 0.327$), and duration of diabetes ($P = 0.325$) between those with the polymorphism to those without it. Our results did not show a significant correlation between the severity of retinopathy and *ALR2* polymorphism (P value for CC, 0.523; CT, 0.714; and TT, 0.687) as shown in Table 5.

Hypertension, ischemic heart disease, and dyslipidemia were found to be significantly higher in diabetic patients whether they had retinopathy or not than in healthy controls. BMI was significantly higher in diabetic patients with or without DR than in healthy controls. The C106T polymorphism of *ALR2* did not cause an effect on the age of onset of diabetes in patients with or without DR. The age

Table 1: Clinical characteristics of controls and diabetic patients

Characteristic	Control (*n*=95)	Diabetic without DR (*n*=100)	Diabetic with DR (*n*=82)	*P**
Gender (male:female)	59:36 (62.1:37.9)	39:61 (39.0:61.0)	41:41 (50.0:50.0)	0.138
Age (years)	55.16±12.92	60.25±8.17	62.01±8.26	0.896
BMI (kg/m^2)	27.69±4.64	31.50±6.16	31.90±5.67	0.781
Hypertension	22 (20.8)	67 (67.0)	58 (71.6)	0.508
Ischemic heart disease	6 (5.7)	21 (21.0)	14 (17.3)	0.532
Dyslipidemia	21 (19.8)	78 (78)	55 (67.1)	0.099
HbA_{1C} (%)	UA	7.07±1.19	7.74±1.13	0.062
Duration of diabetes (years)	NA	7.01±4.27	10.78±4.08	0.0001

Quantitative date are represented as mean ± SD. Qualitative data are represented as counts. Percentages are shown in parenthesis. **P* value compares diabetics with DR to diabetics without DR. BMI=Body mass index; UA=Unavailable; NA=Not applicable; DR=Diabetic retinopathy

Table 2: Distribution of *ALR2* genotype and allele frequencies among diabetic patients and controls

	Controls (*n*=95)	Diabetic cases (*n*=178)	*P*
Genotype			
CC	45 (47.9)	80 (44.9)	
CT	37 (39.4)	77 (43.3)	0.588
TT	12 (12.8)	21 (11.8)	1
Alleles			
C	127 (66.8)	237 (66.6)	
T	61 (32.11)	119 (33.4)	0.807

Data are represented as counts. Percentages are shown in parenthesis

Table 3: Distribution of *ALR2* genotype and allele frequencies among diabetic patients with or without retinopathy

	Diabetic cases without retinopathy (*n*=100)	Diabetic cases with retinopathy (*n*=78)	*P*
Genotype			
CC	54 (54.0)	26 (33.3)	
CT	38 (38.0)	39 (50.0)	0.024
TT	8 (8.0)	13 (16.7)	0.014
Alleles			
C	146 (73.0)	91 (58.3)	
T	54 (270.)	65 (41.7)	0.007

Data are represented as counts. Percentages are shown in parenthesis

Table 4: Clinical characteristics of diabetic patients according to *ALR2* genotypes

Characteristic	CC (*n*=26)	CT (*n*=39)	TT (*n*=13)
Gender (male:female)	11:16	24:18	7:9
Age (years)	62.7±9.2	61.0±8.0	63.3±8.1
BMI (kg/m^2)	31.5±6.1	32.6±5.3	31.2±5.5
Hypertension	17 (65.4)	30 (76.9)	7 (53.8)
Ischemic heart disease	5 (19.2)	6 (15.4)	3 (23.1)
Dyslipidemia	18 (69.2)	27 (69.2)	7 (53.8)
HbA_{1C}	7.7±1.2	7.8±1.0	7.7±1.2
Duration of diabetes (years)	10.9±4.3	10.2±4.0	11.0±4.4

Quantitative data are represented as means±SD. Qualitative data are represented as counts. Percentages are shown in parenthesis. BMI=Body mass index; SD=Standard deviation

Table 5: *ALR2* genotype and the severity of diabetic retinopathy

Retinopathy severity	CC (*n*=26)	CT (*n*=39)	TT (*n*=13)
Mild	4 (15.4)	11 (28.2)	5 (38.4)
Moderate	9 (34.6)	10 (25.6)	4 (30.8)
Severe	5 (19.2)	9 (23.1)	2 (15.4)
Proliferative	8 (30.8)	9 (23.1)	2 (15.4)

Data are represented as counts. Percentages are shown in parenthesis. Grades of severity according to the worse eye

of onset of diabetes did not show a statistically significant correlation with the severity of DR ($P = 0.196$).

DISCUSSION

DR is the second major cause of blindness among Jordanians.[14] The prevalence of DR in Jordanian diabetic patients is 64.1% with proliferative complications present in 9.3% of DR patients.[15,16] The clinical data of the subjects included in this study had excluded the effect of risk factors such as obesity, hypertension, dyslipidemia, and glucose level control on the development of DR. However, the duration of diabetes was significantly longer in diabetic patients with DR than those without it.

We were able to show an association between promoter C106T polymorphism of *ALR2* and DR. The T allele of the promoter C106T polymorphism is significantly more common in diabetic patients who developed retinopathy as a complication. There is significantly more CT and TT genotype carriers among diabetic patients with retinopathy when compared to patients who did not show this complication. This is the first study of *ALR2* polymorphisms in Jordanian diabetic patients. It involved diabetic patients with DR and compared them to those without this complication and to normal controls. The frequency of this polymorphism in the 3 groups of subjects was determined for the first time in Jordan. Although many epidemiological studies were conducted worldwide in the past few decades concerning the relationship between *ALR2* gene C106T polymorphism and DR, only a few ones had correlated this polymorphism with the severity of DR. Other studies classified disease severity into proliferative and nonproliferative, while our study included more degrees of disease severity within nonproliferative type. An Iranian study showed that the C106T polymorphism in the *ALR2* gene is a potential risk factor for the development of a retinal microvascular complication of diabetes in their population.[6] Studies on English population demonstrated the same association but in Type 1 diabetic patients.[5] Studies on Chinese patients with Type 2 diabetes showed both a significant and a nonsignificant association of C106T polymorphism of the *ALR2* gene with retinopathy.[12,17] A study on Egyptians, Australians, and Indonesians of Yogyakarta did not show the association between C106T polymorphism and DR.[18-20] In a meta-analysis of studies performed on Chinese population, the overall analysis demonstrated a nonsignificant association between the *ALR2* C106T polymorphism and DR, whereas, subgroups stratified by ethnicity analyzed in this study showed significantly increased risks for DR in *ALR2* C106T variants of the Chinese Han population.[21] Another meta-analysis that involved 3512 diabetic patients with DR and 4319 diabetic patients without it found that ALR2 C106T polymorphism was not associated with a higher risk to develop DR, however, ALR2 C106T polymorphism increased the risk of DR in Type 1 diabetic patients but not in Type 2 diabetics.[22] In a recent meta-analysis that involved 23 studies, a significant association between the *ALR2* gene C106T polymorphism and DR susceptibility was shown. In addition, they found a significantly higher risk

for DR in Type 1 DM, East Asian populations, and Middle Eastern populations.[15] The possible explanations for the inconsistent findings between different populations may be attributed to variability in sample size and population structure, bias, liability of genetic-association studies to statistical errors, a failure to control for confounders, differences in the ethnicity, diversity of genetic and environmental backgrounds, or simply chance. In addition, population-based genotypes may have different effects in different populations.

Apart from retinopathy, the association of *ALR2* C106T polymorphism with other microvascular complications of diabetes such as diabetic nephropathy was reported in patients with Type 1 and Type 2 diabetes. The *ALR2* gene was reported as a candidate locus for diabetic nephropathy in Pima Indians.[23] The C106T polymorphism in the *ALR2* gene was shown to increase the risk for developing diabetic nephropathy in Type 2 diabetic patients with poor glycemic control in Polish population.[24] Other studies showed an association between T allele and nephropathy in Caucasian Type 1 diabetic patients.[25] Another study also suggested that the C106T polymorphism of *ALR2* gene might be associated with higher expression of ALR in Chinese population, even though this needs confirmation.[4]

Other studies demonstrated that non-Caucasian diabetic patients have a higher risk of retinal complications than Caucasian populations.[26] This may affect our results since Jordanian community at the ethnic level is a mixed stock that mostly descended from villagers and Bedouins of the Arabic Peninsula. Even though our results did not demonstrate an association between *ALR2* C106T polymorphism and the severity of retinal complication, a larger sample size may show this relationship suggesting that genetic factors might be of importance to those with severe or proliferative complications.

A possible undefined regulatory element of *ALR2* or another gene near *ALR2* that contributes to the development of diabetic complications might be present although we found some significant associations with C106T *ALR2* polymorphism. After the Human Genome Project was completed, up to 30% of the single nucleotide polymorphisms showed variations among different racial groups.[27] In addition, growing evidence suggests that haplotypes of variants within a gene may provide more information in disease prediction and regulation of gene function than a single gene variant.[28]

The limitations of this study include the relatively small sample size and the difficulty of matching DR cases and controls on some variables such as, disease duration. In addition, healthy recruits (controls) were not tested to confirm that they were free of diabetes or retinal diseases at the time of recruitment, nor were follow-up tests performed later. Another limitation of this study is the presence of other *ALR2* polymorphisms such as, (AC)n dinucleotide repeat and G > A substitution (rs9640883) in intergenic region of *ALR2* gene that we did not study, hence, no haplotype analysis can be performed for the three polymorphisms. DNA sequencing could have been done to confirm results, but financial constraints were behind the inability to perform sequencing or to investigate the three polymorphisms and their haplotype analysis. However, to the best of our knowledge, our study is the first study of *ALR2* polymorphisms in Jordanian and Arab diabetic patients. This study not only compared DR patients to diabetic patients without DR but also compared diabetic patients to normal control healthy individuals. It also subdivided severity level of the nonproliferative type of DR into several ones. In addition to that, case–control association studies can be helpful in the identification of disease biomarkers and in the analysis of multiple potential factors for complex diseases such as DR.

CONCLUSION

Our study revealed statistical associations that need confirmation and elucidation of their nature by larger sample size, prospective and family-based studies, studies on different ethnic groups, functional studies and long-term follow-up studies.[29] However, our results have demonstrated the presence of an effect of *ALR2* C106T genetic polymorphism on the risk of DR in Jordanian population. *ALR2* can be tested as a marker that may help in providing better prevention for high-risk diabetic patients.

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Conflicts of interest

There are no conflicts of interest.

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