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Functional differences between the susceptibility *Z−2/C−106* and protective *Z+2/T−106* promoter region polymorphisms of the aldose reductase gene may account for the association with diabetic microvascular complications

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Abstract

Studies have shown that polymorphisms located at positions −106 and approximately −2100 base pairs (5′*ALR2*) in the regulatory region of the aldose reductase gene are associated with susceptibility to microvascular complications in patients with diabetes. The aim was to investigate the functional roles of these susceptibility alleles using an in vitro gene reporter assay. Susceptibility, neutral and protective 5′*ALR2*/*−106* alleles were transfected into HepG2 cells and exposed to excess D-glucose (D-glucose at final concentrations 14 or 28 mmol/l). Transcriptional activities were determined using a dual luciferase reporter gene assay. The "susceptibility alleles" *Z−2* with *C−106* had the highest transcriptional activity when compared with the "protective" combination of *Z+2* with *C−106* alleles (58.7 ± 9.9 vs. 10.1 ± 0.7; $P<0.0001$). Those constructs with either the *Z* or *Z−2* in combination with the *C−106* allele had significantly higher transcriptional activities when compared to those with the *T−106* allele (*Z/C−106*, 37.4 ± 5.4 vs. *Z/T−106* 7.7 ± 1.6, $P<0.003$; *Z−2/C−106*, 58.7 ± 9.9 vs. *Z−2/T−106* 10.9 ± 0.6, $P<0.0001$). These results demonstrate that the *Z−2/C−106* haplotype is associated with elevated transcriptional activity of the aldose reductase gene. This in turn may explain the role of these polymorphisms in the susceptibility to diabetic microvascular complications.

Keywords: Aldose reductase gene; Diabetic microvascular complication; Polyol pathway; Hyperglycaemia; Hypertonicity

1. Introduction

It is becoming clear that there is a genetic as well as hyperglycaemic component for the development of microvascular complications in patients with either Type 1 or Type 2 diabetes mellitus. Recent studies have shown that a AC dinucleotide repeat microsatellite in the promoter region of the aldose reductase gene (AKR1B1 or ALR2) are associated with the susceptibility to microvascular complications in patients with either Type 1 or Type 2 diabetes mellitus who have diverse ethnic origins [1–6]. ALR2 is the first and rate-limiting enzyme of the polyol pathway and converts glucose to sorbitol in an NADPH-dependent reaction. Several studies have shown that the ALR2 mRNA and protein levels in blood cells are increased in patients with diabetic microvascular complications [7–10]. The *Z−2* 'susceptibility' allele (5′*ALR2*) that is located 2.1 kilobase (kb) upstream in the promoter region of ALR2 gene has been shown to be linked with increased mRNA levels in peripheral blood mononuclear cells (PBMC) of patients with diabetic nephropathy and Type 1 diabetes mellitus [11]. Further, when PBMC of patients with nephropathy and Type 1 diabetes mellitus are exposed to hyperglycaemia in vitro for 5 days, there is a marked induction of ALR2 mRNA with those individuals with the *Z−2* allele having more than eightfold increase in expression. In marked contrast, the PBMC of patients with Type 1 diabetes mellitus and no complications after 20 years duration of diabetes as well as normal healthy controls showed no change in the expression of ALR2 when exposed to hyperglycaemia [12].

A *C−106T* substitution in the basal promoter region has been shown to be associated with susceptibility to diabetic

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retinopathy [4,13]. These studies have suggested that the *Z − 2/C − 106* combination is a higher risk susceptibility haplotype for microvascular complications compared to *Z+ 2/T* or, *Z+ 2/C* haplotypes although this has not been confirmed in other studies [14,15]. The aim of this study was to investigate the functional role of these susceptibility and protective haplotypes using an in vitro gene reporter assay.

2. Materials and methods

2.1. Cloning of the ALR2 promoter gene into the pGL3-enhancer vectors

An aliquot of high molecular weight DNA that had previously been typed for the *5′ALR2/C − 106T* polymorphic sites were used as template to amplify the promoter region of the ALR2 gene using Expand High Fidelity PCR System (Roche, East Sussex, UK). The region from − 2223 to + 60 was amplified with the amplimers 5′-CATACGCGTGTGCAGGCCTGGGACTAAGGGAAG-3′ and 5′-CGGCTCGAGTGAGCAGGAGACGGCTTGCCATGG-3′ containing *Mlu*I and *Xho*I restriction sites. The following haplotypes were amplified: *Z − 2/C − 106*, *Z − 2/T − 106*, *Z+ 2/C − 106*, *Z+ 2/T − 106*, *Z/C − 106*, *Z/T − 106*, *Z − 4/C − 106* and *Z − 4/T − 106* (Fig. 1). The products were cloned into the pCR-XL-TOPO vectors (Invitrogen, The Netherlands) following the manufacturer's instruction. The inserts were excised by digesting with the restriction endonuclease *Xho*I and *Mlu*I, which were then subcloned into the *Xho*I and *Mlu*I sites of the pGL3-enhancer luciferase reporter vectors (Promega, Southampton, UK), which lack a promoter sequence. The *5′ALR2* and *C − 106T* sites were confirmed by microsatellite analysis for the *5′ALR2* site and digestion with *Bfa*I for the *C(− 106)T* polymorphism. In addition, both strands of the inserts of all recombinants used in this study were sequenced to confirm the haplotypes (MWG, Milton Keynes, UK). For each haplotype, at least three separate recombinants were constructed and used in the following transfection and luciferase assays (Table 1).

Table 1
Number of recombinants for each haplotype

Haplotypes	No. recombinants × no. transfection
Z+ 2/C	5 × 2[a]
Z+ 2/T	3 × 2
Z/C	4 × 2
Z/T	3 × 2
Z − 2/C	5 × 2
Z − C/T	4 × 2
Z − 4/C	3 × 2
Z − 4/T	4 × 2

[a] Transfection was performed twice for each recombinant. Therefore, each mean of transcription activity in luciferase assays was the number of recombinants times two for each haplotype under normal, hyperglycaemic and hypertonic conditions.

2.2. Transfection assays using ALR2-luciferase constructs

The recombinant plasmids were purified using QIAGEN-tip 100 columns (QIAGEN, Dorking, UK). HepG2 cells (ECACC, Salisbury, UK) maintained in complete culture medium, which contains Eagle's minimal essential medium supplemented with 10% feotal bovine serum (FBS), 2 mmol/l glutamine, 1% nonessential amino acids and suitable antibiotics were used for transfection. The cells were seeded at 106 per well of 24-well plates and cultured for 24 h until 80% confluent at 37 °C incubator. For each well, the following mixture was prepared in a 1.5-ml microcentrifuge tube, 675 ng of recombinant plasmid and 75 ng of pRL-TK

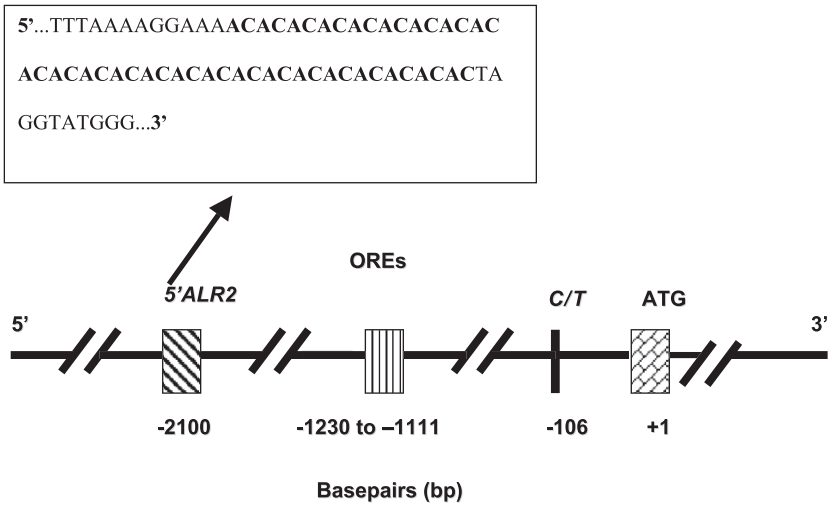

Fig. 1. Structure of the promoter region of the human aldose reductase gene. The (*AC*)*n* dinucleotide repeat polymorphism region (*5′ALR2*) is located at − 2.1 kb upstream in the promoter region. *Z+ 2*, *Z*, *Z − 2* and *Z − 4* alleles are equal to $(AC)_{25}$, $(AC)_{24}$, $(AC)_{23}$ and $(AC)_{22}$ repeats. A *C − 106T* polymorphism is present in the basal promoter region. There are several osmotic response elements (OREs) located between the *5′ALR2* and *C − 106T* sites.

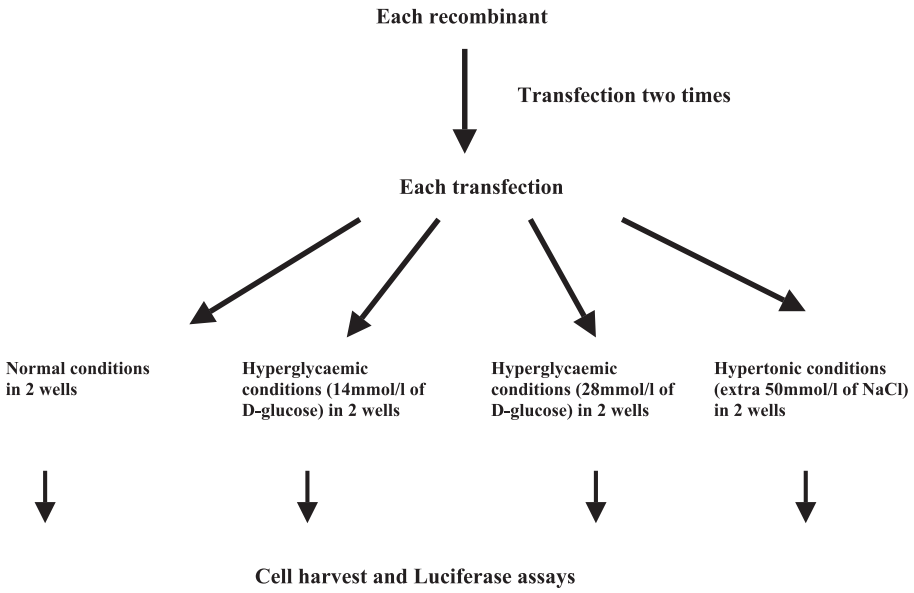

Fig. 2. This graph shows the number of transfection and luciferase assays for the recombinants. For each individual recombinant, transfection was performed twice. For a single transfection reaction, eight wells were prepared and divided into four groups in duplicate. Group A was the normal control. The cells in groups B and C were stressed under hyperglycaemic conditions (final D-glucose concentration, 14 and 28 mmol/l respectively). Group D contained cells exposed to hypertonic conditions (extra 50 mmol/l of NaCl). At the end of incubation, the cells were harvested, lysed and a luciferase assay was performed (see Sections 2.2 and 2.3).

control plasmid (10:1 ratio) were mixed with the complete culture medium without FBS to a final volume of 197.8 μl. Then, 2.2 μl of the Tfx-20 reagent (Promega) was added to make final volume of 200 μl at charge ratio 2:1. The tubes were incubated at room temperature for 15 min and the 24-well plates containing the HepG2 cells were removed from the incubator and the medium aspirated and replaced by the mixture in the 1.5-ml tubes. The plates were then returned to the incubator for 1 h before each well was overlaid with 1 ml of the complete medium and the plates returned to the incubator.

For each individual recombinant, transfection was performed twice. For each time's transfection (i.e. for each single recombinant), eight wells were prepared and divided

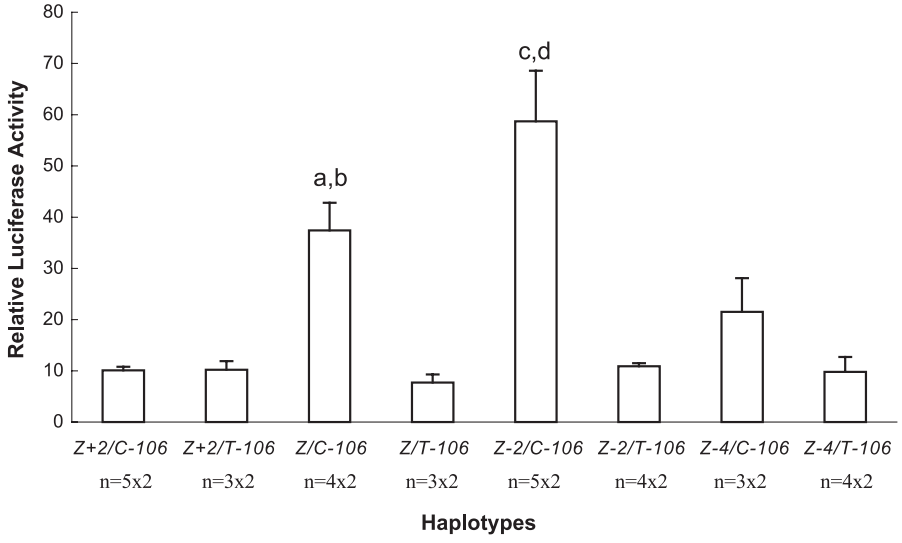

Fig. 3. The effect of the (*AC*)*n* repeats and *C(−106)T* polymorphisms on luciferase transcription in the pGL3-enhancer vectors. Firefly luciferase activity normalised by the activity of *Renilla* luciferase is presented as relative luciferase activity, which is expressed as means ± S.E. Each mean represented transcriptional activities in a number of independent recombinants for each haplotype under normal conditions. The number of recombinants, transfection and luciferase assays is shown under each bar for each haplotype. For each recombinant, repeated transfection was performed and expressed as × 2 under each bar. Comparison of the relative luciferase activity in all haplotypes by one-way ANOVA showed a statistically significant difference ($P < 0.0001$). The post hoc Bonferroni test revealed significant differences between recombinants and the differences were indicated as above. (a) $P < 0.0001$ vs. *Z+ 2/C − 106* and *Z − 2/T − 106*; (b) $P < 0.003$ vs. *Z+ 2/T − 106*, *Z/T − 106* and *Z − 4/T − 106*; (c) $P < 0.04$ vs. *Z/C − 106*; (d) $P < 0.0001$ vs. all others apart from *Z/C − 106*.

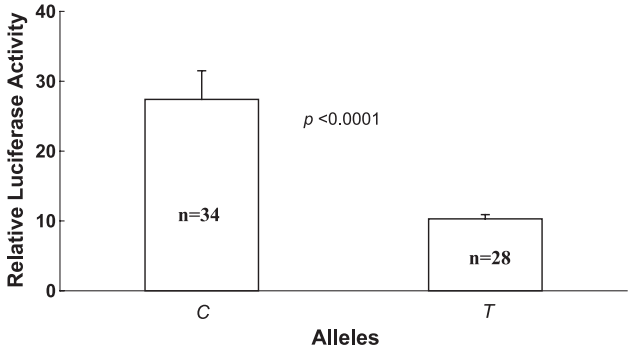

Fig. 4. This shows the relative luciferase activity in recombinants with either the *C – 106* or the *T – 106* allele in cells exposed to normal conditions. There is a highly significantly increase in transcriptional activity in recombinants containing the *C – 106* allele. Each mean represented transcriptional activities for haplotypes containing either *C – 106* or *T – 106* allele (Table 1).

into four groups in duplicate. After 24 h, Group A was the normal control. To either Group B or Group C, 9.2 or 18.4 μl of 20% D-glucose was added, respectively (final D-glucose concentration, 14 and 28 mmol/l, respectively). Group D contained cells exposed to hypertonic conditions created by adding 50 mmol/l of NaCl to the culture medium (Fig. 2). All plates were incubated for a further 20 h before they were lysed using 100 μl of lysis buffer (Promega) and the lysates were stored at − 80 °C.

2.3. Luciferase assay

Luciferase activity was measured using Dual-luciferase reporter assay system (Promega) in a MLX luminometer (Dynex Technologies Inc., USA). The density was used to determine the transcriptional activity. The relative activity was defined as luciferase activity normalised by the activity of the internal control (*Renilla* luciferase).

2.4. Statistical analysis

The data were expressed as the means ± standard error of the mean (S.E.). Post hoc analysis was performed using the nonparametric Bonferroni [5] to compare the relative luciferase activities between different groups after analysis of variance (ANOVA). The *t*-test was used to compare the relative luciferase activity between two groups. A *P* value of less than 0.05 was considered significant.

3. Results

There was a highly significant difference in the luciferase activities between all the recombinants when the transfected HepG2 cells were grown under normal conditions when analysed by one way ANOVA ($P<0.0001$) (Fig. 3). The reporter construct containing the *Z – 2/C – 106* ALR2 alleles had the highest relative luciferase activity (58.7 ± 9.9) among all the constructs. The relative luciferase activity in the *Z – 2/C – 106 ALR2* promoter (58.7 ± 9.9) was higher than the activity in any of the other constructs: *Z + 2/C – 106* (10.1 ± 0.7, $P<0.0001$), *Z/C – 106* (37.4 ± 5.4, $P<0.04$), *Z – 4/C – 106* (21.5 ± 6.6, $P<0.001$) and all those with the *Z + 2*, *Z*, *Z – 2* and *Z – 4* together with the *T – 106* allele ($P<0.0001$). There was nearly a fivefold increase in transcriptional activity of the

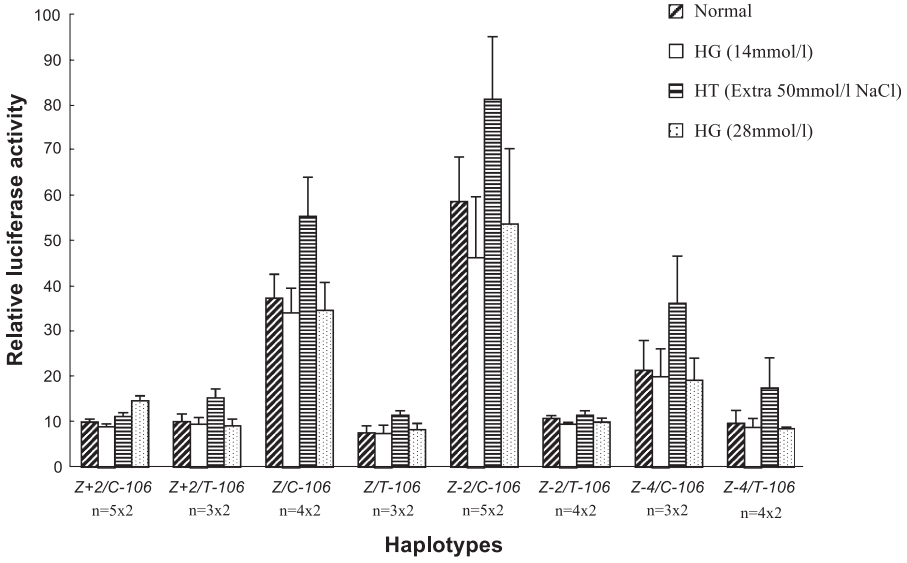

Fig. 5. Effects of the *(AC)n* repeats and *C/T – 106* on luciferase transcription in the pGL3-enhancer vectors under hyperglycaemic (14 or 28 mmol/l D-glucose) or hypertonic (extra 50 mmol/l of NaCl) conditions. No significant difference in transcriptional activity was found when the cells were exposed to either hyperglycaemic or hypertonic conditions when compared to normal conditions for an individual haplotype. Each mean represented transcriptional activities in a number of independent recombinants for each haplotype under normal, hyperglycaemic or hypertonic conditions. The number of recombinants, transfection and luciferase assays is shown under bars for each haplotype. For each recombinant, repeated transfection was performed and expressed as × 2 under bars.

Z − 2/C − 106 "susceptibility haplotype" when compared to the *Z+ 2/C − 106* and *Z+ 2/T − 106* "protective haplotypes". Indeed, these "protective haplotypes" had the lowest luciferase activities (10.1 ± 0.7 and 10.2 ± 1.7, respectively). The *Z/C − 106 ALR2* promoter construct had the second highest luciferase activity (37.4 ± 5.4) and was significantly different from the *Z+ 2/C − 106 ALR2* promoter construct (10.1 ± 0.7, $P < 0.0001$) as well as the following combinations; *Z+ 2*, *Z*, *Z − 2* and *Z − 4* with the *T − 106* ($P < 0.002$). Furthermore, there was a highly significant difference in the transcriptional activity of all ALR2 haplotypes containing the *C − 106* allele compared to those with the *T − 106* allele (27.4 ± 4.2 vs. 10.3 ± 3.1, $P < 0.0001$) (Fig. 4).

When the HepG2 cells that had been transfected with the ALR2 promoter region constructs were exposed to hypertonicity, the transcriptional activities were increased in all haplotypes (Fig. 4), but these were not statistically significantly different when compared with normal conditions. When the cells were exposed to hyperglycaemia, there were no significant changes in luciferase activity for any of the haplotypes (Fig. 5).

4. Discussion

Several studies have suggested that the *Z − 2* and *C − 106* alleles are associated with the microvascular complications whilst the *Z+ 2* and *T − 106* may be protective factors [1–4,13,16–18]. To determine whether these haplotypes have a functional role in modifying transcription of the ALR2 gene, we cloned the promoter region containing the various combinations of *Z+ 2*, *Z − 2*, *Z* and *Z − 4* allele (a risk allele in Japanese study) [5] with either the *C − 106* or *T − 106* alleles into the pGL3-enhancer vectors, respectively. The HepG2 cell line was selected for the transfection experiments as it has previously been shown that it expresses ALR2 and this can be induced by stress following transfection [19–22]. This was confirmed by studies in this laboratory (data not shown). The results demonstrate that the susceptibility haplotype *Z − 2/C − 106* had the highest relative transcriptional activity compared to all other haplotypes. This strongly supports the previous genetic studies showing that the *Z − 2/C − 106* alleles and haplotype contribute to the genetic background for diabetic microvascular complications. These results also concur with previous studies showing that the *Z − 2* allele is associated with significantly higher ALR2 mRNA levels as well as enzyme activity in those patients with nephropathy compared to those with neutral or protective allelic combinations [11,12,18]. Increased expression of the ALR2 gene has been demonstrated in human retinal pigment epithelial cell lines that are homozygous *C − 106* alleles compared to those with the *T − 106* or heterozygous for these alleles [23]. Our study showed that those recombinants with the *Z+ 2/T − 106* and *Z+ 2/C − 106* haplotypes had the lowest transcriptional activity of all. Thus, there is evidence that not only the *Z − 2* allele, but also the *C − 106* together would be risk factors for diabetic complications. This was supported by previous studies that demonstrated that the *Z − 2* and *C − 106* alleles are in strong linkage disequilibrium [4,13] although there have been conflicting reports [14,15]. The results from this study strongly support the suggestion that the *Z − 2/C − 106* is the susceptibility haplotype rather than other combinations of alleles proposed by other studies. The biological basis of the conflicting results is not clear. Neamat-Allah et al. [14] have mentioned that variations in haplotype frequencies and patterns cannot be ruled out as a source of variability in the results. They also explained that there may be more than one haplotype carrying the disease-predisposing variant, leading to differences in the degree of association of *Z − 2* haplotype with diabetic complications in different study groups. Other possible explanations could be that association studies have limitations such as sample sizes, bias of populations, diagnosis criteria, and differences in the ethnical backgrounds and methods of genotyping. Therefore, collaborative studies throughout the world to standardise methods for genotyping and clinical data analysis are needed.

The haplotype construction containing the *Z/C − 106* had the second highest relative luciferase activity among all recombinants. This may reflect the normal baseline level of ALR2 expression as '*Z*' is the most common allele in the normal population. Those constructs containing the "protective allele" *Z+ 2* with either the *C − 106* or *T − 106* allele had a lower transcription activity and this may contribute to the mechanisms protecting cells from hyperglycaemia. In contrast, the "susceptibility allele" *Z − 2* with the *C − 106* would lead to increased flux through the polyol pathway thereby contributing to the metabolic and vascular abnormalities and oxidative stress and development of the diabetic complications. The exact mechanism for the higher transcription in the *Z − 2/C* haplotype is not clear. A Japanese group also confirmed that the transcription of the ALR2 gene is enhanced or suppressed by the number of dinucleotide repeats although they claimed that the *Z − 4* allele has a highest transcription activity [5]. Other studies also support a role for dinucleotide repeats on enhancing transcriptional activities, such as the human type 1 collagen alpha2 (COL1A2) gene [24] as well as the matrix metalloproteinase-9 gene both in human and mouse [25,26]. The mechanism by which dinucleotide repeats upstream of genes regulate transcription may be due to modification of DNA conformation, DNA helical and backbone parameters, DNA structures and stability [27,28]. For the *C(− 106)T* polymorphism, the plausible explanation is that this polymorphism is located in the basal promoter region of the ALR2 gene and close to the CCAAT promoter element, which is present between − 98 and − 94 and appears to be functionally active [19]. In this study, they also demonstrated that the CCAAT motif is recognised by a nuclear factor from the Hep G2 cells. Therefore, different alleles may

affect the CCAAT element function by changing nuclear factor binding activity and finally change the basal transcriptional level of the ALR2 gene. The *Z − 2* allele may enhance the increased transcriptional level of the ALR2 gene, which is with the *C − 106* allele because these two alleles are tightly linked each other [4,13].

Our results have shown that the hyperglycaemic conditions did not increase transcriptional activity in all recombinants. Whilst the OREs were present in the inserts, it is unclear where the glucose-response elements reside. Further, the cells were only briefly exposed to hyperglycaemia and this may be too short a period to increase the ALR2 gene expression. A previous study in rat Schwann cells exposed to hyperglycaemia also found no increase in ALR2 mRNA although this study did not take into account any possible role of polymorphisms within the rat ALR2 gene [29].

In conclusion, we have found that the susceptibility *Z − 2*/*C − 106* haplotype had a greater transcriptional activity when compared with any of the other possible haplotypes. This finding strongly supports the role of this haplotype in the development of diabetic microvascular complications.

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