Association analysis of $-429\text{T/C}$ and $-374\text{T/A}$ polymorphisms of receptor of advanced glycation end products (RAGE) gene in Malaysian with type 2 diabetic retinopathy

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ABSTRACT

Conflicting results have been reported in different populations on the association between two particular RAGE gene polymorphisms ($-429\text{T/C}$ and $-374\text{T/A}$) and retinopathy in diabetic patients. Therefore this study was designed to assess the association between both gene polymorphisms with retinopathy in Malaysian diabetic patients. A total of 342 type 2 diabetic patients [171 without retinopathy (DNR) and 171 with retinopathy (DR)] and 235 healthy controls were included in this study. Genomic DNA was obtained from blood samples and the screening for the gene polymorphisms was done using polymerase chain reaction-restriction fragment length polymorphism approach. Overall, the genotype distribution for both polymorphisms was not statistically different ($p > 0.05$) among the control, DNR and DR groups. The $-429\text{C}$ minor allele frequency of DR group (12.0%) was not significantly different ($p > 0.05$) when compared to DNR group (16.1%) and healthy controls (11.3%). The $-374\text{A}$ allele frequency also did not differ significantly between the control and DNR ($p > 0.05$), control and DR ($p > 0.05$) as well as DNR and DR groups ($p > 0.05$). This is the first study report on RAGE gene polymorphism in Malaysian DR patients. In conclusion, $-429\text{T/C}$ and $-374\text{T/A}$ polymorphisms in the promoter region of RAGE gene were not associated with Malaysian type 2 DR patients.

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1. Introduction

Diabetic retinopathy (DR) is fast emerging as the leading cause of blindness in persons with diabetes mellitus with a total of 4.8% already affected across the globe [1]. Though clinical risk factors such as duration of diabetes, poor control of disease, systemic hypertension and proteinuria have been reported previously for proliferative diabetic retinopathy (PDR), these factors account only for small portion of the severe forms of the disease [2]. Genetic factors, independent of the environment and clinical factors have been found to contribute to its pathology [3-5]. The receptor of advanced glycation end product (RAGE) gene is one of the genes related to retinopathy [6,7]. This gene is located on chromosome 6p21.3 at the major histocompatibility complex locus in class III region. It is composed of 11 exons and a 3'UTR and is expressed by endothelium, smooth muscle, messengial cells and monocytes [8]. In vivo and in vitro studies have shown that RAGE contributes to the pathogenesis of diabetic microvascular complications [9,10].
RAGE recognizes a wide range of endogenous ligands including advanced glycation end products (AGE) which is known to be accumulated in DR patients [11]. AGE are complex, heterogenous molecules formed from non-enzymatic glycation and oxidation of protein, lipids and nucleic acids [6,7]. The sustained interaction of AGE with RAGE in retinopathy patients leads to a positive feedback loop that enhances the expression of RAGE in the retina and causes a plethora of deleterious effects [10], mainly due to the activation of proinflammatory transcription factor NF-κB [6,7,12]. The variants of RAGE gene could alter the above mentioned pathway of events by changing the expression of RAGE and indirectly affect the disease development.

The focus of this research was to study the –429T/C and –374T/A gene polymorphisms since most of the identified retinopathy RAGE gene polymorphisms are either located at non-coding regions or rarely affect the coding changes [8,13]. In general, –429T/C and –374T/A are two common functional gene polymorphisms that are located at the promoter region of the RAGE gene which could affect RAGE mRNA and protein expression. Association studies between these two gene polymorphisms and patients with retinopathy carried out so far in other populations showed conflicting results [9,14–16]. To date, this is the first report on RAGE gene polymorphism in Malaysian diabetic patients with retinopathy.

2. Materials and methods

2.1. Sample collection

A total of 342 consecutive patients with type 2 diabetes (118 Malay, 67 Chinese, 157 Indian) aged 57.9 ± 9.8 years (mean ± SD; range, 35–79 years) (198 men, 144 women) were recruited from both Diabetic and Ophthalmology clinics at the University of Malaya Medical Centre (UMMC), Malaysia. Diabetes was diagnosed previously according to World Health Organization criteria. Patients with type 2 diabetes and with more than one year duration of retinopathy were included in the study. Written informed consent was obtained from each patient and detailed medical and ophthalmologic histories as well as sociodemographic factors and lifestyle variables were noted. There were 171 diabetic without retinopathy (DNR) (63 Malay, 28 Chinese, 80 Indian) and 171 diabetic with retinopathy (DR) patients (55 Malay, 39 Chinese, 77 Indian). A total of 235 non-diabetic unrelated healthy volunteers (106 Malay, 90 Chinese, 39 Indian) aged 52.2 ± 3.6 years (mean ± SD; range, 44 to 60 years) (134 men, 101 women) were recruited during blood donation campaigns held in Kuala Lumpur, Malaysia as controls for this study.

All the DR patients underwent a complete eye examination that included dilated retinal examination and 7-field stereoscopic Diabetic Retinopathy Study retinal photographs [17]. The color fundus photographs were graded for DR severity in a masked fashion by two independent ophthalmologists at the University of Malaya Eye Research Center in Kuala Lumpur. The modified Early Treatment of Diabetic Retinopathy Study Airlie House classification of DR was used to grade the retinopathy [18,19]. The DR patients consisted of 27 mild non-proliferative retinopathy, 72 moderate non-proliferative, 9 severe non-proliferative retinopathy patients and 63 proliferative retinopathy patients. Six millilitre of blood was obtained from each subject for the single-nucleotide polymorphism (SNP) study. Three millilitre of the collected blood was sent for routine blood examination in Clinical Diagnostic Laboratory at the University Malaya Medical Centre. The study was performed in adherence to the principles of the Declaration of Helsinki and approved by the Medical Ethics Committee of the UMMC.

2.2. DNA extraction and polymerase chain reaction (PCR)

Genomic DNA was obtained from the whole blood samples using a simplified phenol/chloroform extraction method as stated previously [20,21]. The gene polymorphism analysis was carried out using the polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) approach. PCR was used to amplify the RAGE 5’ promoter regions containing –374T/A (rs1800624) and –429T/C (rs1800625) polymorphisms as described in a previous study with some modifications [22]. In silico PCR analysis as stated in other studies was also carried out to predict the amplicon size prior to actual PCR [23–25].

PCR was performed with 100 ng genomic DNA in a final volume of 20 μl containing 12.5 pmol of each primers (forward primer 5’ GGG GCA GTT CTC TCA CT 3’ and reverse primer 5’ GTG TCA GCC CAG ACT GTT GT 3’), 1 U Taq DNA polymerase (Fermentas, Lithuania), 0.2 mM dNTP mix, 1× Taq buffer, and 1.5 mM MgCl₂ and appropriate amount of distilled water. PCR was carried out in a thermal cycler (MyCycler™, Bio-Rad Laboratories Inc., USA). The cycling program was set according to our standard protocol as stated in previous study [26]. The PCR amplicons (250 bp) were then visualized on a 3% (w/v) agarose gel stained with ethidium bromide.

2.3. Restriction enzyme analysis and sequencing

The change of nucleotide (T>C) at position –429 of 5’ promoter region of the RAGE gene resulted in the formation of Alul restriction site (AAG/CTC) whereas the change of nucleotide (T>A) at –374 position diminished Mfe restriction site (C/AATTG). Restriction enzyme digestion was performed with all PCR products using 5 U of restriction enzymes, 1× buffer and appropriate amount of distilled water in a total volume of 20 μl, followed by incubation overnight at 37 °C. Alul and Mfe (Fermentas, Lithuania) were used for –429T/C for –374T/A analysis, respectively. The digested products were directly electrophoresed on 3% (w/v) agarose gel with ethidium bromide and the results were recorded using a gel documentation system. Overall, digestion with Alul revealed fragments of 162 bp and 88 bp for mutated minor allele –429C while 250 bp for wild type major allele –429T. The wild type major allele –374T gave fragments of 215 bp and 35 bp after digestion by Mfe whereas 250 bp was detected for mutated minor allele –374A. Five representative samples from each genotype were further sequenced to confirm the overall genotyping results.

2.4. Statistical analysis

The unpaired t-test and one-way analysis of variance (ANOVA) with Tukey’s post hoc test were used for the evaluation of
biochemical parameter differences among the groups. For the evaluation of RAGE gene polymorphism, the Hardy–Weinberg equilibrium (HWE) was examined in the control, diabetic without retinopathy (DNR) and diabetic retinopathy (DR) groups using chi-squared test with one degree of freedom. The statistical significance of differences in genotype and allele frequencies between the groups was tested by two-tailed Fisher’s exact test. A p-value (p < 0.05) was considered to be statistically significant. GraphPad Prism® for Windows® version 5.02 (GraphPad Software Inc., CA, USA) was used for statistical analysis in this study.

3. Results

The demographic data of healthy control, DNR and DR patients is listed in Table 1. Both DNR and DR patients displayed significantly (p < 0.05) higher level of HbA1c, total cholesterol, high-density lipoprotein (HDL-C), low-density lipoprotein (LDL-C), systolic blood pressure and number of subjects with hypertension as compared to healthy control. Comparison between DNR and DR groups revealed significant differences (p < 0.05) in age, higher level of HbA1c, total cholesterol, longer diabetes duration and lower number of smoker in DR patients. No significant differences (p > 0.05) were observed in body mass index (BMI), triglyceride, alanine aminotransferase (ALT), aspartate aminotransferase (AST) level, diastolic blood pressure (DBP), number of subjects with alcohol intake and with hypertension.

A representative PCR-RFLP result is shown in Fig. 1. Overall, genotype frequencies for both polymorphisms in healthy controls, DNR and DR did not differ from the Hardy–Weinberg equilibrium except for –374 polymorphism of control group. Table 2 shows the genotype and allele frequency distributions for –429T/C and –374T/A polymorphisms on studied control, DNR and DR groups. The genotype distribution for both polymorphisms was not statistically different (p > 0.05) among the control, DNR and DR groups. The minor allele

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Table 1 – Demographic data of healthy control, DNR and DR groups.

<table>
<thead>
<tr>
<th>Demography</th>
<th>Healthy control (n = 235)</th>
<th>DNR (n = 171)</th>
<th>DR (n = 171)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (male/female)</td>
<td>198/144</td>
<td>100/71</td>
<td>98/73</td>
</tr>
<tr>
<td>Races (Malay/Chinese/Indian) (%)</td>
<td>45.1/38.3/6.6</td>
<td>36.8/16.4/46.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>32.2/22.8/45&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Age (years)</td>
<td>55.2 ± 5.0</td>
<td>59.2 ± 9.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>56.1 ± 10.1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>BMI (Kg/m&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>25.6 ± 4.8 (n = 183)</td>
<td>27.2 ± 4.4</td>
<td>26.4 ± 5.3</td>
</tr>
<tr>
<td>HbA&lt;sub&gt;1c&lt;/sub&gt; (%)</td>
<td>5.6 ± 0.4 (n = 183)</td>
<td>7.9 ± 1.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.0 ± 2.1&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>3.8 ± 0.6 (n = 183)</td>
<td>4.5 ± 1.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.9 ± 1.6&lt;sup&gt;b,c&lt;/sup&gt;</td>
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<tr>
<td>Triglyceride (mmol/l)</td>
<td>1.8 ± 1.3 (n = 183)</td>
<td>1.6 ± 0.7</td>
<td>1.7 ± 0.9</td>
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<tr>
<td>HDL-C (mmol/l)</td>
<td>1.0 ± 0.3 (n = 183)</td>
<td>1.2 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.3 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>LDL-C (mmol/l)</td>
<td>2.1 ± 0.5 (n = 183)</td>
<td>2.5 ± 0.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.8 ± 1.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>ALT (IU/l)</td>
<td>30–65&lt;sup&gt;a&lt;/sup&gt;</td>
<td>39.8 ± 17.5</td>
<td>36.0 ± 18.6</td>
</tr>
<tr>
<td>AST (IU/l)</td>
<td>15–37&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22.0 ± 14.0</td>
<td>21.0 ± 14.3</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>124.0 ± 8.0 (n = 183)</td>
<td>136.5 ± 19.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>141.0 ± 22.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>83.0 ± 7.0 (n = 183)</td>
<td>79.0 ± 10.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>80.1 ± 12.6</td>
</tr>
<tr>
<td>Diabetes duration (years)</td>
<td></td>
<td>10.4 ± 7.9</td>
<td>14.8 ± 8.6&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Current smoker (yes/no)</td>
<td>43/192</td>
<td>29/142</td>
<td>14/157&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Alcohol intake (yes/no)</td>
<td>70/165</td>
<td>24/147&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18/153&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hypertension (yes/no)</td>
<td>11/224</td>
<td>134/37&lt;sup&gt;b&lt;/sup&gt;</td>
<td>137/34&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data were expressed as mean ± SD. Dichotomous variables are given in absolute numbers and tested with chi-square test. In table, ALT: alanine aminotransferase; AST: aspartate amino transferase; BMI: body mass index; DBP: diastolic blood pressure; DNR: diabetic non-retinopathy; DR: diabetic retinopathy; HbA<sub>1c</sub>: glycated hemoglobin; HDL-C: high density lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol; SBP: systolic blood pressure.

<sup>a</sup> Normal value range provided.
<sup>b</sup> p < 0.05 versus healthy control.
<sup>c</sup> p < 0.05 versus DNR.

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Fig. 1 – PCR-RFLP results of –374 and –429 RAGE gene polymorphisms. Lane 1: 50bp DNA ladder; Lane 2: DNA blank; Lane 3: homozygote –374TT; Lane 4: heterozygote –374TA; Lane 5: homozygote for –374AA; Lane 6: homozygote –429TT; Lane 7: heterozygote for –429TC; Lane 8: homozygote –429CC. Note: digested product of 35 bp DNA fragment is too small to be seen in Lane 3 and 4 on the gel.
frequency of −429C in DNR group (16.1%) was comparable to healthy control group (11.3%). The −429C allele frequency of DR group was not significantly different (p > 0.05) when compared to both healthy control and DNR group. The −374A minor allele frequency also did not differ significantly between the control and DNR (p > 0.05), control and DR (p > 0.05) as well as DNR and DR group (p > 0.05).

4. Discussion

Muller cells (retinal glia) contribute to the regulation of vital features of early retinal vasculature damage such as homeostasis and vascular permeability [27]. Predominant expression of RAGE was discovered in the retinal glia (Muller cells) of rats with diabetic retinopathy compared to non-diabetic rats. Thus, RAGE may act as regulator for diabetic retinopathy [27]. The accumulation of AGE could increase vascular permeability, causing vascular leakage. This will in turn stimulate the production of vascular endothelial growth factor through the AGE–RAGE interaction, leading to neo-vascularisation and angiogenesis which inevitably results in retinopathy [28]. Previously, Hudson et al. had demonstrated that −429T/C and −374T/A polymorphisms increase the transcriptional activity and protein expression of RAGE gene. In addition, the −374A allele also influences the binding affinity of the transcription factor [14]. This finding suggests that the both gene polymorphisms are involved in RAGE gene regulation and thus may influence the pathogenesis of diabetic vascular complication.

In 2003, Globocnik Petrovic et al. found that the −374T/A polymorphism was associated with proliferative retinopathy in a Caucasian population [15]. The study was further supported by Lindholm et al. who reported significantly higher frequency of −374AA and TA genotypes in Caucasians with retinopathy [16]. Ramprasad et al. also showed a modest association of the −374T/A polymorphism in Indian subjects with non-proliferative retinopathy [29]. In the following year, the −374A allele was reported to play an important role in the increased risk of sight threatening retinopathy in Caucasians [30]. Studies carried out in the Asian Chinese population showed contradictory results. JiXiong et al. reported that the −374T/A polymorphism was not associated with retinopathy and Lu and Feng speculated that the −374T/A polymorphism was a potential protective factor for vascular complications [9,31]. Conflicting results were also found in previously reported −429T/C polymorphism studies. Both Hudson et al. and Globocnik Petrovic et al. showed a significant increase of −429C allele in Caucasians with retinopathy especially with proliferative retinopathy whereas JiXiong et al. reported that this polymorphism was not associated with the Asian Chinese retinopathy group [9,14,15].

In this study, we have investigated the association between the RAGE gene promoter polymorphisms at position −429 and −374 and retinopathy in a Malaysian population. We found that both the −429C and −374A minor alleles in DR group were not significantly different when compared to DNR group and healthy control (Table 2). This finding indicated both −429T/C and −374T/A polymorphisms in the RAGE gene promoter region were not associated with type 2 DR patients. Our results are in contrast with a previous study which showed that −429C allele increased the transcriptional activity and protein expression of RAGE gene in vitro [14]. A possible explanation for this could be that the −429C allele may be in linkage disequilibrium with adjacent regions nearby the RAGE gene that are associated with diabetic complications as shown in other previous studies [12,30,32]. In addition, Kankova et al. has recently used an in vitro method to demonstrate that the transcriptional activity of −429C allele increased only in normoglycemia as compared to −429T allele and it did not increase in hyperglycemia [33]. More in vitro studies are required to confirm the variations of RAGE gene expression activity in different metabolic milieu. The lack of association of both −429T/C and −374T/A RAGE gene polymorphisms with Malaysian DR in the present finding might reflect the existence of differences in the pathogenesis of DR in our population compared to other populations as shown in previous studies which investigated similar locus [9,15,16,29,30].

In conclusion, this study showed that −429T/C and −374T/A polymorphisms in the promoter region of RAGE gene were not associated with Malaysian type 2 diabetic retinopathy.

Conflict of interest

The authors declare that they have no conflict of interest.
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