Investigation of SLC2A1 26177A/G gene polymorphism via high resolution melting curve analysis in Malaysian patients with diabetic retinopathy

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ARTICLE INFO

Article history:
Received 9 August 2011
Received in revised form 21 May 2012
Accepted 21 May 2012
Available online 12 July 2012

Keywords:
Diabetic retinopathy
GLUT1
HRM
Polymorphism
SLC2A1

ABSTRACT

Purpose: In this study, we aimed to investigate the possible association between SLC2A1 26177A/G polymorphism and diabetic retinopathy (DR) in Malaysian patients with type 2 diabetes.

Methods: Genomic DNA was extracted from 211 Malaysian type 2 diabetic patients (100 without retinopathy [DNR], 111 with retinopathy) and 165 healthy controls. A high resolution melting assay developed in this study was used to detect SLC2A1 26177A/G polymorphism followed by statistical analysis.

Results: A statistically significant difference in 26177 G minor allele frequency between healthy controls (19.7 %) and total patient group (26.1 %) (p < 0.05, Odd ratio = 1.437, 95% Confidence interval = 1.015–2.035) as well as between healthy controls (19.7 %) and DNR patients (27.5 %) (p < 0.05, Odd ratio = 1.546, 95% Confidence interval = 1.024–2.336) was shown in this study. However, when compared between DR and DNR patients, there was no significant difference (p > 0.05).

Conclusions: This is the first study which shows that SLC2A1 26177G allele is associated with type 2 diabetes in Malaysian population but not with DR.

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1. Introduction

Diabetic retinopathy (DR) is not only a common complication of diabetes due to the blood vessel damage in the retina, but it is also the greatest fear among diabetic related ocular complications as it can lead to blindness and disability (Uthra et al., 2008). To date, many environmental and clinical factors have been proposed to affect the development of DR (Klein, Klein, Moss, Davis, & DeMets, 1984). However, clinical studies on diabetic subjects have revealed a wide variation in responses to the onset and severity of DR which cannot be fully explained by the known clinical risk factors (Liew, Klein, & Wong, 2009). The genetic influence on the DR development has been demonstrated through the findings of racial differences in the prevalence of DR and the existence of familial aggregation of severe DR among the siblings, twins and family member of diabetic patients, independent of known clinical risk factors (Leslie & Pyke, 1982; Rema, Saravanan, Deepa, & Mohan, 2002). A recent study by Roy, Hallman, Fu, Machado, and Hanis (2009) has reported single nucleotide polymorphism in solute carrier family 2 (SLC2A1) gene (rs841846) (involved in glucose transport and metabolism, hypertension, retinal development, neurotransmission and angiogenesis) is significantly associated with severe DR in African–Americans with type 1 diabetes.

SLC2A1 or previously known as facilitated glucose transporter (GLUT1) is a member of a family of sodium-independent glucose transporter proteins (Kumagai, Glasgow, & Partridge, 1994). The gene that encodes for this protein is located on chromosome 1 and is expressed in the tissues possessing barrier functions, including the blood–brain barrier and the blood–retina barrier that contains endothelial cells and non-vascular cells such as glia, ganglion cells and photoreceptors (Roy et al., 2009). Previous study had shown that patients with DR have high expression of GLUT1 in the endothelial cells (Takagi, King, & Aiello, 1998). One of the cardinal signs of DR is the retinal blood vessel leakage due to the functional loss of blood–retinal barrier where GLUT1 is the predominant glucose transporter (Kumagai et al., 1994). Since hyperglycemia is one of the important clinical risk factors for DR (Klein et al., 1984), GLUT1 certainly plays an important role in DR development.

High-resolution melting (HRM) was recently introduced as a simple, inexpensive and rapid tool for genotyping (Liew et al., 2009). The principle of this assay involved the change in fluorescence caused by the release of DNA binding dye from DNA duplex during denaturation by increasing temperature (Erali, Voelkerding, & Wittwer, 2008). To the best of our knowledge, this is the first report in literature on the association study between SLC2A1 gene polymorphism and DR in type 2 diabetes. Therefore we have developed an HRM assay in this study to detect an SLC2A1 26177A/G polymorphism and further investigated the association of this gene polymorphism with DR in Malaysian patients with type 2 diabetes.
2. Materials and methods

2.1. Sample collection

A total of 211 type 2 diabetic patients (100 without retinopathy [DNR], 111 with retinopathy [DR]) and 165 selected age and sex matched healthy controls with no history of diabetes and DR referred by the Diabetic clinic for eye examination at the ophthalmology clinic in the University of Malaya Medical Centre, Malaysia were recruited for this study. Patients with other comorbidities other than diabetes and retinopathy were excluded from this study. Patients with other types of retinopathy (non-diabetic origin) such as hypertensive retinopathy and retinopathy of prematurity were not included in this study. All the patients underwent a complete eye examination that included dilated retinal examination and 7-field stereoscopic Diabetic Retinopathy Study retinal photographs (The Diabetic Retinopathy Study Research Group, 1981). The colour fundus photographs were graded for DR severity in a masked fashion by two independent ophthalmologists at the University of Malaya Eye Research Centre, Malaysia.

Written informed consent was obtained from each subject prior to this study. Three mL of blood was drawn for single-nucleotide polymorphism (SNP) examination from each subject. The modified Early Treatment of DR Study Airlie House classification of DR was used to grade the DR (Early Treatment and of Diabetic Retinopathy Study Airlie House Programme was set as follows: denaturation at 95 °C for 10 s, annealing started in the same real-time PCR system. The melt curve programme was set according to the optimized protocol.

2.2. DNA extraction and polymerase chain reaction (PCR)

Genomic DNA was obtained from the whole blood samples using a standard extraction method as stated previously (Chua et al., 2009; Tan et al., 2010). The gene polymorphism screening was carried out with a newly developed polymerase chain reaction (PCR) amplification-high resolution melting curve (PCR-HRM) protocol.

<table>
<thead>
<tr>
<th>Demography</th>
<th>Ctrl (n=165)</th>
<th>DNR (n=100)</th>
<th>DR (n=111)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (male/female)</td>
<td>97/68</td>
<td>55/45</td>
<td>64/47</td>
</tr>
<tr>
<td>Age (years)</td>
<td>58.1±5.1</td>
<td>59.0±7.6</td>
<td>59.2±9.5</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.4±3.8</td>
<td>27.0±4.0</td>
<td>27.3±5.0</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.5±0.3</td>
<td>7.9±0.9</td>
<td>8.8±2.0</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>3.7±0.6</td>
<td>4.4±1.0*</td>
<td>4.9±1.1*</td>
</tr>
<tr>
<td>Triglyceride (mmol/L)</td>
<td>1.7±1.0</td>
<td>1.6±0.5</td>
<td>1.7±1.3</td>
</tr>
<tr>
<td>HDL-C (mmol/L)</td>
<td>1.0±0.3</td>
<td>1.1±0.1</td>
<td>1.1±0.2</td>
</tr>
<tr>
<td>LDL-C (mmol/L)</td>
<td>2.0±0.8</td>
<td>2.6±0.9*</td>
<td>2.7±1.2*</td>
</tr>
<tr>
<td>HDL-C/LDL-C ratio</td>
<td>0.6±0.1</td>
<td>0.5±0.1*</td>
<td>0.5±0.2*</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>-</td>
<td>36.8±15.5</td>
<td>37.8±20.6</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>-</td>
<td>21.0±14.0</td>
<td>22.3±16.0</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>125.0±9.0</td>
<td>137.5±15.5*</td>
<td>138.5±22.4*</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>82.0±7.0</td>
<td>79.2±10.5*</td>
<td>78.9±13.1*</td>
</tr>
<tr>
<td>Diabetes duration (years)</td>
<td>-</td>
<td>10.0±7.9</td>
<td>15.9±9.1*</td>
</tr>
<tr>
<td>Current smoker (yes/no)</td>
<td>32/133</td>
<td>23/77</td>
<td>21/90</td>
</tr>
<tr>
<td>Alcohol drinker (yes/no)</td>
<td>29/136</td>
<td>17/83</td>
<td>16/95</td>
</tr>
</tbody>
</table>

Data were expressed as mean±SD. Dichotomous variables are given in absolute numbers and tested with chi-square test. In the table, ALT: alanine aminotransferase; AST: aspartate amino transferase; BMI: body mass index; Ctrl: healthy controls; DBP: diastolic blood pressure; DNR: diabetic non-retinopathy; DR: diabetic retinopathy; HbA1c: glycated hemoglobin; HDL-C: high density lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol; SBP: systolic blood pressure; *P<0.05 versus Ctrl; 1P<0.05 versus DNR.

In general, the primer set targeting SLC2A1 intron regions containing rs841846 SNP was designed and checked through using in-silico PCR as described previously (Teh, Chua, & Thong, 2010; Thong, Lai, Teh, & Chua, 2011). Optimization of the PCR was done in a conventional thermal cycler to make sure the newly designed primers are able to produce the desired PCR amplicons prior to the commencement of the actual experiments.

PCR-HRM reaction mixture contained 20 ng of genomic DNA, 0.3 µM of each primer (forward primer 5′GAATAGGACCATCATCAGCCATCA 3′ and reverse primer 5′TGGGCAGACCTCACCTCCT3′), 10 µL of 2× MeltDoctor™ HRM Master Mix (Applied Biosystem Inc., CA, USA) and appropriate amount of PCR grade water in a final volume of 20 µL. The PCR and high resolution melting step were carried out in a 7500 Fast real-time PCR system (Applied Biosystem Inc., CA, USA). The PCR cycling program was set according to the optimized protocol. In brief, the reaction was started with enzyme activation (95 °C for 10 min) and followed by amplification comprising 40 cycles (each cycle consists of denaturation step at 95 °C for 15 s, primer annealing and elongation step at 60 °C for 60 s).

2.3. High resolution melting (HRM) curve analysis

After the PCR amplification step, PCR melt curve/dissociation was started in the same real-time PCR system. The melt curve programme was set as follows: denaturation at 95 °C for 10 s, annealing at 60 °C for 60 s, high resolution melting at 95 °C for 15 s and a final annealing step at 60 °C for 15 s. In this process, the PCR amplicons (118 bp) were allowed to denature and re-anneal before the high resolution melting. The change in fluorescence signal in melting step was monitored real-time. High resolution melting curve profile was then analyzed using HRM analysis software for Windows® version 2.0.1 with fluorescence normalization.

2.4. DNA sequencing

Confirmation of the three genotypes (wild type AA, homozygous mutant GG and heterozygous AG) which served as positive controls was required prior to the screening of all studied samples using this approach. Initially, 20 randomly selected DNA samples were screened with PCR-HRM. PCR amplicons which displayed distinct curve shapes and melting temperature (Tm) were purified using a HiYield™ Gel/PCR DNA Fragment purification kit (RBC Real-biotech, Taiwan) followed by DNA sequencing to confirm the genotypes. Five representative samples from each genotype were sequenced to validate the overall genotyping results obtained by PCR-HRM genotyping method.

2.5. Statistical analysis

The continuous variables were checked for normality prior to the statistical analysis. Unpaired t-test was used for the evaluation of
differences between groups. Comparison of subgroups was performed with ONE-WAY analysis of variance and Tukey’s post hoc test. Dichotomous variables and Hardy–Weinberg equilibrium (HWE) were examined in the healthy controls, DNR and DR patients using chi-squared test with one degree of freedom through online freeware (http://www.oege.org/software/hwe-mr-calc.shtml) (Rodriguez, Gaunt, & Day, 2009). The statistical significance of differences in allele frequencies among the three groups was tested by two-tailed Fishers’ exact test. For each odd ratio, 95% confidence interval (CIs) was calculated. A p-value (p < 0.05) was considered to be statistically significant. GraphPad Prism® for Windows® version 5.02 (GraphPad® Software Inc., CA, U.S.A) was used for all the statistical analyses.

3. Results

The demographic data of healthy controls, DNR and DR patients are listed in Table 1. Both DNR and DR patients had significantly (p < 0.05) higher levels of total cholesterol, low density lipoprotein (LDL-C), glycated haemoglobin (HbA1c) and systolic blood pressure (SBP), lower diastolic blood pressure (DBP) and HDL-C/LDL-C ratio than the healthy controls. The number of hypertensive subjects was significantly higher when compared to the healthy controls. Besides, DR patients had significantly higher levels of HbA1c, total cholesterol and longer diabetes duration when compared to the DNR patients. No significant difference was observed in age, gender, body mass index (BMI), high density lipoprotein (HDL-C), triglyceride, alanine aminotransferase (ALT), aspartate aminotransferase (AST), number of smokers and alcohol drinkers.

Fig. 1 shows the PCR amplification plot of three DNA samples, representing wild type (AA), homozygous mutant (GG) and heterozygous (AG). Genotype distribution and allele frequency of 26177A/G polymorphism in the healthy controls, DNR and DR patients are shown in Table 2. Distribution of the genotype did not deviate from the Hardy–Weinberg equilibrium in any of the group. The genotype and allele frequencies for 26177A/G polymorphism were AA 64.8%, AG 30.9%, GG 4.3% (A 80.3%, G 19.7%) for healthy controls, AA 49.0%, AG 47.0%, GG 4.0% (A 72.5%, G 27.5%) for DNR patients and AA 55.0%, AG 40.5%, GG 4.5% (A 75.2%, G 24.8%) for DR patients. A statistically significant difference in 26177 G minor allele frequency was found between healthy controls (19.7%) and total patient group (26.1%) (p < 0.05, Odd ratio = 1.437, 95% Confidence interval = 1.015–2.035) as well as between healthy controls (19.7%) and DNR patients (27.5%) (p < 0.05, Odd ratio = 1.546, 95% Confidence interval = 1.024–2.336). However, when comparison was made between DR and DNR patients, there was no significant difference (p > 0.05). The results showed that 26177G minor allele has a significant presence in the DNR patients compared to the healthy controls.

4. Discussion

Five distinct isoforms of mammalian GLUT, each possessing different distribution and functional properties have been identified previously (Maher, Vannucci, & Simpson, 1994). GLUT1 is the most widely distributed glucose transporter and is predominantly expressed by both retinal capillary endothelial cells and pigment epithelial cells (Takagi et al., 1998) that maintain the blood–retina barrier. Previous study has shown that the loss of selective permeability in blood–retina barrier of proliferative diabetic retinopathy was associated with the absence of facilitated glucose transport (Kumagai et al., 1994). It is noteworthy that polymorphisms in GLUT1 gene have so far been linked with diabetic nephropathy but not with DR (Tarnow, Grarup, Hansen, Parving, & Pedersen, 2001; Warpeha & Chakravarty, 2003).

However, a recent report by Roy et al. indicated that SLC2A1 (GLUT1) polymorphism rs841846 was associated with progression of DR in African-Americans with type 1 diabetes (Roy et al., 2009). In contrast to the finding by Roy et al., our study showed that rs841846 polymorphism was not associated with DR but 26177G minor allele was significantly present in DNR patients. This contradictory finding could be explained by the differences in genetic background and population history between African-Americans and Malaysians. Besides, the different pathological features between type 1 diabetes (Roy et al., 2009) and type 2 diabetes in this study could also contribute to this contrast finding. This study provides further evidence that GLUT1 polymorphism might contribute to susceptibility to type 2 diabetes (Baroni et al., 1992; Pontiroli et al., 1996; Tao et al., 1995). It had been shown that mouse diabetic gene (db) mutations are in a gene cluster containing GLUT1 gene (Bahary, Leibel, Joseph, & Friedman, 1990). The syntenic region of this gene (db) in human would most probably fall between C1p31 to C1p34 where the GLUT1 gene is present (Pontiroli et al., 1996). However, further analysis is required to identify the causal variants in this region.

The principle behind high resolution melting (HRM) analysis is the assessment of different melting behaviour of PCR DNA amplicon due to base pair changes in the presence of high saturating DNA binding dye (SYTO®9). The new DNA binding dye allows saturation of PCR products, exhibits minimal redistribution during melting and does not inhibit PCR (Erali et al., 2008). As the temperature increases during melting, the double-stranded DNA unwinds to become single-stranded and releases the bound dye, leading to a decrease in fluorescence intensity. The melting temperature (Tm) refers to temperature at which 50% of the DNA is double-stranded and can be estimated from the derivative melt curve (Fig. 2B). In HRM, the unique pattern of melting curve, derivative plot and

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**Fig. 1.** Amplification plot of DNA samples with 26177A/G polymorphism. Wild type AA (red), heterozygous AG (green) and homozygous mutant GG (blue).

**Fig. 2.** Melt curve analysis of 26177A/G polymorphism in sample with wild type AA (red), heterozygous AG (green) and homozygous mutant GG (blue).
difference plot can be used to differentiate the PCR amplicons (Fig. 2). Homozygous genotypes are best differentiated through Tm difference while heterozygous genotype is easily identified by curve shape. Prior to the invention of HRM, many techniques for SNP genotyping which require an additional separation step such as restriction fragment length polymorphism analysis, single-strand conformational polymorphism analysis, single nucleotide extension, oligonucleotide ligation and sequencing were available (Liew et al., 2004). However, this simple close-tube system applied in HRM reduces the risk of contamination. In addition, HRM does not require sample processing and/or separation after PCR. It is also much more cost-effective.

In conclusion, besides the RAGE gene investigation studies on Malaysian patients with DR (Ng et al., 2012a; Ng, Kuppupsumy, Tajunisah, Fong, & Chua, 2012b, Ng et al., 2012c), this is the first study which shows that SLC2A1 26177A/G polymorphism is associated with the development of type 2 diabetes but not with retinopathy in these patients. A replication study in other populations is required to substantiate our findings. The subjects investigated in this study are only limited to diabetic retinopathy patients. The association of this SNP with other types of retinopathy (non-diabetic origin) should be investigated in future. Nevertheless, the HRM assay that we established herein could become a useful technique to speed up the polymorphism screening process and provide important SNP information.

Acknowledgment

This study was supported by University of Malaya Research Grant, RG155-09HTM and University of Malaya Postgraduate Research Fund, PS239/2010A.

References


