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Lack of association between Gly82Ser, 1704G/T and 2184A/G of RAGE gene polymorphisms and retinopathy susceptibility in Malaysian diabetic patients

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ABSTRACT. Diabetic retinopathy is the most common diabetic eye disease, occurring in about 60% of type 2 diabetic patients. Other than known clinical risk factors, the influence of genes has been suggested as part of the development of diabetic retinopathy. We investigated the association of Gly82Ser, 1704G/T and 2184A/G polymorphisms in the RAGE gene with retinopathy in type 2 diabetic patients in Malaysia. Ninety-eight unrelated retinopathy patients and 185 unrelated healthy controls from all over Malaysia were recruited in this study. The allele and genotype frequencies of the three gene polymorphisms were investigated using PCR-RFLP. The allele frequency of the three polymorphisms did not differ significantly between the control and the retinopathy group ($P > 0.05$). Analysis of the frequency of GA+AA, GT+TT and AG+GG in the retinopathy group did not reveal significant differences ($P > 0.05$) compared to

the control group. We conclude that RAGE gene Gly82Ser, 1704G/T and 2184A/G polymorphisms are not associated with retinopathy development in the Malaysian population.

Key words: RAGE; Gene polymorphism; Retinopathy

INTRODUCTION

Diabetic retinopathy (DR) is the most common diabetic eye disease, occurring in about 60% of type 2 diabetic patients (Esteves et al., 2008). It remains the second leading cause of blindness, accounting for 4.8% blindness in the working-age group worldwide (Uthra et al., 2008). Strong evidence suggests that longer duration of diabetes, poor glycemic control, systemic hypertension, and presence of proteinuria are risk factors for developing retinopathy (Klein et al., 1984). However, clinical studies on diabetics have revealed the existence of variations in the onset and severity of DR, which are not fully associated with these known risk factors. Hallman et al. (2005) have reported on familial aggregation of severe DR in type 2 diabetic patients, independent of clinical risk factors. Recently, single nucleotide polymorphisms (SNPs) of several genes were proven to be associated with the progression and severity of DR (Roy et al., 2009). The moderate heritability of proliferative diabetic retinopathy risk (Hietala et al., 2008) suggests the influence of genes on the development of DR.

Receptor for advanced glycation end product (RAGE) is a multiligand member of the immunoglobulin superfamily of cell surface molecules, and its gene is located on chromosome 6p21.3 at the major histocompatibility complex locus in the class III region (Bierhaus et al., 2005; Basta, 2008). *In vivo* and *in vitro* studies have proven that RAGE contributes to the pathogenesis of diabetic microvascular complications (JiXiong et al., 2003; dos Santos et al., 2005). RAGE is regarded as one of the candidate genes involved in the development of DR, since it is expressed by critical tissues such as endothelium, smooth muscle, mesangial cells, and monocytes (Hudson et al., 2001). Advanced glycation end products (AGEs), which are well known to accumulate in retinopathy patients (Salman et al., 2009), are one of the endogenous ligands recognized by RAGE. They are complex, heterogenous molecules formed from non-enzymatic glycation and oxidation of proteins, lipids and nucleic acids (Bierhaus et al., 2005; Basta, 2008). The sustained interaction between AGE and RAGE in retinopathy patients causes a positive feedback loop that enhances the expression of RAGE in the retina. This leads to a plethora of deleterious effects (dos Santos et al., 2005), mainly due to the activation of proinflammatory transcription factor NF-κB (Bierhaus et al., 2005; Kalousova et al., 2005; Basta, 2008). The variants of the RAGE gene could alter the above mentioned pathway of events by changing the expression of RAGE and indirectly affecting disease development.

Kankova et al. (2001) have identified 15 SNPs in the RAGE gene. Among these SNPs, the prevalence of exon polymorphism Gly82Ser and intron polymorphisms 1704G/T and 2184A/G, associated with DR, is high in the human population. To the best of our knowledge, there is no report in the literature on the relationship between Gly82Ser, 1704G/T and 2184A/G polymorphisms in the RAGE gene and DR in Malaysians. Therefore, the aim of this study was to investigate the association of these three RAGE gene polymorphisms with retinopathy in Malaysians with type 2 diabetes.

MATERIAL AND METHODS

Subjects

A total of 98 retinopathy patients (57 men and 41 women) aged 55.4 ± 10.2 years (mean ± SD, range = 34 to 78 years) were recruited in this study from ophthalmology clinics in the University Malaya Medical Centre (UMMC), Malaysia. Patients with type 1 diabetes mellitus (DM) and type 2 DM without retinopathy or with less than one year duration of retinopathy were excluded from the study. Detailed medical and ophthalmologic histories of each patient were recorded. A complete eye examination and 7-field stereoscopic Diabetic Retinopathy Study retinal photography were performed on all patients (The Diabetic Retinopathy Study Research Group, 1981). The color fundus photographs were graded for DR severity in a masked fashion by two independent ophthalmologists at University Malaya Eye Research Centre, Kuala Lumpur. The modified Early Treatment of Diabetic Retinopathy Study Airlie House classification of DR was used to grade the retinopathy into four categories: mild non-proliferative retinopathy (mild NPDR), moderate non-proliferative retinopathy (moderate NPDR), severe non-proliferative retinopathy (severe NPDR) and proliferative retinopathy (PDR) (ETDRS, 1991a,b). Among the retinopathy patients, there were 13 mild NPDR, 41 moderate NPDR, 5 severe NPDR, and 39 PDR. The non-retinopathy group (control) was recruited from Kuala Lumpur and Selangor, Malaysia. It consisted of 185 unrelated healthy subjects from different ethnicities (108 men, 77 women) in the age range of 30 to 58 years. The study was performed in adherence to the principles of the Declaration of Helsinki and approved by the Medical Ethics Committee of the UMMC (Reference No. 744.12). Informed consent was obtained from each individual prior to this study.

Genotyping

Genomic DNA was extracted from 3 mL whole blood sample by the conventional phenol/chloroform extraction method. The three gene polymorphisms were detected using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method, as previously described (Kankova et al., 2001; Kalousova et al., 2007). Gly82Ser (rs2070600) polymorphism in exon 3 of the RAGE gene was amplified with forward primer 5'-GTA AGC GGG GCT CCT GTT GCA-3' and reverse primer 5'-GGC CAA GGC TGG GGT TGA AGG-3'. Forward primer 5'-GGA GCC AGA AGG TGG AGC AGT AG-3' and reverse primer 5'-GTC TCA CCG ATG ATG CTG ATG ATG-3' were used to amplify the intron 7 region containing 1704G/T (rs184003) polymorphism, whereas 2184A/G (rs3134940) polymorphism in the intron 8 region was amplified by forward primer 5'-GGC CTC AGG ACC AGG GAA CCT ACA-3' and reverse primer 5'-TTG GTC AGG CTG GTC TCG AAC TCC-3'. All amplifications were performed in a final volume of 20 μL containing 100 ng genomic DNA and appropriate PCR mixture, as previously described (Chua et al., 2009). Following that, PCR was performed in a thermal cycler according to the conditions described by Chua et al. (2010).

Restriction enzyme digestion

The nucleotide change (guanine to adenine) at position 557 in exon 3 (Gly82Ser)

and adenine to guanine at position 2184 in intron 8 resulted in the formation of *Alu*I restriction site (AG/CT) and *BsmF*I restriction site (GGGAC[N]$_{10}$/…), respectively. The nucleotide change (guanine to thymine) at position 1704 in intron 7 diminished the restriction site of *Bfa*I (C/TAG). Restriction analysis was performed with all PCR products using 5 U restriction nucleases, *Alu*I (MBI Fermentas, Vilnius, Lithuania) for Gly82Ser, *Bfa*I (MBI Fermentas) for 1704G/T and *BsmF*I (MBI Fermentas) for 2184A/G, with overnight incubation at 37°C (20-µL reaction). The digested products were then electrophoresed on a 3% agarose gel stained with ethidium bromide and visualized under UV-light (Figure 1). Digestion with *Alu*I revealed fragments of 181, 67 and 149 bp for mutated minor allele 557A (82Ser), while 248 and 149 bp for wild-type major allele 557G (Gly82). The wild-type major allele 1704G gave fragments of 240, 143 and 42 bp after digestion with *Bfa*I, whereas 240 and 185 bp were detected for mutated minor allele 1704T. Subsequent digestion with *Bsm*I revealed fragments of 174, 92 and 136 bp for mutated minor allele 2184G. Wild-type major allele 2184A showed 266 and 136 bp fragments, since it lacks the restriction site.

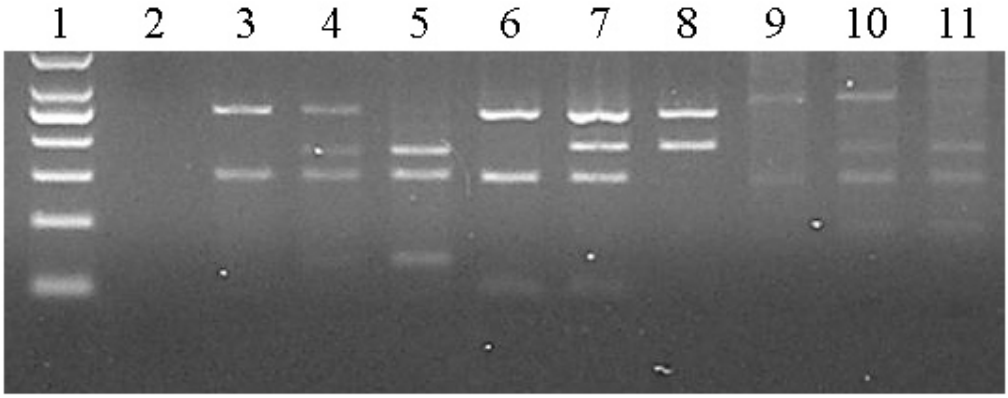

Figure 1. Digested PCR products of 557 exon 3, 1704 intron 7 and 2184 intron 8. *Lane 1* = 50-bp DNA ladder; *lane 2* = DNA blank; *lanes 3-5* = "wild-type" homozygote 557GG (248 and 149 bp), heterozygote 557GA (248, 181, 149, and 67 bp) and "mutated" homozygote 557AA (181, 149 and 67 bp), respectively. *Lanes 6-8* = "wild-type" homozygote 1704GG (240, 143 and 42 bp), heterozygote 1704GT (240, 185, 143, and 42 bp) and "mutated" homozygote 1704TT (240 and 185 bp), respectively. *Lanes 9-11* = "wild-type" homozygote 2184AA (266 and 136 bp), heterozygote 2184AG (266, 174, 136, and 92 bp) and "mutated" homozygote 2184GG (174, 136 and 92 bp), respectively.

Statistical analysis

For the evaluation of the RAGE gene polymorphism, Hardy-Weinberg equilibrium was examined in both the control and retinopathy groups using the chi-squared test with one degree of freedom. The statistical significance of differences in allele and genotype frequencies between the retinopathy and control groups was tested by the two-tailed Fisher exact test. For each odds ratio, 95% confidence intervals were calculated. $P < 0.05$ was considered to be statistically significant. GraphPad Prism® for Windows® version 5.02 (GraphPad® Software Inc., CA, USA) was used for all statistical analyses.

RESULTS

Genotype frequencies for the three polymorphisms in the control and retinopathy groups were in accordance with the Hardy-Weinberg equilibrium. Table 1 shows the allele and genotype frequency distribution for Gly82Ser, 1704G/T and 2184A/G polymorphisms in control and retinopathy groups. The distribution of Gly82Ser polymorphism genotype frequency was the same as the genotype frequency of 2184A/G polymorphism in the control group. The mutated minor genotype, 2184AA, in retinopathy group was not detected in this study. The allele frequency of the three polymorphisms did not differ significantly between the control and retinopathy group ($P > 0.05$). Since the frequencies of the AA genotype in Gly82Ser, TT genotype in 1704G/T and GG genotype in 2184A/G were low, the enrolled subjects were divided into two groups: GG and GA+AA, GG and GT+TT, as well as AA and AG+GG, respectively. However, analysis of the frequencies of GA+AA, GT+TT and AG+GG in the retinopathy group did not differ significantly ($P > 0.05$) compared to the control group.

Table 1. Allele and genotype frequency distribution of three polymorphisms in control and retinopathy groups.

Gly82Ser polymorphism							
Clinical group	GG	GA	AA	GA+AA	% G	% A	HWE (χ^2)
Control (N = 185)	147 (79.5%)	34 (18.4%)	4 (2.1%)	38 (20.5%)	88.6	11.4	1.39
Retinopathy (N = 98)	79 (80.6%)	17 (17.3%)	2 (2.1%)	19 (19.4%)	89.2	10.8	0.85
1704G/T polymorphism							
Clinical group	GG	GT	TT	GT+TT	% G	% T	HWE (χ^2)
Control (N = 185)	121 (65.4%)	57 (30.8%)	7 (3.8%)	64 (34.6%)	80.8	19.2	0.01
Retinopathy (N = 98)	56 (57.1%)	37 (37.8%)	5 (5.1%)	42 (42.9%)	76.0	24.0	0.12
2184A/G polymorphism							
Clinical group	AA	AG	GG	AG+GG	% A	% G	HWE (χ^2)
Control (N = 185)	147 (79.5%)	34 (18.4%)	4 (2.1%)	38 (20.5%)	88.6	11.4	1.39
Retinopathy (N = 98)	73 (74.5%)	25 (25.5%)	0 (0%)	25 (25.5%)	87.2	12.8	2.09

Data are reported as number with percent in parentheses. HWE = Hardy-Weinberg equilibrium; χ^2 = chi-squared value.

DISCUSSION

Previously, several polymorphisms in the RAGE gene have been described as candidates for DR. Polymorphism Gly82Ser occurs in exon 3 of the RAGE gene. It is believed to have a functional impact on the AGE-RAGE interaction, as it occurs at a predicted N-linked glycosylation site in the same immunoglobulin variable domain as the AGE binding site (Hudson et al., 1998). The functional and quantitative impact of the 1704G/T polymorphism occurring at intron 7 on RAGE expression is not yet known. Soluble RAGE (sRAGE) is a naturally occurring inhibitor of signaling pathways induced by RAGE, and it is produced by alternative splicing of RAGE mRNA, which involves regions between intron 7 and 9 (Schlueter et al., 2003). 2184A/G polymorphism in intron 8 of the RAGE gene could hypothetically be involved in the regulation of the sRAGE production.

The present investigation showed the absence of a significant contribution of Gly82Ser, 1704G/T and 2184A/G RAGE gene polymorphisms to DR in Malaysia. To date, there are only two reports on the positive association of RAGE gene polymorphisms with DR in the Asian

population. Kumaramanickavel et al. (2002) reported a significant association of the Gly82Ser RAGE gene polymorphism with DR in Asian Indians. Zhang et al. (2009) recently proved that the Gly82Ser polymorphism was associated with DR and that haplotypes containing 1704G and 82Ser could be a genetic marker for DR in type 2 diabetic Asian Chinese. On the other hand, other reports do not support a significant association of RAGE gene polymorphisms (Gly82Ser, 1704G/T, 2184A/G) with DR in Chinese, Japanese or Caucasian populations (Liu and Xiang, 1999; Yamamoto et al., 2000; Kankova et al., 2002; Yoshioka et al., 2005). Positive association of 82Ser with microvascular dermatoses of diabetic late complications, as well as an influence of 1704G/T and 2184A/G on the level of oxidant stress, were established by Kankova et al. (2001). The lack of association of the RAGE gene polymorphism with DR in the present finding may reflect the heterogeneity in organ susceptibility to late complications of diabetes. A larger scale prospective association cohort study with combination of other related genes is needed to identify the genetic background of diabetic retinopathy.

In conclusion, our results suggest that Gly82Ser, 1704G/T and 2184A/G polymorphisms in the RAGE gene are not involved in the development of retinopathy in the Malaysian population.

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