Relation between Polymorphisms G1704T and G82S of RAGE Gene and Diabetic Retinopathy in Japanese Type 2 Diabetic Patients

Keiji YOSHIOKA, Toshihide YOSHIDA*,**, Yasuto TAKAKURA*, Tsunekazu UMEKAWA*, Akinori KOGURE*, Hitoshi TODA*** and Toshikazu YOSHIKAWA****

Abstract

Objective To clarify whether polymorphisms G1704T and G82S of the RAGE gene were related to diabetic retinopathy, we performed a case-control study in Japanese type 2 diabetic patients.

Patients and Methods Two hundred and sixty-eight patients with type 2 diabetes were examined for polymorphisms G1704T and G82S of the RAGE gene. The genotypes of G1704T and G82S of the RAGE gene were determined with a fluorescent allele-specific DNA primer assay system. Diabetic retinopathy (DR) was diagnosed in a masked manner by independent ophthalmologists using fundus photographs and was classified as non-diabetic retinopathy (NDR), non-proliferative retinopathy (NPDR), and proliferative retinopathy (PDR).

Results The T allele frequency of G1704T and S allele frequency of G82S in patients with DR did not significantly differ from those without retinopathy. There were no differences among the genotypes of G1704T and G82S of the RAGE gene regarding age, duration of diabetes, BMI, HbA1c, blood pressure, and lipids levels.

Conclusion These data suggest that polymorphisms G1704T and G82S of the RAGE gene are not related to DR in Japanese type 2 diabetic patients.

Key words: RAGE gene, single nucleotide polymorphism (SNP), diabetic retinopathy

Introduction

The development of diabetic retinopathy (DR) shows variations among individuals, since genetic factors may contribute to the development of DR as well as metabolic control. Its putative etiology is attributable to prolonged exposure to hyperglycemia leading to formation of advanced glycation end products (AGEs) (1, 2), that act through specific receptors, particularly the receptor for AGEs (RAGE) (3). In diabetes, sustained AGE-RAGE interaction (4, 5) mediates activation and secretion of various cytokines via activation of transcription factors such as nuclear factor-κB (6). Genetic polymorphism in RAGE gene may alter the reactions following the AGE binding to RAGE, and thereby may influence the development of diabetic microvascular complications. In Asian populations, Kumaramanickavel et al (7) reported only positive association of the Gly82Ser polymorphism in the RAGE gene for DR in Indian patients. On the other hand, other reports (8–11) do not support significant linkage between polymorphisms (G82S, G1704T, A2184G, G2245A, -T429C, -T374A) of the RAGE gene and DR in Chinese or European populations. In Japanese, however, the relation between G1704T and G82S polymorphisms of the RAGE gene with DR is not known. We therefore investigated this association in Japanese type 2 diabetic patients.
tients with type 2 diabetes participating in a multi-center research protocol. The inclusion criteria were: age at diagnosis of diabetes ≥30 years, and known duration of diabetes of ≥5 years. Diabetes was diagnosed according to 1999 WHO criteria (12). DR was diagnosed in a masked manner by independent ophthalmologists using fundus photographs and was classified as non-diabetic retinopathy (NDR), non-proliferative retinopathy (NPDR), and proliferative retinopathy (PDR). NDR denotes no signs of diabetic retinopathy; NPDR denotes signs of microaneurysm, intraretinal hemorrhage, exudates, macular edema, venous dilatation, soft exudates, peripheral ischemia on fluorescein angiography, intraretinal microvascular abnormalities, and diffuse intraretinal hemorrhage; and PDR denotes signs of neovascularization at the optic disc, neovascularization elsewhere, vitreous hemorrhage, fibrovascular proliferation, and rubeosis iridis. Control subjects comprised 98 non-diabetic healthy individuals without retinopathy. The patients were treated with diet alone (30 kcal/kg standard body weight per day), with diet in combination of oral hypoglycemic agents or with diet in combination with insulin therapy. The study protocol complied with the appropriate guidelines approved by the governmental agencies on physical and biological containment procedures. It was also approved by the Institutional Ethics Committee, and all patients gave informed consent.

Genotyping

The genomic DNA was extracted from peripheral blood. The genotypes of G1704T of the RAGE gene were determined with a fluorescent allele-specific DNA primer assay system as described elsewhere (13). Briefly, the polymorphic region of the RAGE gene was amplified by the polymerase chain reaction (PCR) with G allele-specific sense primers labeled at the 5′ end with fluorescein isothiocyanate (5′-GG TAGGGTTAGGCTACTxGC3′) or T allele-specific sense primers labeled at the 5′ end with Texas red (5′-GG TAGGGTTAGGCTACTxTC3′) and an antisense primer labeled at the 5′ end with biotin (5′-TTTCCCTCGTTAGC CCTCCTG-3′). The reaction mixture (25 μl) contained 20 ng of DNA, 5 pmol of each primer, 0.2 mmol/l each deoxynucleoside triphosphate, 2.5 mmol/l MgCl2, and 1 U of DNA polymerase (rTaq; Toyobo, Osaka, Japan) in DNA polymerase buffer. For determination of the genotype, amplified DNA was incubated in a solution containing streptavidin-conjugated magnetic beads in plate wells at room temperature, and measured for fluorescence with a microplate reader (Fluoroscan Ascent; Dainippon Pharmaceutical, Osaka, Japan) at excitation and emission wavelengths of 485 and 538 nm, respectively and for fluorescein isothiocyanate at 584 and 612 nm, respectively, for Texas red. The genotypes of G82S of the RAGE gene were amplified by the polymerase chain reaction (PCR) with G allele-specific sense primers labeled at the 5′ end fluorescein isothiocyanate (5′-CTCGTGTTCTTTCCAAxGG-3′) or A allele-specific sense primers labeled at the 5′ end with Texas red (5′-CTCGTG TCTTTCCAAxAG-3′) and an antisense primer labeled at the 5′ end with biotin (5′-ACAGCCGGAAGGAGGAGG-3′). The following procedure was the same as that for G1704T of the RAGE gene.

Statistical analysis

Statistical analysis was performed using StatView version 5.0 (Abacus Concept, Berkeley, CA). Clinical characteristics were analyzed by ANOVA. Genotype and allele frequencies between different groups were assessed by χ² test. A p value of less than 5% was considered significant. Multiple logistic regression analysis was used to determine the independent factors associated with DR.

Results

Genotype G1704T and G82S frequencies of the RAGE gene were in accordance with the Hardy-Weinberg equation in diabetic patients and non-diabetic healthy controls. The T allele frequency of G1704T and the S allele frequency of G82S in diabetic patients were 9.8% and 10.8%, respectively, which were similar to those (T allele of G1704T: 8.7%; S allele of G82S: 17.3%) in non-diabetic healthy controls. Table 1 shows the clinical characteristics in the study groups. The PDR group showed higher age and a longer diabetic duration than the NPDR group. Diabetic duration and glycosylated hemoglobin (HbA1c) level in the PDR group were significantly higher than those in the NDR group. Because the frequencies of the TT genotype in G1704T and SS genotype in G82S were low, we divided the enrolled subjects into two groups: GG and GT + TT, and GG and GS + SS, respectively. Table 2 shows the relation between the cumulative incidence of retinopathy assessed by diabetic duration and G1704T and G82S genotypes of the RAGE gene. Even though the duration of diabetes was under consideration, the frequency of the GT + TT genotype in both NPDR and PDR groups was not significantly different from that in the NDR group. The frequency of the GS + SS genotypes of RAGE G82S in the NPDR and PDR groups was also similar to that in the NDR group. Among the G1704T and G82S genotype groups, age, diabetic duration, BMI, HbA1c, plasma total cholesterol, plasma triglyceride, and HDL-cholesterol levels showed no statistical differences in diabetic subjects (data not shown). In addition, multiple logistic regression analysis identified the disease duration (χ²=4.013, p=0.0466) and HbA1c (χ²=5.662, p=0.0173) as independent risk factors for DR, whereas this was not the case for both RAGE G1704T (χ²=0.146, p=0.7026) and RAGE G82S (χ²=1.662, p=0.1974).

Discussion

There may be a genetic influence on the development of diabetic complications, since familial clustering of diabetic microangiopathy exists (14). Many genetic studies have been reported on the association with the development of DR. For example, proposed genes are angiotensin-converting enzyme...
gene (15, 16), plasminogen activator inhibitor-1 gene (17, 18), tumor necrosis factor gene (19, 20), aldose reductase gene (21, 22), nitric oxide synthase gene (23, 24), vascular endothelial growth factor gene (25, 26), transforming growth factor-beta gene (27), methylenetetrahydrofolate reductase gene (28, 29), fatty acid binding protein 2 gene (30) and so on. However, the linkage of these gene polymorphisms with DR is still controversial because some specific genotypes of genes are associated with a more rapid course of DR, whereas others do not increase the frequency of DR.

AGE is one of the key components causing DR (1, 2). In animal models of diabetes, the role of RAGE (3) and an AGE-RAGE interaction (4, 5) in this process is thought to occur, since the blockade of AGE/RAGE binding by soluble RAGE, a scavenger preventing ligand binding to RAGE, prevents the underlying cellular changes associated with diabetic microvascular dysfunction (31), whereas RAGE-overexpressing mice develop nephropathy more rapidly than wild type mice (32).

Several polymorphisms in the RAGE gene have been described as candidates for DR. Polymorphism G1704T of the RAGE gene occurs in intron, of which functional impact of

gene (15, 16), plasminogen activator inhibitor-1 gene (17, 18), tumor necrosis factor gene (19, 20), aldose reductase gene (21, 22), nitric oxide synthase gene (23, 24), vascular endothelial growth factor gene (25, 26), transforming growth factor-beta gene (27), methylenetetrahydrofolate reductase gene (28, 29), fatty acid binding protein 2 gene (30) and so on. However, the linkage of these gene polymorphisms with DR is still controversial because some specific genotypes of genes are associated with a more rapid course of DR, whereas others do not increase the frequency of DR.

AGE is one of the key components causing DR (1, 2). In animal models of diabetes, the role of RAGE (3) and an AGE-RAGE interaction (4, 5) in this process is thought to occur, since the blockade of AGE/RAGE binding by soluble RAGE, a scavenger preventing ligand binding to RAGE, prevents the underlying cellular changes associated with diabetic microvascular dysfunction (31), whereas RAGE-overexpressing mice develop nephropathy more rapidly than wild type mice (32).

Several polymorphisms in the RAGE gene have been described as candidates for DR. Polymorphism G1704T of the RAGE gene occurs in intron, of which functional impact of

### Table 1. Clinical Characteristics of Diabetic Subjects and Non-diabetic Control Subjects

<table>
<thead>
<tr>
<th></th>
<th>Diabetic subjects (n=268)</th>
<th>Control subjects (n=98)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NDR group (n=189)</td>
<td>NPDR group (n=39)</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>62.0±10.5</td>
<td>59.1±9.4</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>124/65</td>
<td>26/13</td>
</tr>
<tr>
<td>Duration of diabetes (yrs)</td>
<td>13.7±7.1</td>
<td>13.4±6.6</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.2±3.0</td>
<td>23.8±3.4</td>
</tr>
<tr>
<td>HbA₁c (%)</td>
<td>7.3±1.3</td>
<td>7.6±1.4</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>132±16</td>
<td>129±13</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>78±10</td>
<td>77±10</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>205±34</td>
<td>198±31</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>130±88</td>
<td>129±78</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>57±14</td>
<td>58±12</td>
</tr>
</tbody>
</table>

Data are means±SD. SBP: systolic blood pressure, DBP: diastolic blood pressure, NDR: non-diabetic retinopathy, NPDR: non-proliferative retinopathy, PDR: proliferative retinopathy, *p<0.05 versus NDR,

### Table 2. Relation between Accumulative Incidence of Retinopathy Assessed by Diabetic Duration and G1704T and G82S Genotypes of RAGE Gene

<table>
<thead>
<tr>
<th>Duration of diabetes</th>
<th>≤12 years (n=132)</th>
<th>≤22 years (n=233)</th>
<th>≤42 years (n=268)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NDR</td>
<td>NPDR</td>
<td>PDR</td>
</tr>
<tr>
<td>RAGE 1704</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>78%</td>
<td>14.4%</td>
<td>7.6%</td>
</tr>
<tr>
<td>GT+TT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>χ²</td>
<td>0.223</td>
<td></td>
<td>0.8943</td>
</tr>
<tr>
<td>p</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RAGE 82</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>76%</td>
<td>15%</td>
<td>8%</td>
</tr>
<tr>
<td>GS+SS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>χ²</td>
<td>0.372</td>
<td></td>
<td>0.8302</td>
</tr>
<tr>
<td>p</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are n unless otherwise indicated. NDR: non-diabetic retinopathy, NPDR: non-proliferative retinopathy, PDR: proliferative retinopathy.
the intron polymorphisms on RAGE expression is not known as yet. The G82S polymorphism in exon 3 of the RAGE gene occurs in the AGE binding domain (33), and its effect on receptor function has been proposed. In Asian populations, Kumaramanickavel et al (7) reported only a positive association of the Gly82Ser polymorphism in the RAGE gene for DR in Indian patients. On the other hand, other reports (8–11) do not support significant linkage between polymorphisms (G82S, G1704T, A2184G, G2245A, T429C, T374A) of the RAGE gene and DR in Chinese or Caucasian populations. To our knowledge, there is no report in the English language literature on the relation between G1704T and G82S polymorphisms of the RAGE gene with DR in Japanese. In the present study, we identified no significant contribution of polymorphisms both G1704T and G82S of the RAGE gene to DR. In addition, multiple logistic regression analysis revealed that independent risk factors for DR were disease duration and HbA1c, but not polymorphisms G1704T and G82S of the RAGE gene. The present findings are in accordance with other reports (8–10), but are different from the previous report by Kumaramanickavel et al (7). Because the HbA1c levels were much higher in their study (8.9±0.7%) than in our study, the longer exposure of hyperglycemia may have influenced the development of DR in their study and may explain the discrepancy between their study and ours.

Regarding the relation with diabetic nephropathy, Matsunaga-Irie et al (34) have recently shown a weak association of polymorphism G1704T of the RAGE gene on diabetic nephropathy. In their study, the T allelic frequency (26%) of G1704T of the RAGE gene in patients with diabetic nephropathy is much higher than that (9.8%) in our study. On the other hand, Poirier et al (35) showed no linkage of G82S polymorphism with nephropathy. Differences of ethics or study design such as duration of diabetes and glycemic control may explain the different results.

Reactive oxygen species (ROS) are generated by (RAGE)-environment (glycation) interaction through stimulation of membrane-bound NADPH-oxidase (36), and it may contribute to the development of diabetic microangiopathy. This reaction is mainly dependent on environment (duration of exposure to hyperglycemia or hyperglycemia itself), and is secondary on the RAGE gene or other related genes (36). Nonetheless, cautious interpretation is necessary since many previous studies as well as ours are cross-sectional investigations on the association between polymorphisms of the RAGE gene and DR. Further, a prospective association study on the relation between RAGE gene and related genes and DR is needed to clarify how genetic aspects may be involved in the development of DR.

In conclusion, G1704T and G82S polymorphism of the RAGE gene did not contribute to DR in Japanese type 2 diabetic patients.

Acknowledgements: This work was supported by a Grant-in-Aid for Scientific Research (C) (No.14571106) (T. Yoshida) from The Ministry of Education, Culture, Sports, Science and Technology of Japan.

References