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Association study of G1704T and G82S polymorphisms of *RAGE* gene for microalbuminuria in Japanese type 2 diabetic patients

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Abstract

To clarify whether polymorphisms G1704T and G82S of the *RAGE* gene were related to microalbuminuria, we performed a case-control study in Japanese type 2 diabetic patients. Polymorphisms G1704T and G82S of the *RAGE* gene were examined with genomic DNA obtained from 116 type 2 diabetic patients with microalbuminuria (urinary albumin/creatinine ratio between 30 and 300 mg/g of creatinine) (microalbuminuria group), and 232 patients with normoalbuminuria (urinary albumin/creatinine ratio <30 mg/g of creatinine) (normoalbuminuria group). The genotype distribution and T allele frequency of G1704T (9.9%) and S allele frequency of G82S (14.2%) in the microalbuminuria group did not significantly differ from those (T allele frequency, 8.4%; S allele frequency, 12.3%) in the normoalbuminuria group. There were no differences among the genotypes of G1704T and G82S of the *RAGE* gene regarding age, duration of diabetes, body mass index, glycosylated hemoglobin (HbA_{1c}), blood pressure, and serum lipid levels. These data suggest that G1704T and G82S polymorphisms of the *RAGE* gene are not related to microalbuminuria in Japanese type 2 diabetic patients.

1. Introduction

The etiology of diabetic microvascular complications is attributable to prolonged exposure to hyperglycemia leading activation of protein kinase C and formation of advanced glycation end products (AGEs) [1,2], which act through specific receptors, particularly 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 after the AGE binding to RAGE, and thereby may influence the development of diabetic microvascular complications. Several reports [7,8] suggested that polymorphisms in the *RAGE* gene were associated with diabetic retinopathy in type 2 diabetes (T2DM), whereas others [9-12] did not support such significant linkage. With regard to diabetic nephropathy (DN), one report [13] showed an association of *RAGE* polymorphism (−374T/A) with DN whereas another report [14] found no relation in type 1 diabetes. In T2DM, little is known about the association of *RAGE* gene polymorphism with DN although Matsunaga-Irie et al [15] have recently reported a weak relation of the G1704T polymorphism in small sample size of Japanese diabetic patients. We therefore conducted this study to investigate whether *RAGE* gene polymorphism (G1704T and G82S) was related to microalbuminuria in Japanese patients with T2DM.

2. Materials and methods

A case-control study was conducted in 348 Japanese patients with T2DM participating in a multicenter research protocol. Diabetes was diagnosed according to 1999 WHO

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Table 1
Clinical and laboratory characteristics of type 2 diabetic patients in the normoalbuminuria group and microalbuminuria group

	Normoalbuminuria group	Microalbuminuria group	*P*
N	232	116	
Sex (male/female)	148/84	65/51	NS
Age (y)*,**	59.5 ± 10.2	63.4 ± 10.4	<.0012
Known diabetes duration (y)	10.8 ± 8.1	13.3 ± 8.5	<.0080
BMI (kg/m^2)	23.4 ± 3.6	24.1 ± 3.1	NS
SBP (mm Hg)	130 ± 14	134 ± 16	.0279
DBP (mm Hg)**	77 ± 10	79 ± 11	NS
FPG (mg/dL)	143 ± 40	163 ± 52	<.0001
HbA_{1c} (%)**	7.0 ± 1.3	7.7 ± 1.4	<.001
Total cholesterol (mg/dL)*	204 ± 34	208 ± 40	NS
Triglyceride (mg/dL)	121 ± 85	160 ± 201	.0335
Serum creatinine (mg/dL)*,**	0.71 ± 0.20	0.70 ± 0.20	NS
ACR (mg/g of creatinine)	11.4 ± 10.0	68.5 ± 55.2	<.001
Retinopathy (%)	20.7	36.2	<.0001
Medication (diet/OHA/insulin) (%)	10/64/26	3/57/40	.0010

Data are means ± SD. *P* denotes significance between the normoalbuminuria group and microalbuminuria group.
BMI indicates body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; FPG, fasting plasma glucose; OHA, oral hypoglycemic agents.
* Indicates significant difference between sex in the normoalbuminuria group.
** Indicates significant difference between sex in the microalbuminuria group.

criteria [16]. The diagnosis of presence of microalbuminuria was based on repeated (2 separate days) measurements of urinary albumin/creatinine ratio (ACR). After the pretest urination, patients were asked to avoid exercise for 1 hour, and spot urine was collected and measured for albumin by radioimmunoassay. Urinary creatinine concentration was also measured with the modified Jaffe's method. We subdivided the subjects into 2 groups: normoalbuminuria group, composed of 232 patients with normoalbuminuria (ACR <30 mg/g of creatinine), and microalbuminuria group, composed of 116 patients with microalbuminuria (ACR, 30-300 mg/g of creatinine). Patients with macroalbuminuria (>300 mg/g of creatinine) or elevated serum creatinine levels ≥2.0 mg/dL were excluded from the study. Fasting plasma glucose (coefficient of variation [cv], 1.1%), glycosylated hemoglobin (HbA_{1c}) (cv, 1.2%), total cholesterol (cv, 0.5%), triglyceride (cv, 0.7%), and serum creatinine (cv, 1.1%) levels were determined by routine automated laboratory methods. Institutional Ethics Committee approved the study protocol, and all patients gave informed consent. The patients were treated with diet alone (30 kcal/kg standard body weight per day containing 60% carbohydrate and 25% fat), with diet in combination of oral hypoglycemic agents, or with diet in combination with insulin therapy.

2.1. Genotyping

The genomic DNA was extracted from peripheral blood. The genotypes of G1704T of *RAGE* gene were determined with a fluorescent allele-specific DNA primer assay system as described elsewhere [17]. Briefly, the polymorphic region of the *RAGE* gene was amplified by the polymerase chain reaction with G allele–specific sense primers labeled at the 5′ end with fluorescein isothiocyanate (5′ -GGTAGGGTGAACC-ATAACTxGC3′) or T allele–specific sense primers labeled at the 5′ end with Texas red (5′ -GGTAGGGTGAACCATAACTxTC3′) and an antisense primer labeled at the 5′ end with biotin (5′ -TTTCCCTCGTTAGCCCTCTG-3′). The reaction mixture (25 μL) contained 20 ng of DNA, 5 pmol of each primer, 0.2 mmol/L of each deoxynucleoside triphosphate, 2.5 mmol/L $MgCl_2$, 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 the 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, for fluorescein isothiocyanate and of 584 and 612 nm, respectively, for Texas red. The genotypes of G82S of *RAGE* gene were amplified by the polymerase chain reaction with G allele-specific sense primers labeled at the 5′ end fluorescein isothiocyanate (5′ -CTCGTGTCCTTCCAA-AxGG-3′) or A allele-specific sense primers labeled at the 5′ end with Texas red (5′ -CTCGTGTCCTTCCCAAxAG-3′) and an antisense primer labeled at the 5′ end with biotin (5′ -ACAGCCGGAAG-GAAGAGG-3′). The following procedure was same as G1704T of *RAGE* gene.

2.2. Statistical analysis

Statistical analysis was performed using StatView version 5.0 (Abacus Concept, Berkeley, Calif). Comparisons of clinical and laboratory characteristics between the normoalbuminuria group and microalbuminuria group were done by unpaired Student *t* test, or χ^2 test as appropriate, and skewed data were logarithmically transformed before analysis. Genotype distribution in the 2 groups was compared by χ^2 test. A *P* value less than 5% was considered significant. Multiple logistic regression analysis was used to determine the independent factors associated with microalbuminuria. Data were shown as mean ± SD.

3. Results

The microalbuminuria group showed older age, longer diabetic duration, greater levels of fasting plasma glucose,

Table 2
Association analysis of G1704T and G82S polymorphisms of *RAGE* gene for microalbuminuria in Japanese patients with T2DM

	Normoalbuminuria group (n = 232)	Microalbuminuria group (n = 116)	χ^2	*P*
RAGE 1704				
GG	196 (84%)	95 (82%)		
GT + TT	36 (16%)	21 (18%)	0.378	.5389
RAGE 82				
GG	183 (79%)	86 (74%)		
GS + SS	49 (21%)	30 (26%)	0.991	.3196

Data are n unless otherwise indicated.

HbA_{1c}, triglyceride and higher systolic blood pressure, and greater ACR than normoalbuminuria group (Table 1). Proportion of subjects with diabetic retinopathy was significantly higher in the microalbuminuria group than the normoalbuminuria group. Patients with microalbuminuria were more frequently treated with insulin than those with normoalbuminuria. In the normoalbuminuria group, women's age (61.8 ± 7.7 vs 58.2 ± 11.2 years, $P = .009$) and total cholesterol levels (214 ± 31 vs 199 ± 34 mg/dL, $P = .0014$) were significantly higher, and serum creatinine levels (0.6 ± 0.2 vs 0.8 ± 0.2 mg/dL, $P < .0001$) were significantly lower than men's parameters. In the microalbuminuria group, greater HbA_{1c} levels (8.1 ± 1.5 vs 7.4 ± 1.2%, $P = .0089$) and lower serum creatinine levels (0.6 ± 0.2 vs 0.8 ± 0.2 mg/dL, $P < .0001$) were observed in female subjects compared to male subjects. Other parameters did not show significant sex differences (data not shown).

The genotype distributions of *RAGE* G1704T and G82S are shown in Table 2. The genotype frequencies in both groups were in Hardy-Weinberg equilibrium. The frequency of the GG, GT, and TT genotypes of the *RAGE* G1704T gene were 82%, 16%, and 2% in the microalbuminuria group, respectively, and 84%, 14%, and 2% in the normoalbuminuria group, respectively. The allele frequency of the T allele was 9.9% in the microalbuminuria group vs 8.4% in the normoalbuminuria group, respectively. Because the frequency of the TT genotype was low, we divided the enrolled subjects into 2 groups: GG and GT + TT. The frequency of the GT + TT genotype in the microalbuminuria group was not significantly different from that in the normoalbuminuria group. On the other hand, the frequency of the GG, GS, and SS genotypes of the *RAGE* G82ST gene were 74%, 23%, and 3% in the microalbuminuria group, respectively, and 79%, 18%, and 3% in the normoalbuminuria group, respectively. The allele frequency of the S allele was 14.2% in the microalbuminuria group vs 12.3% in the normoalbuminuria group, respectively. The frequency of GS + SS genotypes of *RAGE* G82S in the microalbuminuria group was also similar to that in the normoalbuminuria group. In addition, multiple logistic regression analysis identified the current age (odds ratio [OR], 1.044; 95% confidence interval [CI], 1.014-1.074, $P = .0035$), diabetes duration (OR, 1.015; 95% CI 1.010-1.061, $P = .0113$), HbA_{1c} (OR, 1.419; 95% CI 1.169-1.722, $P = .0004$), and triglyceride (OR 1.004; 95% CI, 1.001-1.008, $P = .0143$) as independent risk factors for microalbuminuria, whereas this was not the case for both *RAGE* G1704T ($P = .7358$) and *RAGE* G82S ($P = .1353$). When analyzed separately in men and women, both *RAGE* G1704T (male, $P = .7023$; female, $P = .6648$) and *RAGE* G82S (male, $P = .1879$; female, $P = .9362$) did not contribute to microalbuminuria in either sex.

4. Discussion

Advanced glycation end products (AGEs) are now recognized as playing a role in the pathogenesis of microvascular and macrovascular complications in diabetes [1,2]. Furthermore, the role for RAGE [3] and AGE-RAGE interaction in this process is now emphasized in animal models of diabetes, because 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 [18], whereas RAGE-overexpressing mice develop DN more rapidly than wild-type mice [19]. As familial clustering of DN exists [20], there may be a genetic influence on the development of diabetic complications. Polymorphisms in *RAGE* gene have been supposed as a candidate for involvement in the pathogenesis of diabetic complications, and a variety of genetic variations in *RAGE* gene have been described [21]. However, the relevance of these alterations with respect to diabetic microvascular complications is still controversial particularly in DN.

In the current study, we found no relation between the 2 polymorphisms (G82S and G1704T) of *RAGE* gene and microalbuminuria that was defined as early DN. There are a few reports on association between G82S polymorphism of *RAGE* gene and diabetic complications such as skin complications [7] and diabetic retinopathy [8], but others did not find such influence of this gene polymorphism on macrovascular [21] and microvascular complications [22]. The G82S polymorphism in exon 3 of the *RAGE* gene occurs in the AGE-binding domain [21], and its effect on receptor function has been supposed. However, it is not involved in a possible genetic modification of susceptibility to microalbuminuria in our study.

Polymorphism G1704T of the *RAGE* gene occurs in intron, of which functional impact of the intron polymorphisms on *RAGE* expression is not known yet. In this study, we identified no significant contribution of polymorphism G1704T of the *RAGE* to microalbuminuria. Furthermore, multiple logistic regression analysis revealed that independent risk factors for microalbuminuria were current age, diabetes duration, HbA_{1c} level, and triglyceride level, but not polymorphisms G1704T and G82S of the *RAGE* gene. Our findings are, however, at conflict with a recent study by Matsunaga-Irie et al [15], showing a weak association of polymorphism G1704T of the *RAGE* gene on DN. The T allelic frequency of G1704T of the *RAGE*

gene in patients with DN in their study (26%) was higher than ours (9.9%). In addition, their sample size was smaller and the duration of disease was longer with higher HbA_{1c} levels than our study. Therefore, longer duration of exposure to hyperglycemia (longer duration of disease and higher HbA_{1c} levels) may influence the development of DN in their study and may explain for the discrepancy between their study and ours.

Other polymorphisms (−374T/A and −429T/C) in the *RAGE* promoter, not examined in this study, have also been reported on correlation with DN in type 1 [13] and T2DM patients [23]. In contrast, other studies found no association of −374T/A with DN [24] and retinopathy [9,11]. Differences of ethics, study design, and type of diabetes may account for the discrepancies of these studies.

Microalbuminuria is considered to be a manifestation of renal injury or as a marker of generalized endothelial dysfunction. Gene (*RAGE*)-environment (glycation) interaction induces the generation of reactive oxygen species through stimulation of membrane-bound NADPH oxidase [25] that is one of the mechanisms involved in the endothelial damage, leading to diabetic microangiopathy. This reaction is mainly dependent on environment (duration of exposure to hyperglycemia or hyperglycemia itself) and secondary on *RAGE* gene or other related genes such as p22phox C242T [15]. Because many previous studies as well as ours were cross-sectional investigations on association between polymorphisms of the *RAGE* gene and diabetic complications, cautious interpretation is necessary. To clarify how genetic aspects may be involved in the more rapid development of DN from microalbuminuria stage, further prospective genomewide association (haplotype) study on the relation between *RAGE* gene and related genes and DN is needed.

In conclusion, G1704T and G82S polymorphism of the *RAGE* gene is not related to microalbuminuria in Japanese patients with T2DM.

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