Polymorphism 2184A/G in the *AGER* gene is not associated with diabetic retinopathy in Han Chinese patients with type 2 diabetes

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Abstract

Objective: To assess the association between the 2184A/G polymorphism in the advanced glycosylation end product-specific receptor (*AGER*) gene and the susceptibility to diabetic retinopathy (DR) in Han Chinese patients with type 2 diabetes mellitus (T2DM).

Methods: This cross-sectional genotyping study included patients with T2DM with and without DR. Genotype and allele frequencies of the 2184A/G polymorphism were detected using polymerase chain reaction–restriction fragment-length polymorphism analysis.

Results: This study included 943 patients with T2DM (285 with DR [DR group] and 658 without DR [NDR group]). There were no significant differences in age, sex, body mass index, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, diastolic blood pressure, glycosylated haemoglobin, fasting blood glucose, postprandial 2-hour blood glucose, and triglycerides between the two groups. The duration of T2DM and systolic blood pressure were significantly increased in the DR group compared with the NDR group. No significant differences were found in allele (A and G) and genotype (AA, AG and GG) frequencies of the 2184A/G polymorphism between the two groups.

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Conclusion: The 2184A/G polymorphism in the *AGER* gene is not associated with DR in Han Chinese patients with T2DM.

Introduction

The increasing prevalence of type 2 diabetes mellitus (T2DM) in China is a cause for concern, with approximately 92.4 million adults aged $\geq$20 years reported to be affected in 2010 according to the Chinese Diabetes Society.[1,2] Diabetic retinopathy (DR) is a common microvascular complication of diabetes mellitus; occurring in approximately 35.7% of patients with T2DM in China and being the principal cause of vision loss and blindness in the adult population.[3]

Hyperglycaemia results in the formation and accumulation of advanced glycation end-products (AGEs) as well as upregulated expression of their cellular receptor (RAGE) in the diabetic vasculature.[4–6] The AGE/RAGE interaction plays an important role in the pathogenesis of diabetic microvascular complications,[7] including retinal capillary degeneration, which is a characteristic of retinopathy in diabetic animal models and patients.[8] Retinal capillaries are the targets of AGE-induced toxicity,[9] including the induction of retinal pericyte apoptosis and the osteoblastic differentiation and calcification of pericytes.[10,11] The AGE/RAGE interaction in retinal microvascular endothelial cells results in cellular activation, including: (i) the activation of nuclear factor-κB;[4] (ii) increased expression of cytokines, such as vascular endothelial growth factor, which is associated with blood–retinal barrier dysfunction;[12] (iii) upregulation of adhesion molecules, such as intercellular adhesion molecule, which mediates retinal capillary leukocyte adherence and inner blood–retinal barrier breakdown;[13] and (iv) the induction of oxidative stress;[6] all of which are involved in the pathogenesis of DR.[14–16] Furthermore, the inhibition of RAGE blocks the development of DR lesions.[15]

Genetic polymorphisms in the advanced glycosylation end product-specific receptor (*AGER*) gene may alter its activity following AGE binding, which in turn might influence the development of diabetic vascular complications. Soluble RAGE (sRAGE) protein is a naturally occurring competitive inhibitor of the pathological effects induced by RAGE; it is produced by alternative splicing of the RAGE mRNA, which involves the regions between intron 7 and 9.[17,18] The 2184A/G polymorphism, which is located on intron 8 of the *AGER* gene, has been postulated to be involved in the regulation of sRAGE production.[18] The 2184A/G polymorphism was reported to be associated with antioxidant status.[19] It was also reported to be associated with diabetes-associated microvascular dermatoses in patients with T2DM.[20]

In light of the strong association between DR and retinal oxidant status and microvascular lesions,[8,9] it can be hypothesized that the 2184A/G polymorphism may contribute to the pathogenesis of DR by

influencing the AGE–sRAGE balance. The aim of this present study was to investigate the association between the 2184A/G polymorphism and DR in Han Chinese patients with T2DM. This information will be important in understanding the influence of ethnic background on the association between *AGER* gene polymorphisms and DR, which might provide a basis for understanding the mechanism by which RAGE contributes to DR.

Patients and methods

Study population

This cross-sectional genotyping study enrolled consecutive Han Chinese patients with T2DM in the Department of Endocrinology and Metabolism, First Affiliated Hospital of Nanchang University, Nanchang, Jiangxi Province, China between June 2010 and December 2012. Family trees were used to ensure their Han Chinese ethnic background. T2DM was diagnosed according to the 2003 American Diabetes Association diagnostic criteria for diabetes,[21] based on a fasting blood glucose (FBG) >7 mmol/l, a causal blood glucose >11.1 mmol/l, or a postprandial 2-h blood glucose (2hBG) >11.1 mmol/l following a 75-g oral glucose tolerance test, or a history of therapy for diabetes. All patients underwent a complete eye examination that included dilated retinal examination and fundus photography or fundus fluorescein angiography using a TRC-50DX Mydriatic Retinal Camera (Topcon Medical Systems, Oakland, NJ, USA), and DR was diagnosed as previously reported.[22] The patients with T2DM were divided into two subgroups: those with retinopathy (DR) and those without retinopathy (NDR). Demographic and clinical data including age, sex, body mass index, duration of T2DM, systolic and diastolic blood pressure were recorded for each study participant.

The exclusion criteria were: (i) those aged <20 or >40 years; (ii) patients with type 1 diabetes mellitus, acute infection, the acute stage of a chronic infectious disease, inflammatory disease, uterine disorders, severe liver or renal disease; (iii) patients with eye diseases, spontaneous miscarriages or embryo transplant failures on more than three occasions; (iv) patients taking drugs or therapies that might affect reproductive or metabolic functions; (v) patients with endometrial thickness <7 mm on the secretory transformation day; (vi) patients who were unable to comply with the study protocol. The reproductive system-related exclusion criteria were necessary because fundus fluorescein angiography is contraindicated in pregnant women.

This study was approved by the Ethics Committee of the First Affiliated Hospital of Nanchang University, Nanchang (no. CDYFY2010029) and written informed consent was obtained from all study participants. This study was performed in adherence to the principles of the Declaration of Helsinki.

Biochemical analyses

Venous blood samples were drawn from each patient by venipuncture after at least an 8-h fast for routine blood biochemical analyses that included total triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), fasting blood glucose, and glycosylated haemoglobin (HbA$_{1c}$). TG (intra- and inter-assay coefficients of variation [CVs] of <8% and <9%, respectively), TC (intra- and inter-assay CVs of <6% and <9%, respectively), HDL-C (intra- and inter-assay CVs of <9% and <11%, respectively), LDL-C (intra- and inter-assay CVs of <12% and <14%, respectively) and fasting blood glucose (intra- and inter-assay CVs of <10% and <12%, respectively) were analysed using an

automated Olympus AU5421 high-volume chemistry immuno analyser (Olympus, Tokyo, Japan) according to the manufacturer's instructions. HbA_{1c} (intra- and inter-assay CVs of $<6\%$ and $<9\%$, respectively) was measured using a Bio-Rad D-10™ haemoglobin analyser (Bio-Rad, Hercules, CA, USA). All biochemical analyses were carried out in triplicate.

Genotyping

For genotyping analysis, genomic DNA was extracted from 5 ml of peripheral blood collected in 1.5 mg/ml ethylenediaminetetra-acetic acid using a DNA isolation kit (Bioteke, Beijing, China) according to the manufacturer's instructions. The genomic DNA was subjected to polymerase chain reaction (PCR) with the following primers: forward primer: 5′-TAATTTCCTGCCCC ATTCTG-3′, reverse primer: 5′-CATCGCA ATCTATGCCTCCT-3′ (Shanghai Sangon Biological Engineering Technology & Service, Shanghai, China). The PCR was performed in a 10 µl reaction volume containing 150 ng of genomic DNA, 0.2 µmol/l of forward and reverse primers, 0.225 U *Taq* DNA polymerase (Nearshore Protein Technology, Shanghai, China), 200 µmol/l of each deoxynucleotide triphosphate (Nearshore Protein Technology), and 2.0 mmol/l Mg^{2+} using an Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The amplification protocol consisted of preliminary denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 20 s, annealing at 58°C for 30 s, and elongation at 72°C for 30 s, followed by a final elongation step at 72°C for 5 min.

For restriction fragment-length polymorphism analysis, the PCR products were digested with *Bsm*F1 (New England Biolabs, Beverly, MA, USA) for 2 h at 65°C. Digestion products were resolved on a 2.5% agarose gel by electrophoresis at 220 V for 30 min. The 2184G minor allele mutation introduces a *Bsm*F1 restriction site into the gene;[23] therefore, diagnostic *Bsm*F1 digestion produced fragments of 160 base pairs (bp) and 236 bp for the mutated minor allele 2184G, while the wild-type major allele 2184A, which does not contain this restriction site, produced a single fragment of 396 bp in length. Four representative samples from each genotype were further sequenced to confirm the overall genotyping results.

Statistical analyses

The sample size in this study was established based on a pilot study, which demonstrated genotype frequencies of 2184AG+GG of 0.20 and 0.17 for the NDR and DR groups, respectively. With a sample size ratio between the NDR and DR groups of 2:1, it was calculated that the total number of patients needed to achieve 80% power with a significance level α (two-sided α error) of 0.05 was 862.

All statistical analyses were performed using the SPSS® statistical package, version 17.0 (SPSS Inc., Chicago, IL, USA) for Windows®. Normally distributed continuous variables are presented as mean ± SD and compared with the independent samples *t*-test. Variables that were not normally distributed are presented as median (interquartile range) and compared with the Mann–Whitney *U*-test. Categorical variables and the Hardy–Weinberg equilibrium were analysed using χ^2-test. Intergroup comparisons of genotype and allele distribution were analysed using Fisher's exact test and χ^2-test, respectively. A two-sided *P*-value <0.05 was considered statistically significant.

Results

A total of 943 patients with T2DM were enrolled in the study, including 285 patients

Table 1. Demographic and clinical characteristics of Han Chinese patients with type 2 diabetes mellitus (T2DM) with or without diabetic retinopathy (DR) who participated in a study to investigate the association between the advanced glycosylation end product-specific receptor (*AGER*) gene polymorphisms and DR.

Characteristic	NDR $n = 658$	DR $n = 285$	Statistical significance[a]
Age, years	60.1 ± 11.9	58.3 ± 10.6	NS
Sex, male/female	359/299	147/138	NS
Duration of T2DM, years	6.8 (2.5–15.6)	9.5 (2.2–16.3)	$P = 0.022$
BMI, kg/m^2	23.48 ± 3.16	23.27 ± 3.17	NS
SBP, mmHg	133.9 ± 18.2	136.6 ± 19.5	$P = 0.039$
DBP, mmHg	81.3 ± 11.1	82.9 ± 11.5	NS
HbA$_{1c}$, %	8.88 ± 2.69	8.86 ± 2.48	NS
FBG, mmol/l	7.61 (6.43–11.22)	8.35 (6.21–11.03)	NS
2hBG, mmol/l	12.90 (9.90–15.63)	12.68 (9.82–12.56)	NS
TG, mmol/l	1.32 (0.90–1.93)	1.30 (0.80–1.86)	NS
TC, mmol/l	4.41 (3.83–5.19)	4.42 (3.87–5.19)	NS
LDL-C, mmol/l	2.70 (2.11–3.20)	2.64 (2.33–3.31)	NS
HDL-C, mmol/l	1.14 (0.93–1.38)	1.19 (0.96–1.43)	NS

Data are presented as mean $\pm$ SD, median (interquartile range) or *n* of patients.

[a]Data presented as mean $\pm$ SD were compared using independent samples *t*-test; data presented as median (interquartile range) were compared using Mann–Whitney *U*-test; and categorical variables were compared using χ^2-test.

NDR, no diabetic retinopathy; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA$_{1c}$, glycosylated haemoglobin; FBG, fasting blood glucose; 2hBG, postprandial 2-h blood glucose; TG, triglycerides; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; NS, no significant between-group difference ($P \geq 0.05$).

in the DR group and 658 in the NDR group, which was an adequate sample size based on the power calculation. The demographic and clinical characteristics for the DR and NDR groups are shown in Table 1. There were no significant differences in age, sex distribution, body mass index, TC, DBP, HbA$_{1c}$, FBG, 2hBG, TG, LDL-C, and HDL-C between the two groups. The duration of T2DM and systolic blood pressure were significantly increased in the DR group compared with the NDR group ($P < 0.05$ for both comparisons).

The genotype frequencies of the 2184A/G polymorphism in the DR and NDR groups were in Hardy–Weinberg equilibrium. Table 2 shows the allele and genotype frequency distribution of the 2184A/G polymorphism in the NDR and DR groups. There were no significant differences in the allele (A and G) and genotype (AA, AG,

and GG) frequencies of the 2184A/G polymorphism between the DR and NDR groups.

Discussion

A number of single nucleotide polymorphisms (SNPs) have been identified in the coding and noncoding regions of the *AGER* gene.[24] Many studies have investigated the association between various SNPs in the *AGER* gene with DR, but the results of these association studies have been inconsistent or even directly conflicting. For example, some studies suggested that the *AGER* polymorphisms −429 T/C, −374 T/A, Gly82Ser, 1704G/T, and 2245G/A are associated with DR,[16,25–30] while other studies, including a previous study from our group, did not support a significant association between these polymorphisms

Table 2. Genotype and allele frequency distribution of the advanced glycosylation end product-specific receptor (*AGER*) gene polymorphism 2184A/G in Han Chinese patients with type 2 diabetes mellitus with or without diabetic retinopathy (DR).

Genotypes and alleles	NDR $n = 658$	DR $n = 285$	χ^2-test
Genotype			1.136
AA	540 (82.1)	240 (84.2)	
AG	109 (16.6)	43 (15.1)	
GG	9 (1.4)	2 (0.7)	
Allele			0.937
A	1189 (90.3)	523 (91.8)	
G	127 (9.7)	47 (8.2)	
HWE, χ^2-test	0.111	0.395	

Data presented as *n* of patients (%) or *n* of alleles (%).
No significant between-group differences ($P \geq 0.05$); genotype and allele distribution were analysed using Fisher's exact test and χ^2-test, respectively.
NDR, no diabetic retinopathy; HWE, Hardy–Weinberg equilibrium.

and DR.[31–37] These studies also suggested that these *AGER* gene polymorphisms have different distributions in different ethnic populations, which may be associated with the different contributions of these mutations against different ethnic backgrounds.[35,36] A significant association between the Gly82Ser polymorphism in the *AGER* gene with DR has been reported in Asian Indians[38,39] and Asian Chinese[29] with T2DM. In contrast, an absence of a significant association between the Gly82Ser, 1704G/T, and 2184A/G *AGER* gene polymorphisms and DR has been reported in Malaysian[32] and Japanese[37] populations. For the 2184A/G polymorphism, a few studies reported that 2184A/G polymorphisms were not associated with DR in patients with T2DM.[31,32,40] Of note, a recent report even showed no association between 2184A/G polymorphisms and DR in a Chinese population.[40] As the diverse nationalities within the very large Chinese population

might bring about complexity in the analysis, this present study performed a cross-sectional analysis to observe the correlation between 2184A/G polymorphisms and DR in a Han Chinese population that is representative of the majority of the Chinese population.

In this present study, there were no statistically significant differences in the genotype and allele frequencies of the 2184A/G polymorphism among the DR and NDR groups, indicating that the 2184A/G polymorphism in the *AGER* gene is not associated with DR in Han Chinese patients with T2DM. The results of this present study were similar to those previously reported.[31,32,40] Two important differences exist between this present study and a previous study that also reported on a Chinese population:[40] (i) this present study exclusively assessed a Han Chinese population, while the previous study evaluated a general Chinese population; and (ii) the present study used polymerase chain reaction–restriction fragment-length polymorphism, while the previous study utilized chip-based matrix-assisted laser desorption ionization time-of-flight mass spectrometry for genotypic analysis. Furthermore, another study reported that the 2184A/G polymorphism in the *AGER* gene was associated with diabetes-associated microvascular dermatoses in patients with T2DM.[20] This finding and that of the present study suggest that there is heterogeneity in the genetic susceptibility to diabetes-associated microvascular complications among different organs.

The present study had some limitations. First, it did not measure the serum levels of RAGE protein, so it was not able to determine if the serum levels of RAGE protein changed concomitantly with the presence of the 2184A/G polymorphism in the *AGER* gene. Secondly, because of the limited sample size, it was not possible to classify the patients with DR into non-proliferative retinopathy and proliferative retinopathy for further subgroup investigations. Thirdly, as this

was a cross-sectional study, it was not possible to investigate the progression of DR. Further prospective studies are necessary to confirm the association between the 2184A/G polymorphism in the *AGER* gene and the serum levels of RAGE protein with the presence of DR in patients with T2DM.

In conclusion, the findings of this present study suggest that the 2184A/G polymorphism in the *AGER* gene is not associated with diabetic retinopathy in Han Chinese patients with type 2 diabetes mellitus.

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Declaration of conflicting interests

The authors declare that there are no conflicts of interest.

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