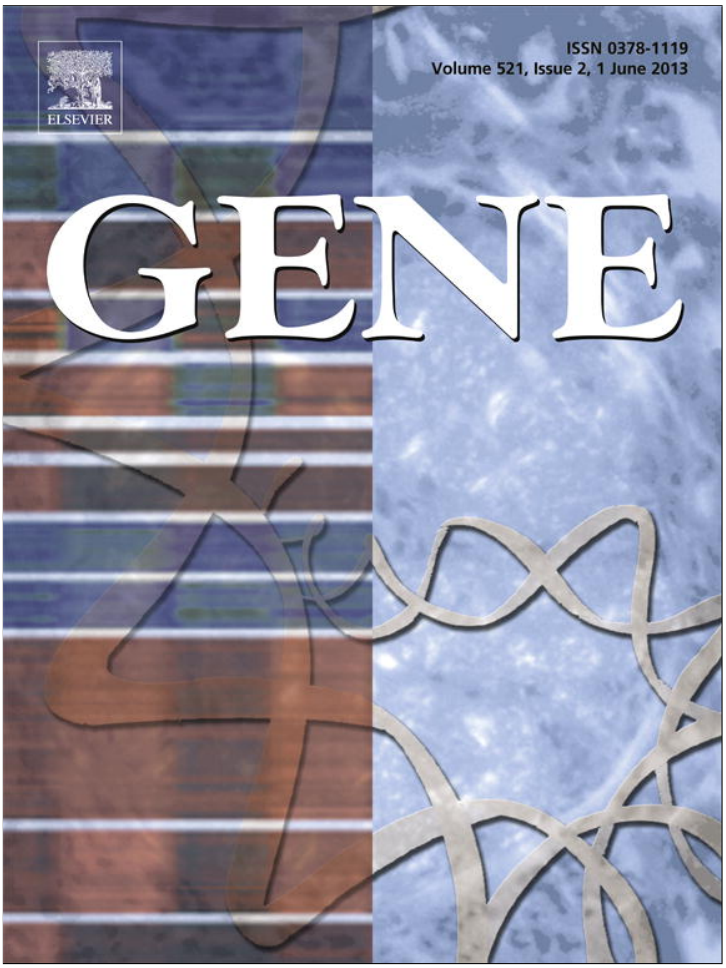

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Receptor for advanced glycation end-product (*RAGE*) gene polymorphism 2245G/A is associated with pro-inflammatory, oxidative-glycation markers and sRAGE in diabetic retinopathy

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ABSTRACT

Background: Receptor for advanced glycation end-product (*RAGE*) gene polymorphism 2245G/A is associated with diabetic retinopathy (DR). However, the mechanism on how it affects the disease development is still unclear.
Aim: This study aims to investigate the relationship between 2245G/A *RAGE* gene polymorphism and selected pro-inflammatory, oxidative-glycation markers in DR patients.
Methods: A total of 371 unrelated type 2 diabetic patients [200 with retinopathy, 171 without retinopathy (DNR)] and 235 healthy subjects were recruited. Genotyping was performed by polymerase chain reaction-restriction fragment length polymorphism method followed by DNA sequencing. The nuclear and cytosolic extracts from peripheral blood mononuclear cells were used for nuclear factor kappa B (NF-κB) p65 and superoxide dismutase activity measurement respectively. Plasma was used for glutathione peroxidase activity, advanced oxidation protein product (AOPP), monocyte chemoattractant protein (MCP)-1, pentosidine and soluble *RAGE* (sRAGE) measurements.
Results: DR patients with 2245GA genotype had significantly elevated levels of activated NF-κB p65, plasma MCP-1, AOPP and pentosidine but lower level of sRAGE when compared to DR patients with wild-type 2245GG.
Conclusion: The *RAGE* gene polymorphism 2245G/A is associated with pro-inflammatory, oxidative-glycation markers and circulating sRAGE in DR patients. Patients with 2245GA *RAGE* genotype could aggravate DR possibly via NF-κB mediated inflammatory pathway.

1. Introduction

Diabetic retinopathy (DR) is a chronic complication of diabetes associated with the presence of microscopic signs of inflammation in retina (Ozturk et al., 2009). It is the second leading cause of vision loss in type 2 diabetic patients (Uthra et al., 2008). The presence of oxidative stress and advanced glycation end products (AGEs) may contribute to the development of DR. AGEs are complex, heterogenous molecules formed from non-enzymatic glycation of protein, lipids and nucleic acids under oxidative stress condition (Basta, 2008). There are increasing evidences that show the involvement of various chemokines and inflammatory cells in the pathogenesis of DR (Ozturk et al., 2009).

Receptor for advanced glycation end product (*RAGE*) plays a role in the pathogenesis of diabetic microvascular complications (Schmidt and Stern, 2000). *RAGE* gene is located on chromosome 6p21.3 at the major histocompatibility complex locus in the class III region which is composed of 11 exons as well as a 3′ untranslated region (Basta, 2008). It is a multiligand member of the immunoglobulin superfamily of cell surface molecules and expressed by critical tissues such as vascular endothelium, epithelium, smooth muscles, monocytes/macrophages, T-lymphocytes, fibroblasts and neuronal cells (Kalea et al., 2009). Its expression is up-regulated in retinopathy patients (Pachydaki et al., 2006). The sustained AGE–*RAGE* interaction in DR could lead to a positive feedback loop that enhances the expression of *RAGE* in the retina.

Abbreviations: AGE, Advanced glycation end-product; ALT, Alanine aminotransferase; AOPP, Advanced oxidation protein product; AST, Aspartate aminotransferase; AU, Arbitrary unit; BMI, Body mass index; DBP, Diastolic blood pressure; DNA, Deoxyribonucleic acid; DNR, Diabetic non-retinopathy; DR, Diabetic retinopathy; ELISA, Enzyme-linked immunosorbent assay; GPx, Glutathione peroxidase; HbA_{1c}, Glycated hemoglobin; HDL-C, High density lipoprotein; HWE, Hardy Weinberg equilibrium; LDL-C, Low density lipoprotein; MCP-1, Monocyte chemoattractant protein-1; NF-κB, Nuclear factor kappa B; NPDR, Non-proliferative diabetic retinopathy; PBMC, Peripheral blood mononuclear cell; PDR, Proliferative diabetic retinopathy; *RAGE*, Receptor for advanced glycation end-product; SBP, Systolic blood pressure; SD, Standard deviation; SNP, Single nucleotide polymorphism; SOD, Superoxide dismutase; sRAGE, Soluble *RAGE*.

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This results in the activation of a pro-inflammatory transcription factor, nuclear factor kappa B (NF-κB) which induces the inflammatory response and causes a series of deleterious effects (Kalousova et al., 2005).

To date, genetic studies have identified an approximate 30 polymorphisms in the *RAGE* gene (Kalea et al., 2009). Previously we have shown that 2245G/A *RAGE* single nucleotide polymorphism (SNP) is associated with DR in Malaysians (Ng et al., 2012b). However, the mechanism on how it affects the disease development is still unclear. We speculate that the genetic polymorphism in the *RAGE* is able to alter the above mentioned biochemical pathways by changing the biological property or the expression of *RAGE* and thereby affect the DR development. Due to the lack of association of other *RAGE* SNPs with DR (Ng et al., 2012a, 2012c), the interest of this study is focused on 2245G/A. In this study, we aimed to investigate the relationship of 2245G/A *RAGE* gene polymorphism with some pro-inflammatory, oxidative-glycation markers, namely the NF-κB p65 transcription factor, monocyte chemoattractant protein-1 (MCP-1), advanced oxidation protein product (AOPP), AGE-pentosidine, soluble *RAGE* (sRAGE) as well as the glutathione peroxidase (GPx) and superoxide dismutase (SOD) activity in the DR patients. To the best of our knowledge, there is no report on the effect of *RAGE* gene polymorphisms on inflammatory markers in DR patients.

2. Materials and methods

2.1. Study population

All the study subjects were the same as reported in Ng et al. (2012b) with the addition of 29 DR patients from University Malaya Medical Centre, Malaysia. All the patients were precisely selected (Fig. 1) prior to carry out this study. Diabetic patients with complications other than retinopathy were excluded from the study. Subjects with previous history of inflammatory-related diseases and those on anti-inflammatory drug treatment and antioxidant supplements were also excluded as these factors would affect the oxidative stress related parameters. A total of 371 unrelated type 2 diabetes patients [171 patients without retinopathy (DNR) and 200 with retinopathy (DR)] (210 men, 161 women) aged 58.2 ± 9.7 years (mean ± SD; range, 40 to 78 years), were recruited in this study. Detailed medical, ophthalmologic histories and socio-demographic factors of each patient were obtained. The healthy controls were blood-donor volunteers, comprising 235 unrelated healthy subjects (134 men, 101 women) aged 57.1 ± 4.1 years (mean ± SD; range, 45 to 65 years). The control subjects and DNR patients were confirmed to be free from any diabetic complication or any other disease by the attending doctors. All the DR patients underwent a complete eye examination that included dilated retinal examination and 7-field stereoscopic Diabetic Retinopathy Study retinal photography (Diabetic retinopathy study, 1981). The color fundus photographs were graded for DR severity in a masked fashion by two independent ophthalmologists at University of Malaya Eye Research Centre, by using the modified Early Treatment of Diabetic Retinopathy Study Airlie House classification (Early Treatment Diabetic Retinopathy Study Research Group, 1991a, 1991b). Among the DR patients, 26 had mild non-proliferative DR (NPDR), 85 had moderate NPDR, 14 had severe NPDR and 75 had proliferative DR. Written informed consent was obtained from each subject prior to blood collection. The study was performed in adherence to the principles of the Declaration of Helsinki and approved by the Medical Ethics Review Committee of University Malaya Medical Centre, Malaysia (IRB reference number: 744.12).

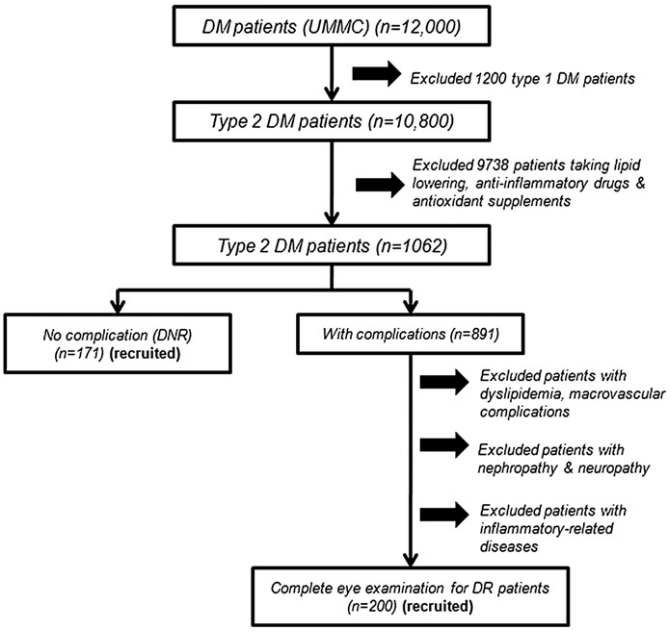

Fig. 1. The inclusion and exclusion criteria for the patients. DM: diabetes mellitus; DNR: diabetic non-retinopathy; DR: diabetic retinopathy; UMMC: University Malaya Medical Centre.

2.2. Sample collection

Nine ml of blood was drawn from patients and control subjects. Three ml of the freshly collected blood was used for the assessment of HbA_{1c} and lipid profile at the Clinical Diagnostic Laboratory at the University Malaya Medical Centre, Malaysia. The whole blood samples (3 ml) in ethylene diamine tetraacetic acid tubes were centrifuged for 15 min at 1000 ×*g*. The plasma was extracted and stored at −80 °C for AGE-pentosidine enzyme-linked immunosorbent assay (ELISA), MCP-1 ELISA, sRAGE ELISA, GPx and AOPP assays. Subsequently, the cell sediments in blood tubes were reconstituted with isotonic phosphate buffered saline solution. The peripheral blood mononuclear cells (PBMC) were isolated as previously described (Boyum, 1968) based on density gradient centrifugation method. Two million PBMCs were disrupted using freeze-thaw method. The cytosolic extract was centrifuged at 14,000 ×*g* for 10 min at 4 °C and the supernatant was used for SOD assay as well as total protein concentration determination as previously described (Bradford, 1976). All assays were performed within 1 month of sample storage.

2.3. Nuclear extraction

The nuclear fraction of isolated PBMC (approximately 4 million cells) was extracted using a commercially available nuclear extraction kit (Cayman Chemical Company, MI, USA), according to the manufacturer's protocol. In brief, PBMCs were first collected in ice-cold phosphate buffer saline in the presence of phosphatase inhibitors which limits dephosphorylation mediated events, namely the transcription factor activation, movement of proteins in or out of the nucleus, proteolysis and new protein expression. The pelleted cells were then resuspended in ice-cold hypotonic buffer to cause cell swelling and this increases cell membrane fragility. Addition of detergent (10% Nonidet P-40) ruptured the cell membranes and released the cytoplasmic fraction while maintaining the integrity of nuclear membrane. The cytoplasmic fraction was separated from the nuclei by brief centrifugation and the pelleted nuclei were lysed in ice-cold extraction buffer containing a mixture of protease and phosphatase inhibitors. The nuclear extract was extracted by centrifugation (14,000 ×*g* for 10 min at 4 °C) and used for NF-κB p65 transcription factor assay.

2.4. Genotyping

The genotype data for the study subjects were obtained from Ng et al. (2012b) with the addition of 29 DR patients in this study. The

2245G/A SNP (rs55640627) was detected using polymerase chain reaction-restriction fragment length polymorphism method as described previously (Ng et al., 2012b). Five representative samples from each genotype were further sequenced to confirm the overall genotyping results.

2.5. Measurement of NF-κB p65 level in PBMC

The level of activated NF-κB p65 in the nucleus of PBMC was measured using a transcription factor assay kit from Cayman Chemical Company, MI, USA. The kit utilized a specific double stranded DNA sequence containing NF-κB response element to specifically bind the activated NF-κB p65 in the nuclear extract. NF-κB p65 was detected by the addition of specific primary antibody directed against NF-κB p65. A secondary antibody conjugated to horseradish peroxidase was added to provide a sensitive colorimetric readout at 450 nm. The nuclear protein concentration was determined as previously described (Bradford, 1976) and the activated NF-κB p65 level was expressed as an arbitrary unit (arbitrary unit [AU]/mg nuclear protein). The inter-assay coefficient of variation was 8%.

2.6. Measurement of PBMC SOD activity

The SOD (E.C. 1.15.1.1) activity in PBMC was measured using a standard kit from Cayman Chemical Company, MI, USA. The assay kit utilized a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. This assay detects all 3 types of SOD (copper/zinc, manganese, and ferrous SOD), with dynamic range of 0.025–0.25 units/ml SOD. Cytosol protein concentration was determined as previously described (Bradford, 1976) and the SOD activity was expressed as U/mg cytosol protein. The inter-assay coefficient of variation was 4.6%.

2.7. Measurement of plasma GPx activity

The plasma GPx (E.C 1.11.1.9) activity was determined using standard kit, EnzyChrom™ GPx, Bioassay Systems, CA, U.S.A. It directly measured nicotinamide-adenine dinucleotide phosphate oxidation in the enzyme coupled reactions. The linear detection range was 12–300 U/L GPx activity. Results were expressed as U/L. The inter-assay coefficient of variation was 5.7%.

2.8. Measurement of plasma AOPP

The plasma level of AOPP was quantitatively determined by using a colorimetric method as described previously (Witko-Sarsat et al., 1996). Chloramine-T was used as the standard and results were expressed as μmol/L. The inter-assay coefficient of variation was 4.6%.

2.9. Measurement of plasma AGE-pentosidine

Plasma AGE-pentosidine was measured with sandwich ELISA standard kit (USCNK Life Science Inc., Wu Han, Republic of China), according to the manufacturer's protocol. The plate was coated with monoclonal antibody against human pentosidine and a polyclonal antibody was used for detection. Mean minimal detectable dose of pentosidine was 0.087 ng/ml. Results were expressed as ng/ml. The inter-assay coefficient of variation was 5.1%.

2.10. Measurement of plasma sRAGE

Plasma sRAGE was measured with sandwich ELISA standard kit (Biovendor Laboratorni medicina a.s., Brno, Czech Republic), according to the manufacturer's protocol. The plate was coated with monoclonal antibody against human sRAGE and a polyclonal antibody was used for detection. Mean minimal detectable dose of sRAGE was 19.2 pg/ml. Results were expressed as pg/ml. The inter-assay coefficient of variation was 3.6%.

2.11. Measurement of plasma MCP-1

Plasma MCP-1 level was quantitatively measured with sandwich ELISA standard kit (Raybiotech® Inc., GA, U.S.A), according to the manufacturer's protocol. The plate was coated with monoclonal antibody specifically against human MCP-1 and a polyclonal antibody conjugated to horseradish peroxidase was used for sensitive colorimetric detection at 450 nm. The mean minimal detectable dose of MCP-1 was typically less than 2 pg/ml. Results were expressed as pg/ml. The inter-assay coefficient of variation was 7.7%.

2.12. Statistical analysis

Hardy–Weinberg equilibrium (HWE) and dichotomous variables were examined using chi-squared test with one degree of freedom. The statistical significance of differences in allele frequencies was tested by two-tailed Fishers' exact test. For each odds ratio, 95% confidence interval (CI) was calculated. 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. Bonferroni correction was applied throughout the analysis. A *p*-value of $p < 0.05$ was considered to be statistically significant. GraphPad Prism® for Windows® version 5.02 (GraphPad® Software Inc., CA, U.S.A) was used for all statistical analyses. Statistical power was calculated using online resource: http://www.stat.ubc.ca/~rollin/stats/ssize/caco.html. With the relative risk of 2.0 for 2245 allele (Ng et al., 2012b), prevalence of DR estimated to be 0.37 (Goh, 2008) and a type 1 error rate of 0.05, the power of the presented results for the 2245G/A SNP was 89% for DNR patients and 93% for DR patients.

3. Results

The demographic data of healthy controls, DNR and DR patients are listed in Table 1. When compared to healthy controls, the patient (DNR and DR) groups had significantly ($p < 0.05$) higher levels of HbA_{1c}, total cholesterol, HDL-C and LDL-C, higher systolic blood pressure (SBP), lower diastolic blood pressure (DBP) and HDL-C/LDL-C ratio. Besides, the number of hypertensive subjects in the patient groups was significantly higher than in the healthy controls. DR patients had longer diabetes duration, lower number of smokers and higher number of subjects on insulin medication when compared to DNR patients. No significant difference was observed in age, gender, body mass index (BMI), levels of triglyceride, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and duration of antihyperlycemic and antihypertensive treatments among all the groups.

Genotype distribution and allele frequency of 2245G/A polymorphism in the studied healthy controls, DNR and DR patients are shown in Table 2. Distribution of the genotype did not deviate from the HWE in any of the groups except in the DR patients. The frequency of 2245A minor allele was significantly higher in DR patients (14.7%) when compared to the healthy controls (5.5%) ($p = 0.0001$) and DNR patients (7.9%) ($p = 0.0032$). The results of various biochemical parameters corresponding to individual genotype for 2245G/A polymorphism in the healthy controls, DNR and DR patients are depicted in Figs. 2–4 respectively. Significant lower plasma GPx activity was detected in DR patients with 2245GA genotype when compared to the patients with 2245GG wild-type genotype (Fig. 4A). Similar phenomenon was also found in the healthy controls (Fig. 2A). In addition, DR patients with 2245GA genotype had significant higher levels of activated NF-κB p65 transcription factor, plasma MCP-1, AGE-pentosidine and AOPP when compared to those with wild-type 2245GG genotype (Figs. 4C–F). However, DR

Table 1
Demographic data of healthy controls, DNR and DR patients.

Demography	Ctrl (n = 235)	DNR (n = 171)	DR (n = 200)
Gender (male/female)	134/101	100/71	110/90
Age (years)	57.1 ± 4.1	59.2 ± 9.6	57.2 ± 9.8
BMI (kg/m^2)	25.6 ± 4.8 (n = 100)	27.2 ± 4.4	26.3 ± 5.0
HbA_{1c} (%)	5.6 ± 0.4 (n = 100)	7.9 ± 1.8 [a]	8.5 ± 2.1 [a]
Total cholesterol (mmol/l)	3.8 ± 0.6 (n = 100)	4.5 ± 1.0 [a]	4.6 ± 1.5 [a]
Triglyceride (mmol/l)	1.6 ± 1.0 (n = 100)	1.7 ± 0.7	1.7 ± 1.0
HDL-C (mmol/l)	1.0 ± 0.3 (n = 100)	1.2 ± 0.3[a]	1.2 ± 0.3[a]
LDL-C (mmol/l)	2.1 ± 0.5 (n = 100)	2.5 ± 0.9[a]	2.8 ± 1.2[a]
HDL-C/LDL-C ratio	0.6 ± 0.2 (n = 100)	0.5 ± 0.2 [a]	0.5 ± 0.2 [a]
ALT (IU/l)	–	37.8 ± 17.5	36.8 ± 24.6
AST (IU/l)	–	22.0 ± 14.0	22.8 ± 16.4
SBP (mm Hg)	124.0 ± 8.0 (n = 100)	136.5 ± 19.5[a]	139.3 ± 22.4[a]
DBP (mm Hg)	83.0 ± 7.0 (n = 100)	79.0 ± 10.5[a]	78.4 ± 13.1[a]
Diabetes duration (years)	–	10.4 ± 5.9	15.7 ± 9.1[b]
Current smoker (yes/no)	43/192	29/142	13/187[a,b]
Alcohol drinker (yes/no)	70/165	24/147[a]	16/184[a]
Hypertension (yes/no)	11/224	104/67[a]	119/81[a]
Antihyperglycemic treatment (years)	–	9.5 ± 5.5	11.5 ± 7.5
Oral antihyperglycemic drugs/insulin	–	107/64	102/98[b]
Antihypertensive treatment (years)	–	7.0 ± 3.5	8.5 ± 4.0
Antihypertensive drugs (yes/no)	–	104/67	119/81

Demographic data of all study subjects were the same as in Ng et al. (2012b) with the addition of 29 DR patients. All DNR and DR patients were on oral antihyperglycemic and insulin medications. Data were expressed as mean ± SD. Dichotomous variables are given in absolute numbers. 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; HbA_{1c}: glycated hemoglobin; HDL-C: high density lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol; SBP: systolic blood pressure.
[a] $p < 0.05$ versus Ctrl.
[b] $p < 0.05$ versus DNR.

patients with 2245GA genotype had significant attenuated level of plasma sRAGE versus those with wild-type 2245GG genotype (Fig. 4G).

4. Discussion

Although the pathogenesis of DR involves multiple hyperglycemia-linked cellular and molecular pathways, the genetic variant of *RAGE* and its interaction with inflammatory markers are the focus of this study. Oxidative stress is reported to play a role in the pathogenesis of DR (Cai and Boulton, 2002). The accelerated formation of AGE and AOPP in oxidative stress is accompanied by the generation of free oxygen radicals and increased retinal endothelial cell permeability. The binding of AGE and AOPP to *RAGE* was shown to be involved in the development of microvascular complications (Hudson et al., 2002; Kankova et al., 2001). The AGE–*RAGE* interaction could induce vascular endothelial growth factor, leading to neovascularisation and angiogenesis commonly seen in DR (Kumaramanickavel et al., 2002).

GPx and SOD are enzymatic antioxidants that protect human tissues (i.e. retinal vascular cells) from excessive oxidative damage. The production of reactive oxygen species under oxidative stress could deplete the enzymatic antioxidants prior to cellular damage (Valko et al., 2007). Previously, 2245A of *RAGE* gene was shown as a risk allele for DR among type 2 diabetic patients (Ng et al., 2012b). In this study, analysis of biochemical parameters corresponding to individual genotype for 2245G/A polymorphism revealed significant lower plasma GPx activity in healthy controls and DR patients with 2245GA genotype when compared to those with wild-type 2245GG genotype. Previous study has revealed several significant *RAGE* genotype-based differences in the circulating non-enzymatic antioxidant level in subjects with diabetes (Kankova et al., 2001). This study provides further evidence that the activity of enzymatic antioxidant was associated with *RAGE* gene polymorphisms. However, the mechanism involved remains to be elucidated. It is feasible to speculate that this polymorphism could affect the receptor-mediated AGE intracellular tissue processing pathway which in turn suppresses the enzymatic antioxidant activity under oxidative stress condition in DR patients.

NF-κB is a "redox-sensitive" nuclear transcription factor which mainly modulates the immune and inflammatory responses. Increased NF-kB expression has been reported in various inflammatory-related diseases (Ho and Bray, 1999). MCP-1 is a chemokine that recruits circulating monocytes to the sites of inflammation (Christiansen et al., 2005). Both NF-κB and MCP-1 were reported to be involved in the pathogenesis of DR (Ng et al., 2013). Interestingly, DR patients with 2245GA genotype showed a significant elevation of activated NF-κB p65 transcription factor, plasma MCP-1, AOPP and AGE-pentosidine levels when compared to DR patients with wild-type 2245GG genotype in this study. NF-κB transcription factor is a key target regulated by *RAGE* signaling (Kalea et al., 2009) and MCP-1 is under the modulation of NF-κB (Christiansen et al., 2005). Previously, −429C and −374A alleles at the promoter of *RAGE* gene were reported to increase the

Table 2
Genotype distribution and allele frequency of 2245G/A polymorphism in healthy controls, DNR and DR patients.

Clinical groups	Genotype distribution			Allele frequency		OR (95% CI)	*P*	HWE
	GG	GA	AA	%G	%A			
Ctrl (n = 235)	209 (88.9)	26 (11.1)	0 (0.0)	94.5	5.5	–	–	0.81
DNR (n = 171)	144 (84.2)	27 (15.8)	0 (0.0)	92.1	7.9	1.46 (0.84–2.56)	0.1392	1.26
DR (n = 200)	141 (70.5)	59 (29.5)	0 (0.0)	85.3	14.7	2.96 (1.82–4.79)	0.0001	5.99
						2.02 (1.25–3.06)[a]	0.0032[a]	

The genotype of the study subjects were the same as in Ng et al. (2012b) with the addition of 29 DR patients. Data are reported as numbers with percent in parentheses, unless otherwise indicated. CI: confidence interval, Ctrl: healthy controls, DNR: diabetic non-retinopathy, DR: diabetic retinopathy, HWE: Hardy–Weinberg equilibrium in chi-squared value, OR: odds ratio, *P*: *p*-value.
[a] Compared with DNR.

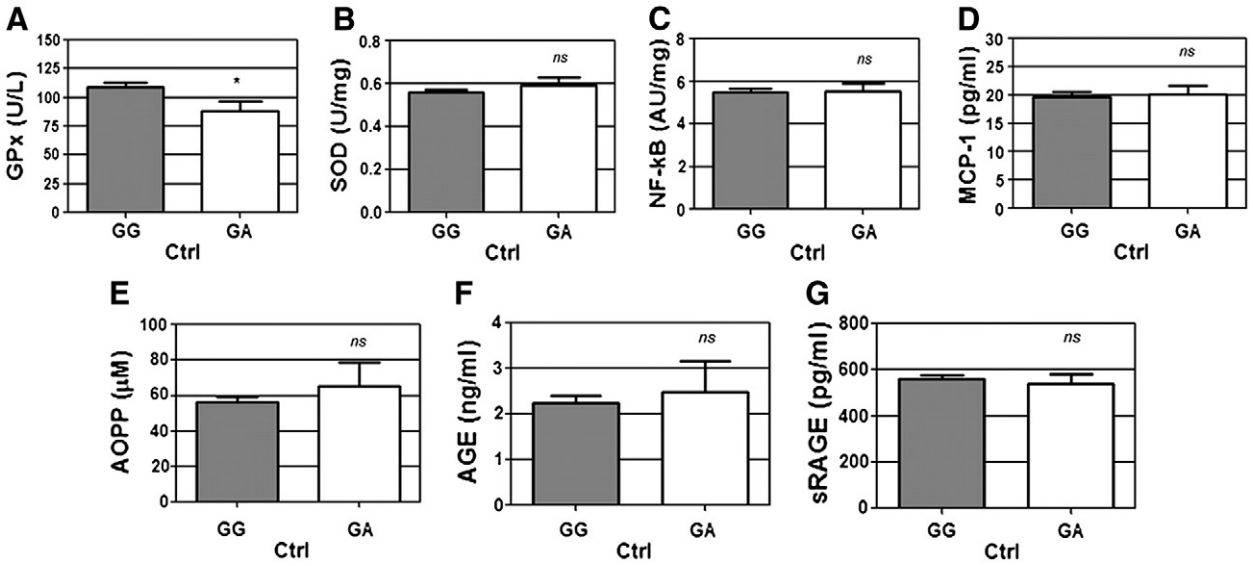

Fig. 2. The biochemical parameters corresponding to 2245G/A individual genotype in the healthy controls (n = 224) (198 for GG and 26 for GA). Only non-hypertensive subjects were included in the analysis. Data are reported as mean ± SD. Unpaired *t*-test with Bonferroni correction (age, gender and metabolic risk factors: BMI, HbA_{1c}, diabetes duration) between homozygote (wild type) and heterozygote in each group of subjects. * indicates $p < 0.05$, ns denotes no significant difference. AGE: advanced glycation end-product (pentosidine); AOPP: advanced oxidation protein product; Ctrl: healthy control; GPx: glutathione peroxidase; MCP-1, monocyte chemoattractant-1; NF-κB: nuclear factor kappa B p65; SOD: superoxide dismutase; sRAGE: soluble form of receptor for AGE.

transcription activity by two and three-fold respectively (Hudson et al., 2001). 2245G/A, an intronic polymorphism of *RAGE* gene, could also affect the transcription sites (acting as enhancer or silencer) and the mRNA stability (Kankova et al., 2001). Thus, the genotype difference in NF-κB and plasma MCP-1 levels observed in this study could possibly be due to the interaction between the promoters and the 2245G/A intron variant in the regulation of transcription activity.

Soluble *RAGE* (sRAGE) is a naturally occurring inhibitor of signaling pathways induced by *RAGE* as it can remove the AGE and AOPP by acting as a decoy and block the AGE–*RAGE* interaction (Schlueter et al., 2003). The inappropriate adherence of leukocytes to the retinal capillaries is a critical event in the pathogenesis of diabetic retinopathy (Moore et al., 2003). Soluble *RAGE* is known to reduce the AGE-induced leukocyte adhesion to endothelial cell monolayer (Moore et al., 2003). Kalousova et al. (2007) reported that a decrease of sRAGE level was associated with complications of diabetes. Soluble *RAGE* is produced by alternative splicing of *RAGE* messenger ribonucleic acid which involves regions between intron 7 and 9 (Moore et al., 2003). The 2245G/A polymorphism (intron 8) could hypothetically be involved in this regulatory process since it lies in the regulatory element binding site within this region. In this study, significant lower level of plasma sRAGE was detected in DR patients with 2245GA genotype versus those with wild-type

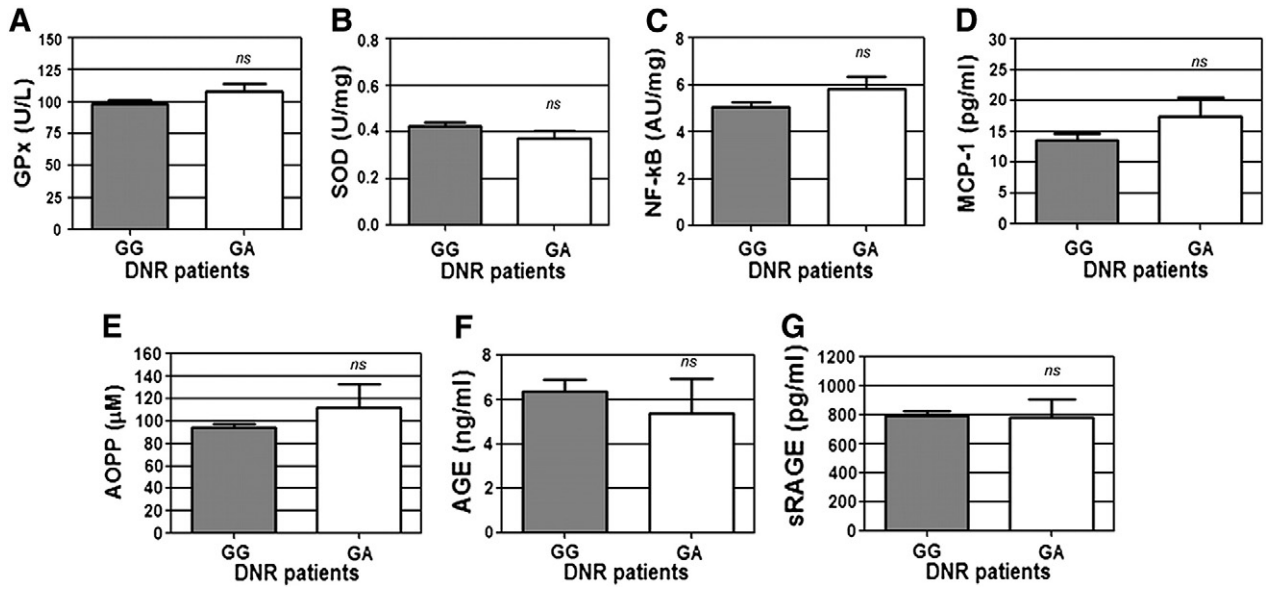

Fig. 3. The biochemical parameters corresponding to 2245G/A individual genotype in DNR patients (n = 67) (42 for GG and 25 for GA). Only non-hypertensive subjects with no history of antihypertensive treatment were included in the analysis. Data are reported as mean ± SD. Unpaired *t*-test with Bonferroni correction (age, gender and metabolic risk factors: BMI, HbA_{1c}, diabetes duration) between homozygote (wild type) and heterozygote in each group of subjects. ns denotes no significant difference. AGE: advanced glycation end-product (pentosidine); AOPP: advanced oxidation protein product; Ctrl: healthy control; DNR: diabetic non-retinopathy; GPx: glutathione peroxidase; MCP-1, monocyte chemoattractant-1; NF-κB: nuclear factor kappa B p65; SOD: superoxide dismutase; sRAGE: soluble form of receptor for AGE.

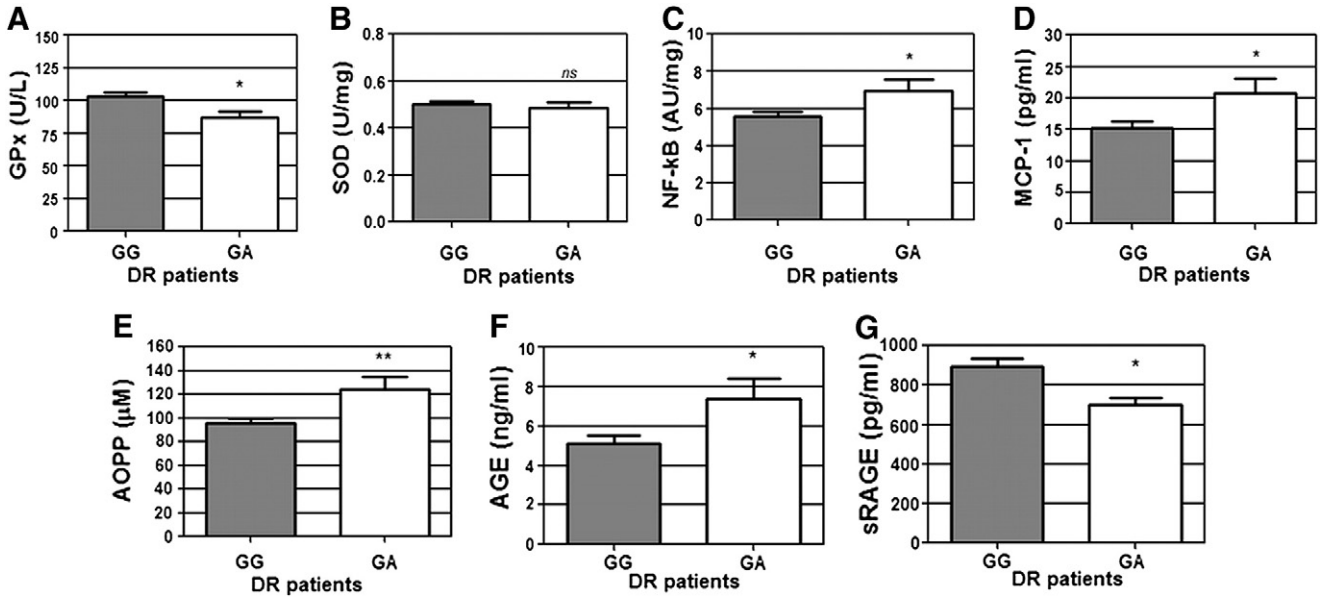

Fig. 4. The biochemical parameters corresponding to 2245G/A individual genotype in DR patients (n = 81) (36 for GG and 45 for GA). Only non-hypertensive subjects with no history of antihypertensive treatment were included in the analysis. Data are reported as mean ± SD. Unpaired *t*-test with Bonferroni correction (age, gender and metabolic risk factors: BMI, HbA_{1c}, diabetes duration) between homozygote (wild type) and heterozygote in each group of subjects. * indicates $p < 0.05$, ** indicates $p < 0.01$, ns denotes no significant difference. AGE: advanced glycation end-product (pentosidine); AOPP: advanced oxidation protein product; DR: diabetic retinopathy; GPx: glutathione peroxidase; MCP-1, monocyte chemoattractant-1; NF-κB: Nuclear factor kappa B p65; SOD: superoxide dismutase; sRAGE: soluble form of receptor for AGE.

2245GG genotype. It should be noted that the significant accumulation of AGE-pentosidine and AOPP in DR patients with 2245GA genotype (Figs. 4E, F) could possibly be due to the low sRAGE level in those patients. Therefore, we hypothesize that individuals with increased 2245A allele frequency could have more AGE–*RAGE* interaction due to low sRAGE level, thus having higher risk of developing DR. However, the possible involvement of some other causative factors (not investigated in this study) that result in linkage disequilibrium should not be ruled out.

5. Conclusion

The *RAGE* gene polymorphism, 2245G/A is associated with oxidative stress, pro-inflammatory (NF-κB and MCP-1) and oxidative-glycation (pentosidine and AOPP) markers as well as the circulating sRAGE in DR patients. Although the development of diabetic late complication such as DR involves a wide range of mechanisms (both environmental and genetic), *RAGE* appears to be one of the pertinent factors. Type 2 diabetic patients with 2245GA *RAGE* genotype could aggravate DR possibly via NF-κB mediated inflammatory pathway. However, a larger scale of prospective association study involving different populations is needed to support and substantiate this study.

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