Association of the receptor for advanced glycation end-products (RAGE) gene polymorphisms in Malaysian patients with chronic kidney disease

Foo Nian Wong, Kek Heng Chua, Umah Rani Kuppusamy, Chew Ming Wong, Soo Kun Lim, Jin Ai Mary Anne Tan

Background. Chronic kidney disease (CKD) is a condition associated with progressive loss of kidney function and kidney damage. The two common causes of CKD are diabetes mellitus and hypertension. Other causes of CKD also include polycystic kidney disease, obstructive uropathy and primary glomerulonephritis. The receptor for advanced glycation end-products (RAGE) is a multi-ligand cell surface receptor of the immunoglobulin superfamily and it has been associated with kidney disease in both non-diabetic and diabetic patients. Presently, data on the association between RAGE polymorphisms and CKD in the Malaysian population is limited, while numerous studies have reported associations of RAGE polymorphisms with diabetic complications in other populations. The present study aims to explore the possibility of using RAGE polymorphisms as candidate markers of CKD in Malaysian population by using association analysis.
Methods. A total of 102 non-diabetic CKD patients, 204 diabetic CKD patients and 345 healthy controls were enrolled in the study. DNA isolated from blood samples were subjected to genotyping of RAGE G82S, -374T/A, -429T/C, 1704G/T and 2184A/G polymorphisms using real-time polymerase chain reaction (PCR). The 63-bp deletion, a polymorphism in the RAGE gene promoter, was genotyped using conventional PCR method and visualized using agarose gel electrophoresis. The collective frequencies of genotypes with at least one copy of the minor alleles of the four polymorphisms were compared between the non-diabetic CKD patients, diabetic CKD patients and healthy controls.
Results. After adjustment of age, gender and ethnic groups in binary logistic regression analysis, the G82S CT + TT genotypes were associated with non-diabetic CKD patients when compared with diabetic CKD patients (p = 0.015, OR = 1.896, 95% CI = 1.132 to 3.176). After further adjustment of CKD comorbidities, the G82S CT + TT genotypes were still associated with non-diabetic CKD patients when compared with diabetic CKD patients (p = 0.011, OR = 2.024, 95% CI = 1.178 to 3.476). However, it cannot be suggested that G82S polymorphism was associated with CKD in non-diabetic patients in this study. This is because there were no significant differences in the frequencies of G82S CT + TT genotypes between non-diabetic CKD patients and healthy controls. In addition, the RAGE

-374T/A, -429T/C, 1704G/T, 2184A/G and 63-bp deletion polymorphisms were also not associated with non-diabetic CKD patients and diabetic CKD patients in this study.
Conclusion. The G82S, -374T/A, -429T/C, 1704G/T, 2184A/G and 63-bp deletion polymorphisms examined in this study were not associated with chronic kidney disease in the Malaysian patients.

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Abstract

Background. Chronic kidney disease (CKD) is a condition associated with progressive loss of kidney function and kidney damage. The two common causes of CKD are diabetes mellitus and hypertension. Other causes of CKD also include polycystic kidney disease, obstructive uropathy and primary glomerulonephritis. The receptor for advanced glycation end-products (RAGE) is a multi-ligand cell surface receptor of the immunoglobulin superfamily and it has been associated with kidney disease in both non-diabetic and diabetic patients. Presently, data on the association between RAGE polymorphisms and CKD in the Malaysian population is limited, while numerous studies have reported associations of RAGE polymorphisms with diabetic complications in other populations. The present study aims to explore the possibility of using RAGE polymorphisms as candidate markers of CKD in Malaysian population by using association analysis.

Methods. A total of 102 non-diabetic CKD patients, 204 diabetic CKD patients and 345 healthy controls were enrolled in the study. DNA isolated from blood samples were subjected to genotyping of RAGE G82S, -374T/A, -429T/C, 1704G/T and 2184A/G polymorphisms using real-time polymerase chain reaction (PCR). The 63-bp deletion, a polymorphism in the RAGE gene promoter, was genotyped using conventional PCR method and visualized using agarose gel electrophoresis. The collective frequencies of genotypes with at least one copy of the minor alleles of the four polymorphisms were compared between the non-diabetic CKD patients, diabetic CKD patients and healthy controls.

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diabetic CKD patients when compared with diabetic CKD patients (p = 0.011, OR = 2.024, 95% CI = 1.178 to 3.476). However, it cannot be suggested that G82S polymorphism was associated with CKD in non-diabetic patients in this study. This is because there were no significant differences in the frequencies of G82S CT + TT genotypes between non-diabetic CKD patients and healthy controls. In addition, the RAGE -374T/A, -429T/C, 1704G/T, 2184A/G and 63-bp deletion polymorphisms were also not associated with non-diabetic CKD patients and diabetic CKD patients in this study.

Conclusion. The G82S, -374T/A, -429T/C, 1704G/T, 2184A/G and 63-bp deletion polymorphisms examined in this study were not associated with chronic kidney disease in the Malaysian patients.

63

64 **Introduction**

65 Chronic kidney disease (CKD) is a general term for heterogeneous renal disorders which is
66 characterized by progressive kidney damage and estimated glomerular filtration rate (eGFR) of
67 less than 60 ml/min/1.73m^2 for three months or more (Levey et al., 2003; Stevens & Levey, 2009).
68 CKD is an increasing health problem in Malaysia. In 2013, the prevalence of kidney failure
69 patients requiring hemodialysis and peritoneal dialysis was 970 per million population (pmp) and
70 95 pmp respectively. In Malaysia, the prevalence of kidney failure has doubled over the last decade
71 (National Renal Registry, 2014). The two common causes of kidney failure in Malaysia are
72 diabetes mellitus and hypertension, with other causes being polycystic kidney disease, obstructive
73 uropathy and chronic glomerulonephritis (National Renal Registry, 2014).

74 Receptor for advanced glycation end-products (RAGE) is a multi-ligand cell surface receptor of
75 the immunoglobulin superfamily. The receptors bind to advanced glycation end-products (AGEs),
76 certain members of S100/calgranulin, amphoterin, amyloid β-sheet fibrils, and advanced oxidation
77 protein products (Kalea, Schmidt & Hudson, 2009; Ramasamy, Yan & Schmidt, 2009). The
78 interaction between RAGE and its ligand triggers signal transduction which results in various
79 cellular effects such as inflammation, oxidative stress, altered gene expression and apoptosis
80 (Kalea, Schmidt & Hudson, 2009; Xie et al., 2013). Furthermore, RAGE has also been studied in
81 association with pathogenesis of kidney diseases in animal models (Myint et al., 2006; Guo et al.,
82 2008; Reiniger et al., 2010).

83 The human RAGE gene is located in the major histocompatibility complex (MHC) class III region
84 on chromosome 6p21.3 (Kalea, Schmidt & Hudson, 2009). Numerous polymorphisms have been

identified in the promoter region, exons and introns of the RAGE gene (Hudson, Stickland & Grant, 1998; Hudson et al., 2001; Kanková et al., 2001). Many studies have reported on the associations of the common RAGE polymorphisms such as G82S, -374T/A, -429T/C, 1704G/T, 2184A/G and 2250G/A with the development of diabetic nephropathy (Matsunaga-Irie et al., 2004; Prevost et al., 2005; Kanková et al., 2005; Lindholm et al., 2006). Moreover, several RAGE polymorphisms have also been investigated in association with lupus nephritis (Martens et al., 2012).

Given the associations of several RAGE polymorphisms with kidney diseases in the published literature, the present study aims to explore the possibility of using RAGE polymorphisms as candidate markers of CKD in Malaysian population by using association analysis. This study investigates the G82S, -374T/A, -429T/C, 1704G/T, 2184A/G and 63-bp deletion polymorphisms based on their effects on RAGE expression and function which potentially affect CKD pathogenesis: (i) G82S polymorphism is a missense mutation in exon 3 of the RAGE gene which potentially regulates RAGE function (Kalea, Schmidt & Hudson, 2009), (ii) the polymorphisms in the transcriptional start site of RAGE such as -374T/A, -429T/C and 63-bp deletion polymorphisms regulate the transcription of RAGE (Hudson et al., 2001), and (iii) the 1704G/T polymorphism and 2184A/G polymorphism may be responsible for alternative splicing that produces endogenous secretory RAGE which is cytoprotective against RAGE ligands (Yonekura et al., 2003; Schlueter et al., 2003).

Materials & Methods

Subject recruitment

CKD patients (40 – 75-year-old) whose eGFR were less than 60 ml/min/1.73m^2 were recruited from University Malaya Medical Centre (UMMC), Kuala Lumpur between September 2013 and November 2014. To further confirm their CKD status, the eGFR of the patients for the past six months were checked to be constantly less than 60 ml/min/1.73m^2. Estimated GFR was determined using the Modified 4-variable Modification of Diet in Renal Disease (MDRD) study equation (Levey et al., 2006). The eGFR was measured on the day of recruitment and the average eGFR of each patient group was calculated. Patients with acute kidney injury which is reversible and kidney transplant recipients whose renal function has reverted to satisfying levels were excluded, as this study aims to investigate kidney diseases which are progressive in nature. Diabetic CKD patients were those with type 2 diabetes (n=204) and non-diabetic CKD patients comprised of patients with hypertension (54), chronic glomerulonephritis (26), obstructive uropathy (9), analgesic nephropathy (7), polycystic kidney disease (4), urate nephropathy (1) and renal tubular acidosis (1). Healthy controls (35 – 65-year-old, n=345) were recruited from blood donors without diabetes, hypertension, heart disease and kidney disease. Peripheral venous blood (3–6 ml) was collected in EDTA tubes for DNA analysis.

This study was approved by the Medical Ethics Committee UMMC (reference number: 982.17) in accordance with the Declaration of Helsinki. Verbal and written informed consent were also obtained from all patients and healthy controls before blood collection.

DNA extraction and genotyping using commercial polymorphism genotyping assays

Blood specimens were kept at -20°C until DNA extraction. DNA was extracted using an in-house modified salting out procedure (Miller, Dykes & Polesky, 1988). Genotyping for the RAGE G82S, -374T/A, -429T/C, 1704 G/T and 2184A/G polymorphisms were performed using the TaqMan® Single Nucleotide Polymorphism (SNP) Genotyping Assays (Life Technologies, USA) (Table 1)

130 according to the manufacturer's instructions. Briefly, 1 µl of diluted DNA sample, 5 µl of 2X
131 TaqMan® GTXpress™ Master Mix (Life Technologies, USA), 0.5 µl of 20X TaqMan® SNP
132 Genotyping Assays and 3.5 µl DNase-free double-distilled water were mixed to obtain a 10 µl
133 SNP genotyping reaction for each DNA sample.

134 Genotypes were determined by real-time polymerase chain reaction (PCR) using Applied
135 Biosystems Fast 7500 Real-Time Thermal Cycler. The pre-PCR stage was 60°C for 1 minute prior
136 to the holding stage at 95°C for 20 seconds. PCR conditions include 40 cycles of denaturation at
137 95°C for 3 seconds followed by annealing and elongation at 60°C for 30 seconds with a final post-
138 PCR stage at 60°C for 1 minute. Results were analyzed using the Applied Biosystems® 7500 Fast
139 Real-Time PCR System and Applied Biosystems® TaqMan® Genotyper Software.

140 *Screening RAGE 63-bp deletion using conventional PCR method*

141 The RAGE 63-bp deletion polymorphism was detected using conventional PCR method. Briefly,
142 10 µl of final PCR reaction mixture contained 1X DreamTaq Buffer (Thermo Scientific), 0.45 U
143 DreamTaq DNA Polymerase (Thermo Scientific), 0.09 mM deoxyribonucleotide triphosphate
144 (dNTP) (Thermo Scientific), 0.22 µM forward primer, 0.22 µM reverse primer and 80 ng sample
145 DNA. The sequences of forward primer and reverse primer are
146 5'- GGGGCAGTTCTCTCCTCACT-3' and 5'-CATGCCTTTGGGACAAGAGT-3' respectively.

147 The PCR was performed with an initial incubation at 94°C for 5 min, followed by 40 cycles
148 consisting of 94°C for 30 s, 63.3°C for 40 s and 72°C for 40 s. One cycle of final extension at 72°C
149 for 5 min was programmed to complete the amplification. The PCR products were visualized using
150 electrophoresis on 1.5% agarose gels.

151 *Statistical analyses*

Hardy-Weinberg equilibrium calculator including analysis for ascertainment bias, a web-tool (http://www.oege.org/software/hwe-mr-calc.shtml), was used to assess Hardy-Weinberg equilibrium (HWE) for each RAGE polymorphism using Chi-squared test to examine the differences in genotype distribution between observed and expected frequencies (Rodriguez, Gaunt & Day, 2009). The significance of HWE deviation was set at $p < 0.05$.

The statistical power of this unmatched case-control study was estimated using Quanto, version 1.2 (Gauderman & Morrison, 2001). In this analysis, the statistical power was calculated for comparing the frequency of genotype with at least one copy of the minor allele of each RAGE polymorphism between a pair of subject groups. The gene only hypothesis and dominance inheritance mode were selected for power calculation.

Chi-squared test was used to detect the significant difference in each categorical variable. The statistical significance of differences in mean values was analyzed using independent *t* test or one-way analysis of variance (ANOVA). Binary logistic regression analysis was used to estimate the probability of CKD development attributed to RAGE polymorphism genotypes by adjusting the covariates such as age, gender, ethnic groups and comorbidities of CKD. Odds ratio and 95% confidence interval were calculated. These statistical analyses were performed using the Statistical Package for Social Sciences, version 20 (SPSS Inc., Chicago, IL). The significance level was set at $p < 0.05$.

Results

The demographic data, eGFR and comorbidities of the study subjects are shown in Table 2. Both non-diabetic CKD ($P_b < 0.001$) (Table 2) and diabetic CKD patients ($P_c < 0.001$) (Table 2) were

174 older compared to the healthy controls while there were no significant differences in categorical
175 variables such as gender and ethnic groups between the study subject groups ($P > 0.05$) (Table 2).
176 Comparison between non-diabetic CKD and diabetic CKD patients showed significant difference
177 in the eGFR levels ($P_a = 0.001$) (Table 2). The CKD patients were also affected with comorbidities
178 such as hypertension, dyslipidemia, ischemic heart disease and stroke. Chi-squared analyses
179 showed that there were significantly more diabetic CKD patients with hypertension ($P_a = 0.043$)
180 (Table 2) and ischemic heart disease ($P_a = 0.002$) (Table 2) than non-diabetic CKD patients.

181 The collective frequencies of genotypes with at least one copy of the minor alleles of G82S, -
182 374T/A, -429T/C, 1704G/T, 2184A/G and 63-bp deletion polymorphisms in the Malaysian CKD
183 patients and healthy controls are shown in Table 3. Genotype distributions of the six
184 polymorphisms in the non-diabetic CKD, diabetic CKD and healthy controls were in HWE except
185 for the non-diabetic CKD patients with -429T/C polymorphism, diabetic CKD patients with G82S
186 and 1704G/T polymorphisms as well as health controls with 2184A/G polymorphism
187 (Supplementary information, Table S1).

188 The frequencies of the genotypes with at least one copy of the G82S T allele (CT + TT genotypes)
189 were significantly higher in the non-diabetic CKD patients than in the diabetic CKD patients
190 (Table 3). Overall, 38.2% of the non-diabetic CKD patients carried at least one copy of the G82S
191 T allele. After adjustment of age, gender and ethnic groups, binary logistic regression analysis
192 indicated that the G82S CT + TT genotypes were associated with non-diabetic CKD patients as
193 compared with diabetic CKD patients ($P = 0.015$, OR = 1.896, 95% CI = 1.132 to 3.176) (Table
194 3, Model 1). Further adjustment of CKD comorbidities showed that the G82S CT + TT genotypes
195 were still associated with non-diabetic CKD patients as compared with diabetic CKD patients (P
196 = 0.011, OR = 2.024, 95% CI = 1.178 to 3.476) (Table 3, Model 2). However, the G82S CT + TT

197 genotypes were not associated with non-diabetic CKD patients and diabetic CKD patients as
198 compared with healthy controls ($P > 0.05$) (Table 3).

199 The genotypes consisting the minor alleles of RAGE -374T/A, -429T/C, 1704G/T, 2184A/G and
200 63-bp deletion polymorphisms were also not associated with non-diabetic CKD patients and
201 diabetic CKD patients ($P > 0.05$) (Table 3). Figure 1 shows the representative gel image of
202 electrophoresed PCR products with or without 63-bp deletion. In this study, the study subjects
203 with 63-bp deletion are heterozygous for the polymorphism and none of the study subjects are
204 homozygous for this particular deletion.

205

206 **Discussion**

207 Published studies have corroborated the role of RAGE in the development of diabetes-associated
208 renal diseases, for example, inhibition of RAGE through pharmacological antagonism or gene
209 deletion showed significant improvements in the pathological features of diabetic nephropathy in
210 animal models (Wendt et al., 2003; Reiniger et al., 2010). In addition to diabetic nephropathy,
211 RAGE is also associated with the pathogenesis of non-diabetic renal diseases, for example,
212 podocyte stress and glomerulosclerosis were elicited in doxorubicin-treated mice, but these
213 features were decreased in the homozygous RAGE-null mice treated with doxorubicin (Guo et al.,
214 2008). In view of the potential pathological role of RAGE in kidney diseases, the associations of
215 RAGE gene polymorphisms with CKD were investigated in this study as the polymorphisms may
216 be fundamentally important in CKD development.

217 In addition to renal diseases, previous human population studies have shown that RAGE
218 polymorphisms were associated with cardiovascular diseases such as ischemic heart disease and

219 stroke (Zee et al., 2006; Poon et al., 2010). In order to investigate accurately the association of
220 RAGE polymorphisms with CKD, the comorbidities such as ischemic heart disease, stroke and
221 their risk factors (hypertension and dyslipidemia) were adjusted in binary logistic regression
222 analysis to eliminate their confounding effects.

223 In the present study, the G82S CT + TT genotypes were associated with non-diabetic CKD patients
224 when compared with diabetic CKD patients, but not with healthy controls. Thus, it cannot be
225 suggested that the T allele is associated with CKD in non-diabetic patients in this study. Studies
226 on the association between G82S polymorphism and kidney disease have showed conflicting
227 results. The frequency of genotype with at least one copy of G82S T allele was significantly higher
228 in type 1 diabetic patients with advanced nephropathy compared with diabetic patients with less
229 severe nephropathy or no nephropathy in France and Belgium (Prevost et al., 2005). On the
230 contrary, this polymorphism was not associated with type 1 diabetic nephropathy and lupus
231 nephritis in Denmark and the Netherlands respectively (Poirier et al., 2001; Martens et al., 2012).

232 The -374T/A, -429T/C, 1704G/T, 2184A/G and 63-bp deletion polymorphisms did not show any
233 association with non-diabetic CKD and diabetic CKD patients in this study. However, published
234 literature have reported contradictory results for these polymorphisms. The -374T/A
235 polymorphism was associated with diabetic complications including diabetic nephropathy in a
236 Swedish population (Lindholm et al., 2006) and has also been implicated in more rapid decline of
237 renal function in Italian CKD patients (Baragetti et al., 2013). In contrast, this polymorphism was
238 protective against albumin excretion and cardiovascular disease in type 1 diabetic Finnish patients
239 (Pettersson-Fernholm et al., 2003).

240 Only limited reports are available on the associations of the RAGE -429T/C, 1704G/T, 2184A/G
241 and 63-bp deletion polymorphisms with kidney diseases. In a linkage disequilibrium analysis, the

frequency of a haplotype containing the -429T/C and 2184A/G polymorphisms were significantly higher in type 2 diabetic nephropathy (Kanková et al., 2005). Furthermore, the 1704 G/T polymorphism showed a significant association with type 2 diabetic patients developing nephropathy in a Japanese population (Matsunaga-Irie et al., 2004). The 2184A/G polymorphism was associated with increased risk for diabetic nephropathy in type 2 diabetic patients from Central Europe (Kaňková et al., 2007) but this polymorphism was reported to play a protective role against diabetic nephropathy in Chinese type 2 diabetic patients (Cai et al., 2015). Another study showed that the type 2 diabetic patients with the 63-bp deletion may be protected from diabetic nephropathy, but the type 1 diabetic patients with 63-bp deletion were at risk of diabetic nephropathy in a German population (Rudofsky et al., 2004). In addition to diabetic nephropathy, the -374T/A, -429 T/C and 2184A/G polymorphisms were also associated with lupus nephritis, worsened proteinuria and decreased renal function in a Dutch population (Martens et al., 2012).

Although the RAGE polymorphisms investigated in this study were not associated with CKD, published studies have demonstrated that some of these polymorphisms are associated with altered expression and function of RAGE which may underlie disease development. Several lines of evidence have suggested the importance of the G82S T allele in the mediation of RAGE-ligand binding and activation of downstream signaling pathways. The G82S T allele renders higher affinity of RAGE towards ligands such as AGEs and S100/calgranulin (Hofmann et al., 2002; Osawa et al., 2007). Another study showed that the RAGE protein with G82S polymorphism promoted *N*-linked glycosylation at the Asparagine 81 site, which was associated with increased ligand binding and pro-inflammatory NF-κB activation (Park, Kleffmann & Hessian, 2011). The deleterious cellular effects resulting from increased ligand binding such as oxidative stress and

inflammation (Kalea, Schmidt & Hudson, 2009) are fundamental to CKD development (Ruiz et al., 2013).

The RAGE -374T/A, -429 T/C and 63-bp deletion polymorphisms which occur in the transcriptional start site of RAGE gene may affect the transcriptional regulation. A published study showed that these polymorphisms resulted in an increase of transcriptional activity (Hudson et al., 2001), indicating an enhanced expression of RAGE which is associated with the progression and severity of renal dysfunction (Wendt et al., 2003; Hou et al., 2004). However, the functional impact of 1704G/T and 2184A/G polymorphisms remain to be elucidated.

Despite the evidence of RAGE polymorphisms associating with kidney diseases, the selected RAGE polymorphisms were not associated with CKD in the present study. A plausible explanation for this discrepancy is the ethnic and regional differences between Malaysian and other populations. Two published reports have provided an example of this concept that the association of 2184A/G polymorphism with type 2 diabetic nephropathy in Central Europe (Kaňková et al., 2007) is contradictory to the decreased risk of type 2 diabetic nephropathy in the Chinese (Cai et al., 2015). This highlights the possible influence of ethno-regional difference on the association between polymorphism and disease. In addition, RAGE polymorphisms may not be CKD-specific in Malaysian patients according to the findings in this study. Given the conflicting findings in the association studies, the relationship between RAGE polymorphisms and kidney diseases should be interpreted with caution.

The main limitation of this study was the subjects who were recruited only from a medical center may not be a representative of the general population. Current sample size determined in *a priori* power analysis could detect significant associations with 80% statistical power. During final analysis, however, most of the comparisons were associated with low statistical power

(Supplementary information, Table S2). The low statistical power could be attributed to the odds ratio which is very close to the null value, small sample size and low frequency of risk allele because statistical power is influenced by effect size, sample size and disease allele frequency (Gordon, 2005; Schneider, 2013). The low statistical power in this study is likely due to the low risk allele frequencies. For example, the risk allele frequencies of several RAGE gene polymorphisms such as -374T/A, -429T/C and 2184A/G are lower in the Malaysian populations compared with the Caucasian populations (Kanková et al., 2005; Kalousová et al., 2007).

Baragetti et al. (2013) demonstrated in a prospective study that the A allele of -374T/A polymorphism was associated with more rapid renal function decline. This suggests that RAGE polymorphisms may affect CKD progression over a longer time span. Therefore, the relationships between RAGE polymorphisms and CKD can be investigated using prospective study design to validate the negative findings in the current study. Furthermore, the polymorphisms of other genes such as transforming growth factor-β1 (TGF- β1), non-muscle myosin heavy chain 9 (MYH9) and apolipoprotein L1 (APOL1) have been shown in associations with kidney diseases (Freedman et al., 2009; Vuong et al., 2009; Langefeld et al., 2015). These candidate genes would serve as promising tools for CKD marker discovery.

Conclusion

Based on the current findings, the RAGE G82S, -374T/A, -429T/C, 1704G/T, 2184A/G and 63-bp deletion polymorphisms are not qualified to be the markers of CKD in Malaysian populations on the account of the null associations between these polymorphisms and CKD. Therefore, it may not be useful to predict CKD using RAGE polymorphisms in Malaysian patients.

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311 **References**

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Table 1. RAGE G82S, -374T/A, -429T/C and 1704G/T polymorphism identification, assay identification and location

Polymorphism	Polymorphism ID	Assay ID *	Location
G82S	rs2070600	C__15867521_20	Chr.6: 32151443
-374T/A	rs1800624	C___3293837_1_	Chr.6: 32152387
-429T/C	rs1800625	C___8848033_1_	Chr.6: 32152442
1704G/T	rs184003	C___2412456_10	Chr.6: 32150296
2184A/G	rs3134940	Custom assay	Chr.6: 32149816

* Assay ID of TaqMan® SNP Genotyping Assay (Life Technologies, USA)

478 **Table 2. Demographic data, estimated glomerular filtration rate and comorbidities of non-diabetic chronic kidney disease**
479 **(CKD) patients, diabetic chronic kidney disease patients and healthy controls**

	Non-diabetic CKD patients n = 102	Diabetic CKD patients n = 204	Healthy controls n = 345	P_a	P_b	P_c
Age (years)	62.98 ± 8.70	64.61 ± 7.44	43.86 ± 6.30	0.141	< 0.001	< 0.001
Gender (male/female)	66/36	129/75	204/141	0.801	0.311	0.341
Ethnic groups (Malay/Indian/Chinese)	33/51/18	84/74/46	127/146/72	0.071	0.387	0.373
eGFR at recruitment (ml/min/1.73m^2)	32.51 ± 14.51	27.04 ± 11.81	Not available	0.001	-	-
Hypertension (%)	73.5	83.3	Not available	0.043	-	-
Dyslipidemia (%)	30.4	26.0	Not available	0.415	-	-
Ischemic heart disease (%)	11.8	27.0	Not available	0.002	-	-
Stroke (%)	3.9	5.9	Not available	0.468	-	-

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481 Data are expressed as mean ± standard deviation, except for categorical variables, which are reported as numbers or percentages. P_a,
482 P-value for non-diabetic CKD patients versus diabetic CKD patients; P_b, P-value for non-diabetic CKD patients versus healthy
483 controls; P_c, P-value for diabetic CKD patients versus healthy controls.

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Table 3. Association of RAGE G82S, -374T/A, -429T/C, 1704G/T, 2184A/G and 63-bp deletion polymorphisms with chronic kidney disease in Malaysian population

Polymorphism	Allele 1/2	Subjects	Genotype 12+22 n (%)	Comparison	Model 1		Model 2	
					P	OR (95% CI)	*P*	OR (95% CI)
G82S	C/T	ND-CKD	39 (38.2)	ND-CKD vs D-CKD	0.015	1.896 (1.132 to 3.176)	0.011	2.024 (1.178 to 3.476)
		D-CKD	51 (25.0)	ND-CKD vs HC	0.749	1.135 (0.522 to 2.467)	0.598	1.440 (0.372 to 5.576)
		HC	90 (26.1)	D-CKD vs HC	0.128	0.533 (0.237 to 1.199)	0.815	1.207 (0.249 to 5.863)
-374T/A	T/A	ND-CKD	22 (21.6)	ND-CKD vs D-CKD	0.644	0.873 (0.490 to 1.554)	0.430	0.787 (0.435 to 1.425)
		D-CKD	48 (23.5)	ND-CKD vs HC	0.408	1.480 (0.585 to 3.743)	0.642	1.451 (0.302 to 6.967)
		HC	83 (24.1)	D-CKD vs HC	0.294	1.556 (0.682 to 3.551)	0.113	4.261 (0.710 to 25.567)
-429T/C	T/C	ND-CKD	16 (15.7)	ND-CKD vs D-CKD	0.134	0.617 (0.328 to 1.160)	0.110	0.588 (0.306 to 1.128)
		D-CKD	46 (22.6)	ND-CKD vs HC	0.735	0.848 (0.326 to 2.205)	0.616	1.533 (0.288 to 8.159)
		HC	81 (23.5)	D-CKD vs HC	0.103	1.948 (0.874 to 4.342)	0.054	5.727 (0.972 to 33.731)
1704G/T	G/T	ND-CKD	42 (41.2)	ND-CKD vs D-CKD	0.284	1.309 (0.799 to 2.143)	0.208	1.385 (0.835 to 2.299)
		D-CKD	70 (34.3)	ND-CKD vs HC	0.619	1.217 (0.562 to 2.636)	0.490	0.604 (0.144 to 2.527)
		HC	125 (36.2)	D-CKD vs HC	0.799	1.100 (0.529 to 2.287)	0.834	0.853 (0.193 to 3.763)
2184A/G	A/G	ND-CKD	16 (15.7)	ND-CKD vs D-CKD	0.134	0.617 (0.328 to 1.160)	0.110	0.588 (0.306 to 1.128)
		D-CKD	46 (22.6)	ND-CKD vs HC	0.725	0.842 (0.324 to 2.189)	0.618	1.530 (0.287 to 8.144)
		HC	82 (23.8)	D-CKD vs HC	0.105	1.941 (0.871 to 4.324)	0.054	5.725 (0.971 to 33.741)
63-bp deletion	_/del	ND-CKD	4 (3.9)	ND-CKD vs D-CKD	0.735	1.244 (0.352 to 4.392)	0.863	1.120 (0.308 to 4.069)
		D-CKD	7 (3.4)	ND-CKD vs HC	0.273	0.303 (0.036 to 2.560)	0.387	0.268 (0.014 to 5.283)
		HC	24 (7.0)	D-CKD vs HC	0.100	0.232 (0.041 to 1.320)	0.423	0.312 (0.018 to 5.393)

Model 1 adjusted for age, gender and ethnic groups. Model 2 extended Model 1 by also adjusting for comorbidities of CKD such as hypertension, dyslipidemia, ischemic heart disease and stroke. Abbreviations in the table – *P*, P-value; OR, odd ratio; CI, confidence interval; ND-CKD, non-diabetic CKD; D-CKD, diabetic CKD; HC, healthy control.

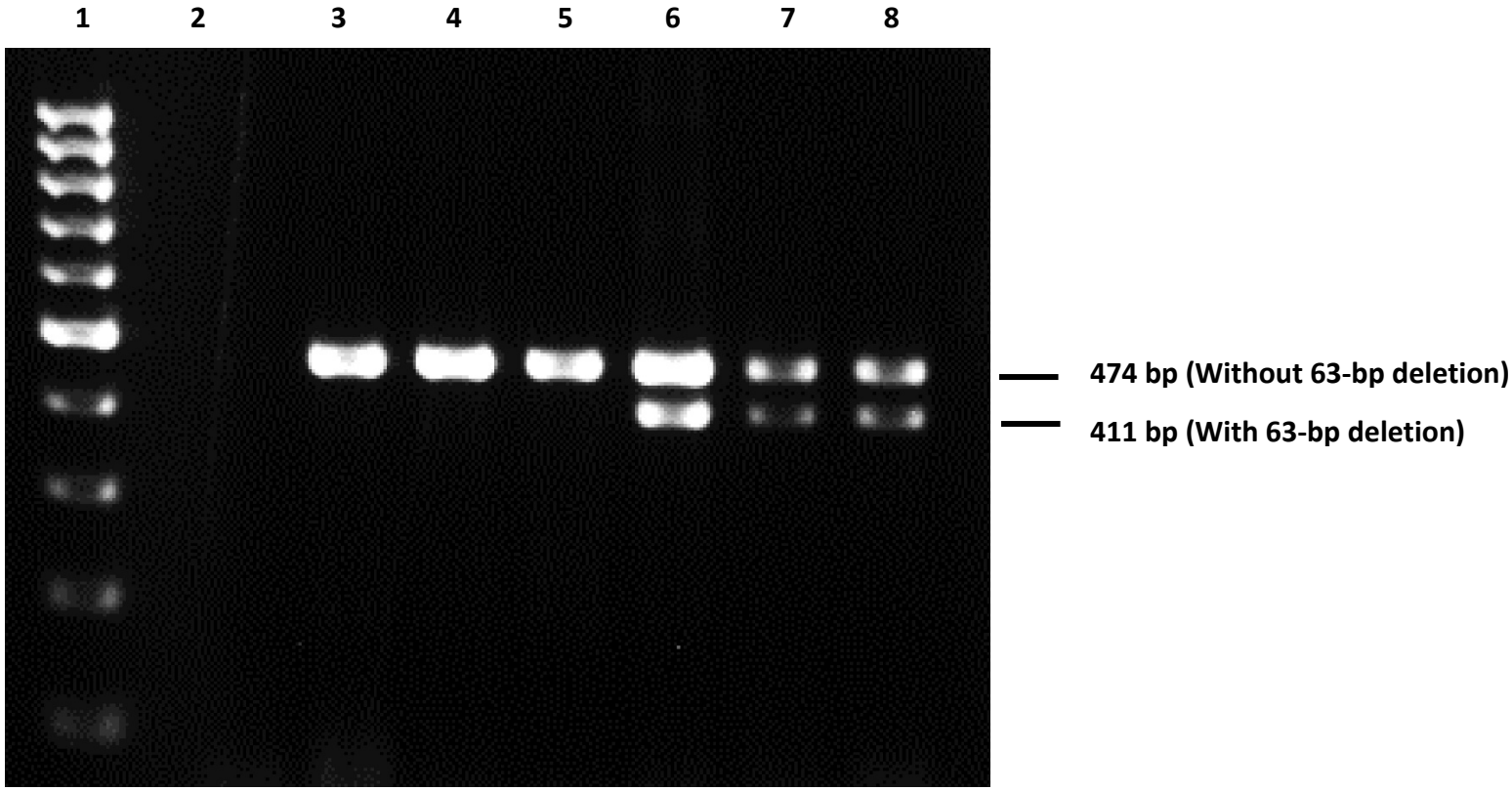

Figure 1. Representative gel image of electrophoresed PCR products with or without 63-bp deletion. Lane 1: 100 bp DNA ladder; lane 2: non-template blank; lanes 3 – 5: selected DNA samples without 63-bp deletion (containing a 474-bp band); lanes 6 – 8: selected DNA samples which are heterozygous for 63-bp deletion (containing a 474-bp band corresponding to sequence without 63-bp deletion and a 411-bp band corresponding to sequence with 63-bp deletion).

Supplementary information

Table S1. Chi-squared values of Hardy-Weinberg equilibrium tests on non-diabetic CKD patients, diabetic CKD patients and healthy controls

Subjects	RAGE polymorphisms					
	G82S	**-374T/A**	**-429T/C**	**1704G/T**	**2184A/G**	**63-bp deletion**
Non-diabetic CKD patients	0.10	0.31	5.85 *	0.45	0.74	0.04
Diabetic CKD patients	6.56 *	0.01	0.00	6.81 *	3.29	0.06
Healthy controls	0.01	2.97	0.06	3.21	6.28 *	0.45

* Hardy-Weinberg equilibrium was violated at $P < 0.05$, corresponding to a Chi-squared values of 3.84 at 1 degree of freedom.

Supplementary information

Table S2. Statistical power of case-control comparison of genotype frequencies

Comparison	RAGE polymorphisms					
	G82S	-374T/A	-429T/C	1704G/T	2184A/G	63-bp deletion
ND-CKD vs D-CKD	78.1	12.8	39.6	26.1	38.0	5.4
ND-CKD vs HC	31.5	26.3	39.4	54.9	38.1	57.9
D-CKD vs HC	15.9	99.9	99.9	13.9	99.9	73.4

Statistical power is expressed in percentages (%). Abbreviations in the table – ND-CKD, non-diabetic CKD; D-CKD, diabetic CKD; HC, healthy control.

Supplementary information

Figure S1. DNA sequences with and without 63-bp deletion. (a) The DNA sequence without 63-bp deletion contains a stretch of nucleotides (63 bp) which is lost in **(b)** the DNA sequence with 63-bp deletion.