Association Between the G82S Polymorphism of the Receptor Gene for Advanced Glycation End-products and Soluble Serum Levels RAGE with Diabetic Nephropathy in the White (Asian) Race

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Introduction. Diabetic nephropathy is one of the most common severe symptoms of diabetes mellitus. Hyperglycemia can lead to tissue damage and inflammation due to mediators such as receptor for advanced glycation end-products (RAGE). Therefore, in this study, we aimed to investigate the association between the G82S polymorphism of the RAGE gene and diabetic nephropathy in diabetic patients.

Methods. In this case-control study, 356 participants (158 men and 198 women) of Asian race, aged 45 to 65 years, who were diagnosed with type 2 diabetes mellitus based on their fasting plasma glucose levels were enrolled. DNA was isolated from the participants' blood samples and genotyped using TETRA -Primer ARMS-PCR. Serum protein concentration of soluble RAGE (sRAGE) was also determined by enzyme-linked immunosorbent assay (ELISA).

Results. Although we found differences in genotyping of participants between homozygous AA and GG and heterozygous GA in the studied groups, the differences were not significant ($P = .568$). In addition, we found no significant correlation between the G82S polymorphism of RAGE and the development of diabetic nephropathy. Serum levels of sRAGE were only slightly decreased in patients with diabetic nephropathy compared with diabetic patients ($P > .05$).

Conclusion. The results of this study indicate no significant association between the G82S polymorphism in the gene RAGE and the development of diabetic nephropathy. Serum levels of sRAGE were only slightly decreased in patients with diabetic nephropathy compared to diabetic patients without nephropathy. Therefore, the study suggests that there is probably no association between the G82S polymorphism in the gene RAGE and the development of diabetic nephropathy.

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Original Paper

INTRODUCTION

As diabetes mellitus progresses, patients experience a greater risk of developing life-threatening and severe complications of the disease, including diabetic nephropathy and retinopathy.[1] Diabetic nephropathy (DN) is one of

the important complications of Diabetes mellitus that can ultimately lead to end-stage kidney disease.[2,3] The interplay between genetic and environmental factors plays a substantial role in the development of this disorder.[2] DN is a severe and common complication of diabetes mellitus that develops because of metabolic and hemodynamic changes.[4,5] In other words, molecular alterations induced by hyperglycemia in diabetes mellitus are responsible for the development of DN in diabetic patients.[6] It has been extensively demonstrated that the formation of advanced glycation end products (AGEs) mediated by glycoxidation contribute to the pathogenesis of DN.[7] Diabetic-induced tissue damage mediated by AGEs has been reported to occur through either receptor-independent or receptor-dependent pathways. Interactions between AGEs and specific cell surface receptors, including RAGE, CD36, 80K-H, OST-48, galectin-3, and macrophage scavenger receptor type II, trigger receptor-dependent pathways.[8,9] The interaction of RAGE-AGE leads to the initiation of an effective signaling pathway that is distinct from that of other receptors because it has primarily a scavenging effect. Therefore, RAGE plays an active role in the persistent inflammation and events that increase tissue damage in diabetes mellitus.[10] In both animal models and human kidney, RAGE has been shown to be expressed in podocytes but not in glomerular endothelium or mesangial cells.[11] In animal models, RAGE has been shown to be involved in glomerular pathology, from thickening of the glomerular basement membrane to inflammation and increased permeability.[12,13] In DN, the interaction of RAGE -AGEs leads to the initiation of a signaling cascade that ultimately activates the VEGF, CTGF, and TGF-β axes and results in renal remodeling.[14]

The gene that expresses RAGE is located on chromosome 6p21.3, near the HLA locus. Researchers have identified at least 30 polymorphisms (9 mutations in the promoter region, 11 mutations in the exon, and 10 mutations in the intron) for RAGE [15]. The single nucleotide polymorphisms (SNPs) in the promoter region -374T/A, 429T/C, and G82S in the exon are the most important ones that have been shown to be effective in creating stable polymorphisms in RAGE expression in various studies.[16,17] G82S is located in exon 3 and results in the replacement of the amino acid glycine with serine at position 82 of the protein. These SNPs have functional effects on the intermediate phenotype,[18] ligand binding,[19] and transcriptional activity.[20] Previous studies have frequently found a significant association between SNPs in RAGE or diabetes mellitus complications such as diabetic retinopathy.[21] However, the number of studies investigating the association between RAGE polymorphisms and DN is limited. Therefore, the aim of this study was to investigate the G82S polymorphism in the gene RAGE and its correlation with DN in the diabetic population and its association with soluble serum levels RAGE.

MATERIALS AND METHODS

Participants

A total of 356 participants (158 men and 198 women) of Asian race with an age range of 45 to 65 years who were diagnosed with type 2 diabetes based on fasting glucose levels were included in this case-study. Diabetic patients with a disease duration between 5 and 10 years, who visit the Razi Hospital, Qazvin, at least once a year for monitoring of diabetic complications, were selected. Participants were divided into three groups: 116 patients suffering from diabetes mellitus with DN, 122 diabetics without DN, and 118 without diabetes. Patients who met the following criteria were included in this case-control study: Diagnosis of type 2 diabetes mellitus based on fasting blood glucose, 2-hour postprandial blood glucose (> 140), and HbA1C levels (> 6.5); a history of diabetes for at least five years; and regular follow-up examinations for diabetes complications performed at least three times per year at Razi Hospital affiliated with Qazvin University of Medical Sciences. Exclusion criteria were individuals who regularly smoked tobacco or consumed alcohol, and individuals with diabetes due to underlying endocrine diseases, including Cushing's syndrome.

Increased Urinary Albumin Excretion

Diabetic nephropathy is characterized by increased urinary albumin excretion (albuminuria) in the early stages. The diagnosis of DN is usually made when urinary albumin excretion is persistently elevated (30 to 300 mg/d) in at least two of three 24 hours urine collection samples collected every three to six months.

Decreased Estimated Glomerular Filtration Rate (eGFR)

As the disease progresses, eGFR, which is a measure of kidney function, decreases. An eGFR of less than 60 mL/min/ 1.73 m² for three or more months indicates chronic kidney disease.[22] Comparability of subjects by sex and age was one of the selection criteria in all groups. All subjects were enrolled in the study after obtaining informed consent.

DNA Extraction and Polymorphism Evaluation

Venous blood samples were collected in EDTA-containing tubes (volume: 3 to 5 mL) and were stored at -70 °C. DNA was extracted from peripheral blood leukocytes with saturated salt from the blood samples of all participants. Subsequently, the G82S polymorphism in the RAGE gene was amplified using the TETRA-primer Amplification Refractory Mutation System PCR (ARMS-PCR) method with the internal and external primers listed in Table 1.

Genotyping

The TETRA-Primer ARMS-PCR technique was used for genotyping by using the extracted DNA. Selection of the inner and outer primer pairs was determined by an online primer-1 software (http://primer1.soton.ac.uk/primer1.html).[23,24] In addition, bioinformatics analysis was performed to ensure the functional accuracy of the primers. The G82S polymorphism of the RAGE gene was examined using the PCR products. The PCR reaction was performed in a total volume of 20 µL containing 1 µL distilled water, 1 µL of each inner and outer primer, 5 µL DNA template, and 10 µL deoxynucleotide triphosphate (dNTP) mix. Temperature conditions were shown in Table 2. To confirm the PCR results, the products were electrophoresed for 30 min at a voltage of 100 V on a 2% agarose gel. UV light from a gel documentation system was used to visualize the bands. To verify the accuracy of the results, several PCR products were sequenced.

Table 2. The PCR Program to Evaluate the G82S Polymorphism

First denaturation temperature	95 ºC for 5 min
Denaturation	95 ºC for 30 min
Annealing	60 ºC
Extension	72 ºC each for 1 min
Final extension	72 ºC for 3 min

Biochemical Analysis and Measurement of Serum Levels of Human Soluble RAGE (sRAGE)

Blood glucose, blood urea nitrogen (BUN), creatinine, total cholesterol, triglycerides, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and serum albumin were measured biochemically using the Selectra XL Autoanalyzer. HbA1C level was determined by turbidimetric immunoassay. The CKD-EPI equation, which considers serum creatinine level, age, sex, and race was used for the estimation of eGFR.[25] The resulting eGFR is expressed in mL/min/ 1.73 m^2. Serum levels of sRAGE protein were measured in all groups by using a sRAGE enzyme-linked immunosorbent assay (ELISA) kit (ZellBio GmbH; Germany). The ZellBio GmbH assay kit uses an ELISA-based biotin double antibody sandwich technology to determine sRAGE. A 24 hours urine (mg/d) sample was used to measure the amount of urine albumin determined by immunoturbidimetry.[26] The concentrations of sRAGE are positively correlated with the intensity of the yellow color. All steps of the test were performed in accordance with the manufacturer instructions.

Statistical Analysis

Demographic data and routine biochemical

Table 1. Characteristics of Primers Used in TETRA-primer ARMS-PCR Method for G82S Polymorphism in RAGE Gene

Gene,SNP,rs ID of polymorphism	Chromosome	Primer sequence	Product size	Annealing temp.
RAGE,G82S,rs 2070600	6:32184665	Forward inner primer (A allele) 474 GACAGTGTGGCTCGTGTCCTTCCCACCA 501	Product size for A : 177 Product size for G : 265 Product size of two outer primer : 388	60
		Reverse inner primer (G allele) 526 CCGACAGCCGGAAGGAAGAGGGATCC 501		60
		Forward outer primer (5´-3´) 262 ACCCCAGCGGCTGGAATGGAAACTGGTAA 290		60
		Reverse outer primer (5´-3´) 649 AAGAGGGAGGCCTTGGAGAAGACCCTGGAA 620		60

parameters were analyzed in the analyses groups. The frequency of G82S in the analyses groups were calculated and compared with the Pearson's Chi-squared test ($P > .05$). RAGE Gene polymorphism was assessed with the Hardy-Weinberg equilibrium (HWE) test. SPSS statistical software version v.22 was used to analyze the results. P values of $< .05$ were considered significant.

RESULTS

The Study Participants

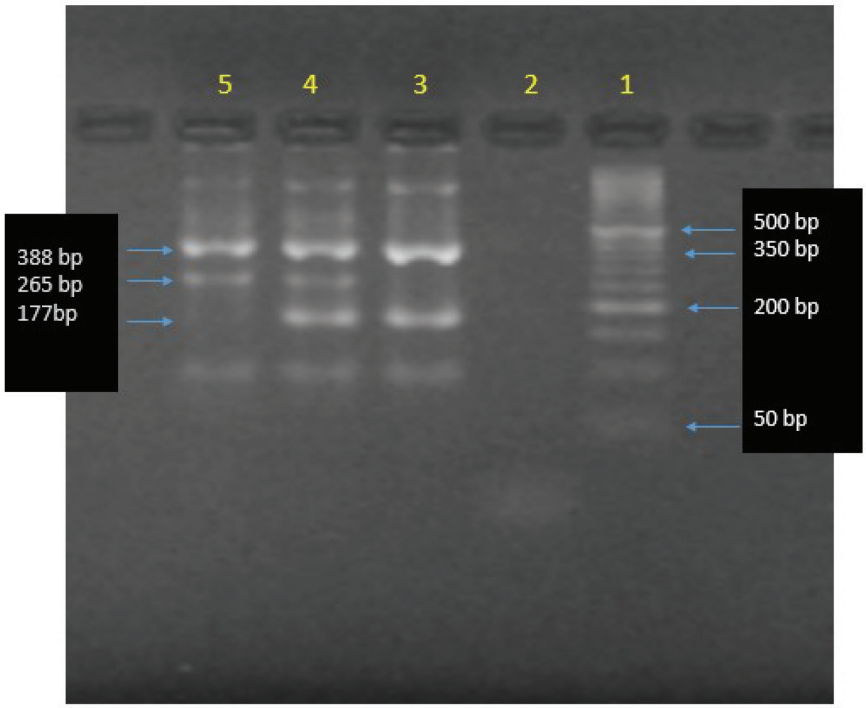
The study sample consisted of 356 participants, including 158 (44.38%) men and 198 (55.61%) women. The clinical and demographic characteristics of the participants in all groups are shown in Table 3. There were no significant differences in age and sex between the groups. Participants with DN had significantly higher serum levels of FBS, HbA1c and creatinine compared to participants without DN ($P < .05$).

Lack of Association Between RAGE rs2070600 Polymorphism and Diabetic Nephropathy

Figure shows the electrophoretic results of genotyping with the TETRA-Primer ARMS PCR molecular method for detection of rs2070600 polymorphism of the RAGE gene. The first column represents the ladder, the second column is the negative control, the third is mutated homozygous with sizes 388, 177, and the PCR products of column 4 with sizes 388, 265, and 177 bp determine the heterozygotes, the PCR products of column 5 with sizes 388 and 265 bp determine the wild homozygotes, and the PCR products with sizes

Table 3. The Laboratory Findings in the Two Groups, With and Without Diabetic Nephropathy, Respectively Designated as Case and Control

Variables	Patients without diabetic nephropathy (122)	Patients with diabetic nephropathy (116)	*P*
FBS, mg/dL	171 ± 64.7	206 ± 78.7	< .0001
HbA1c	7.5 ± 1.15	8.1 ± 1.14	< .001
Creatinine, mg/dL	1.069 ± 0. 14	2.934 ± 2.903	< .0001
eGFR	72.99 ± 9.4	37.83 ± 19.9	< .0001



Genotype analysis of the G82S polymorphism using the TETRA-Primer ARMS-PCR method. PCR products were run on a 2% agarose gel. Lane 1 is DNA molecular marker (ladder), Lane 2 is negative control, lane 3 is mutated homozygous (AA), lane 4 is heterozygote (GA), and lane 5 is wild homozygous (GG).

Table 4. Frequency Distribution of Different Genotypes of G82S Polymorphism of RAGE Gene

G82S polymorphism	Distribution
GG	302 (84.83%)
GA	51 (14.32%)
AA	3 (0.84%)

388 and 177 determine the homozygous mutants.

Statistical analysis of the samples with respect to the genotype distribution of the G82S polymorphism in the gene RAGE (Table 4) determined the following results in three cases: wild homozygous GG (302 cases), heterozygous GA (51 cases) and homozygous mutant AA (3 cases). In addition, the frequency of G82S polymorphism in RAGE gene in different groups was determined. The results of the Hardy Weinberg Equilibrium (HWE) test showed that the G82S polymorphism and allele frequencies had no significant differences among the three groups (P = .58; Table 5). This result indicates that there is no significant association between this variant and the occurrence of DN.

sRAGE Levels in the Serum Samples of Case and Control Groups

In addition to the G82S polymorphism in the gene RAGE, we also examined the protein expression of sRAGE in serum samples from all groups by ELISA. Our results showed that in DN patients, levels of sRAGE in serum compared to diabetic subjects without DN were not significantly different (3.22 ± 0.64 vs. 3.24 ± 0.76 ng/mL, P > .05; Table 6).

DISCUSSION

The study found no significant association between the RAGE rs2070600 polymorphism, and the development of DN. Genotyping results showed no significant differences in genotype distribution or allele frequencies among the three groups. In addition, protein expression levels of sRAGE in serum samples were evaluated, and although patients with diabetes mellitus had lower levels of sRAGE in their serum compared with nondiabetics, these differences were not statistically significant. In addition, there were no significant differences in sRAGE levels between patients with and without diabetic nephropathy.

Table 6. Serum Levels of sRAGE in Experimental Groups

Variables	Patients without diabetic nephropathy (122)	Patients with diabetic nephropathy (116)	*P* total
sRAGE, ng/mL	3.24 ± 0.76	3.22 ± 0.64	> .05

Numerous studies have identified genetic factors, hyperlipidemia, hypertension, and duration of diabetes and poor glycemic control as major risk factors for the occurrence of diabetic nephropathy in diabetic patients.[27,28] It has been suggested that hyperglycemia-mediated formation and accumulation of AGEs in the kidney, which is one of the first tissues affected by these products, plays a crucial role in the pathophysiology of DN, and therefore kidney failure.[28] The interaction of AGEs with their specific receptor (RAGE), and the initiation of a signaling cascade leads to the induction of oxidative stress and a strong overexpression of pro-thrombotic and inflammatory mediators.[29,30] RAGE and its signaling are generally considered to be important in diabetic vascular complications.[30] In addition, recent studies have indicated that the activity of RAGE-AGE is altered by genetic polymorphisms in the RAGE gene, which mainly affects vascular complications in diabetic patients.[31] Several RAGE gene polymorphisms have been found, and their association with microvascular complications of diabetes, such as DN, has been investigated. The G82S polymorphism (located in exon 3 and resulting in the replacement of the amino acid glycine by serine at position 82 of the protein) is the most studied polymorphism in the

Table 5. Frequency of Genotype and Allele of G82S Polymorphism in Experimental Groups

	G82S genotype frequencies			
	Controls	Patients without diabetic nephropathy	Patients with diabetic nephropathy	OR (95% CI)
GG	81.35%	86.88%	85.34%	1.59 (0.77 to 3.24)
GA	17.79%	13.11%	12.93%	0.66 (0.32 to 1.36)
AA	0.84%	0%	1.72%	0 (0 to 0)
Allele frequencies				
G	90.3%	93.5%	91.9%	1.60 (0.81 to 3.16)
A	9.7%	6.5%	8.1%	0.62 (0.31 to 1.22)

disease, as it is relatively common and is related to RAGE-AGE interactions.[32]

Because of the need for AGE-RAGE interaction in the complications of diabetes and also the presence of several RAGE polymorphisms associated with vascular complications[33], in this study we investigated the association between the G82S polymorphism and nephropathy in diabetic patients. According to our results, the GG genotype of the rs2070600 polymorphism in the exon region of the RAGE gene may be associated with an increased risk of diabetic nephropathy and diabetics without DN compared with healthy individuals. In addition, allele G of the aforementioned polymorphism may increase the risk of disease, similar to the aforementioned genotype. Numerous studies have investigated the contribution of RAGE polymorphisms in diabetic complications. For example, Tripathi AK *et al.* examined the relationships between the 374T/C, 429T/C, and G82S polymorphisms of the gene RAGE and diabetic complications in the Indian population.[33] In this study, 176 healthy individuals, 140 individuals with type 2 diabetes without vascular complications, 152 patients suffering from diabetes with microvascular complications, and 135 diabetics with macrovascular complications were studied. A significant association was found between Single Nucleotide Polymorphisms (SNPs) of haplotypes and vascular complications in diabetic patients, which was not consistent with the results of our study and genotyping by PCR-RFLP,[33] this can be due to the difference in the number of samples and the difference in the race studied. In another study by Wong *et al.* the polymorphisms 1704G/T, -429T/C, -374T/A, and G82S of the RAGE gene were investigated in 102 patients without diabetic chronic kidney disease (DCKD), 204 diabetic CKD patients, and 345 healthy volunteers.[24] The Kim *et al.* showed that polymorphism of these four SNPs and deletion of 63 base pairs were not associated with CKD, which is consistent with our findings.[31] Cai *et al.* investigated the association between DN and RAGE gene polymorphism (2184A/G) in Chinese diabetic patients and reported significant associations between these factors.[34] In addition, Wu and colleagues investigated the association between the RAGE gene polymorphism (G82S), sRAGE plasma levels, and chronic periodontitis in 230 participants with diabetes mellitus and 264 nondiabetic subjects. The results showed that the G82S polymorphism of the RAGE gene was associated with chronic periodontitis in the non-diabetic subjects but not in the diabetic subjects.[35] One of the nonsignificant factors in the relationships between the G82S polymorphism of the RAGE gene and the diabetic group could be the effects of different variables, including different diseases and genotyping by RFLP.[35] A study was conducted by Tavakoli *et al.* to investigate the 374T/A and 429T/C polymorphisms of the RAGE gene in the Iranian population of Qazvin.[36] In this study, 150 individuals aged 45 to 65 years (79 individuals with type 2 diabetes and 71 individuals prone to type 2 diabetes without DN) were studied. The study showed that there was no significant association between the 374T/A and 429T/C polymorphisms of the RAGE gene and DN. The Tavakoli's study was performed using TETRA primer ARMS-PCR genotyping and was similar to the present study in terms of race, disease duration, age range, and genotyping. The results of this study was consistent with our findings. Several other studies have investigated the association between RAGE gene polymorphisms and various diseases, including various cancers such as oral, breast, and lung cancer, as well as metabolic diseases. The study by Li *et al.* showed that rs184003 and rs2070600 RAGE gene polymorphisms were associated with gastric cancer.[37] Another study showed that among the polymorphisms of the RAGE gene, SNP rs1800625 is associated with hepatocellular carcinoma.[37] The study by Kalousová *et al.* showed that patients with kidney failure who were treated with hemodialysis (HD) and hemodiafiltration had increased sRAGE levels, which could be due to decreased renal function or protection against AGEs.[39] In addition, studies have indicated that sRAGE levels may be affected by several factors, including treatments and medications such as the use of the angiotensin-converting enzyme inhibitor perindopril, which may increase sRAGE levels, decrease the accumulation of AGE, and increase the production and secretion of sRAGE.[40]

STUDY LIMITATION

The most important limitations of this study included sample collection, difficulty in accessing laboratory materials, and lack of proper patient cooperation.

CONCLUSION

In summary, our results suggest that the frequency of the G82S polymorphism of the RAGE gene does not correlate significantly with the prevalence of DN in the population of Asian origin and that there were no significant changes in serum levels of sRAGE between the study groups. Therefore, it is unlikely that the G82S polymorphism in the RAGE gene increases the risk of DN. However, further studies are needed to investigate larger populations and underlying mechanisms of DN.

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ETHICAL CONSIDERATIONS

This study was conducted with the approval of the Research Ethics Committee of the Qazvin University of Medical Sciences (IR.QUMS. REC.1399.289). The study obtained approval from the Qazvin University of Medical Sciences. Patient confidentiality and data security were concerned at all levels.

AUTHORS' CONTRIBUTIONS

The study was supervised by HP. ARA, SSB and HY conducted the literature search and data collection. HP, IS, SH and SM carried out the data analysis and interpretation. HP and ARA participated in writing the manuscript. HP, MF and ZA participated in the critical revision. All authors approved the final manuscript.

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STATEMENTS AND DECLARATIONS

The author declares no competing interest in the publication of this work.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

CONSENT FOR PUBLICATION

All authors agree to publish this article

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