Journal of Kerman University of Medical Sciences
2022; 29(1): 50-59
http://jkmu.kmu.ac.ir/
10.22062/jkmu.2022.91863
Original Article

Haplotype Analysis of RAGE Gene Polymorphisms and Association with Increased Risk of Diabetic Nephropathy

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ABSTRACT

Background: The present study aimed at evaluating the association between the -429T/C and -374T/A polymorphisms of RAGE (Receptor for Advanced Glycation End Products) gene promoter and diabetic nephropathy as well as examining its possible application as candidate markers of diabetic nephropathy among the population of Qazvin, Iran.

Methods: In this study, the diabetic patients were divided into the two groups of with or without nephropathy. The frequency of genotype and allele were determined using TETRA-Primer ARMS-PCR. Hardy-Weinberg equilibrium test and correlation of polymorphisms, odds ratio (OR), and FAMHAP software were used for haplotype analysis.

Results: Based on our data, the CC genotype of -429T/C polymorphism may play a protective role against the development of nephropathy (OR=0.586, 95%; CI: 0.158-2.167) while, the AA genotype may be associated with increased risk of the disease (OR=1.889, 95%; CI: 0.454-7.854). Allele's analysis revealed that the C allele of -429T/C polymorphism maybe protective against the appearance of nephropathy (OR=0.794, 95%; CI: 0.48-1.314) whereas, the A allele may be related to increased risk for nephropathy (OR=1.452, 95%; CI: 0.783-2.695). Haplotype analysis demonstrated that there was no significant correlation between the two -429T/C and -374T/A SNPs (χ^2=5.125, p value=0.135). However, it was found that the CA haplotype may have a protective effect against the development of nephropathy (OR=0.48, 95%; CI: 0.14-1.64) while, the TA haplotype may increase the risk of the disease (OR=2.06, 95%; CI:1.01-4.23).

Conclusion: Overall, no correlation between the -374T/A and -429T/C polymorphisms and the haplotypes in RAGE gene and the occurrence of diabetic nephropathy, was established.

Keywords: Nephropathy, Type 2 Diabetes, Haplotype, Receptor for Advanced Glycation End Products, SNP, Iran

Citation: Tavakoli A, Salahshourifar I, Hajialilo E, Haghdoost-Yazdi H, Ilghari D, Piri H. Haplotype analysis of rAGE gene polymorphisms and association with increased risk of diabetic nephropathy. Journal of Kerman University of Medical Sciences 2022; 29(1): 50-59. doi: 10.22062/jkmu.2022.91863

Received: 29.08. 2021
Accepted: 20.11. 2021
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Published by Kerman University of Medical Sciences

Introduction

Diabetic nephropathy is a chronic microvascular complication caused by diabetes mellitus which is identified by sustained albuminuria and decreased glomerular filtration rate (GFR) (1). It is well-known that the course and the severity of diabetes mellitus and poor glucose control are of important factors in developing diabetic nephropathy, yet considering the heterogenic nature of this disease, several genetic and environmental factors contribute to the emergence of the disease (2). Although it is claimed that the advanced glycation end products have a possible role in the initiation of diabetic nephropathy with kidneys as significant concentrating location, the exact molecular and genetic mechanisms involved in the development of diabetic nephropathy are still unclear. There are two mechanisms by which AGE (Advanced Glycation End Products) may play a role in the occurrence of nephropathy: 1) a receptor independent mechanism that acts through changing the extracellular matrix proteins and intracellular proteins (3) and 2) activation of AGE receptor which is also called RAGE (Receptor for Advanced Glycation End Products) and considered as the major pathway. This multi-ligand receptor is a member of immunoglobulin superfamily (4) with the potential to bind to different ligands such as AGEs, S100, and HMGB1 (4, 5). The interaction between AGE and RAGE causes the initiation of signal transduction cascades and the expression of transcription factors such as NFκB followed by expression of target genes such as vascular endothelial growth factor (VEGF), proinflammatory cytokines such as TGF-β, IL-6 and IL- 8, tumor necrosis factor (TNF-α), and also the RAGE itself which lead to the generation of prothrombotic components, oxidative stress, and inflammatory responses that eventually cause functional vascular defect (6-8). Relevant studies have shown that the two functional polymorphisms i.e. the SNPs of -429T/C and -374T/A at the promoter region of the gene, have attracted excessive attention due to their important role in increasing the transcriptional level from the RAGE gene, *in vitro* (9). Therefore, based on the previous studies regarding the effect of different polymorphisms of RAGE gene on the development of renal disorders, the present research was aimed at evaluating the association of -429T/C and -374T/A polymorphisms of RAGE gene with diabetic nephropathy and also their possible application as candidate markers of diabetic nephropathy among the study population of Qazvin, Iran.

Materials and Methods

Approval of the Ethics Committee

The present research was approved by the Ethics Committee of Qazvin University of Medical Sciences marked as Project No: IR.QUMS.REC.1396.463.

Study population

A total of 150 patients (58 male and 92 female, aged 45-70 years, white-skinned Iranian) participated in the study. The patients were diagnosed as having typ-2 diabetes mellitus based on three criteria (fasting blood sugar level, 2-hour postprandial blood sugar level, and HbA1C level) (10), a history of diabetes of at least 5 years but not more than 10 years (mean=7.21 year), and regular diabetes complications check-ups of at least 3 times a year at the metabolic diseases clinic of Razi hospital in Qazvin, Iran. Of total patients, 79 ones were with type-2 diabetes mellitus with nephropathy and 71 ones with type-2 diabetes mellitus but without nephropathy. Exclusion criteria were regular tobacco smokers, regular alcohol consumers and those with diabetes due to the presence of underlying endocrine diseases such as Cushing syndrome. Concerning the Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) test, the patients with exogenous insulin injection were also excluded from the study upon calculation.

Sample collection

Samples were taken following the registration of demographic information. Venous blood samples without EDTA were used for biochemical analyses and blood samples with EDTA were collected for HbA1C measurement, CBC, and DNA extraction. Urine specimens were collected for routine urine analysis and urinary micro-albumin assay.

Biochemical analysis

The biochemical measurements performed for blood sugar, BUN, creatinine, total cholesterol, triglycerides, AST, ALT, HDL, LDL, and serum albumin all with Selectra XL autoanalyzer, insulin test was done by ELISA Monobind kit (Insulin-R) and ELISA reader (Anthoans2020), CBC (Sysmex-KX21-N cell

counter), HbA1C was determined by a *latex* particle-*enhanced* turbidimetric *immunoassay*/LTIA (Selectra E autoanalyzer), estimated glomerular filtration rate test (eGFR) value was determined by using the formula "CDK-EPI (Chronic Kidney Epidemiology Collaboration" creatinine equation and resistance to insulin (HOMA-IR) was calculated by using the formula "fasting insulin (µU/L) x fasting glucose (mg/dl)/405 (divided by 405).

Diabetic nephropathy diagnosis

Clinically, diabetic nephropathy is characterized by increasing albumin excretion rate (AER), GFR reduction, and hypertension; however, diabetic nephropathy is recently described by normal albuminuria or microalbuminuria and a reduction in eGFR value (11). The preferred screening tests to diagnose diabetic nephropathy (DN) are serum creatinine level for calculating eGFR and morning random urine sample to determine the AER value (12). In the present study, diabetic nephropathy was diagnosed by the value obtained for eGFR level which was regarded as the principal criterion.

Genomic DNA extraction

Peripheral blood leucocytes were used for DNA extraction according to the protocol recommended by the manufacturer of EURX (Poland) commercial kit. The purity and the concentration of the extracted DNA were assessed by NanoDrop™ instrument which is based on measuring the intensity of light absorption by a specimen at two wavelengths 260 and 280 nm. Also, the integrity of the DNA fragments in the extracted DNA was examined by electrophoresis on 2% agarose gel (13).

Genotyping, Hardy-Weinberg equilibrium (HWE), SNP and haplotype analyses

The extracted DNA was applied for Genotyping using TETRA-Primer ARMS-PCR technique (14). The internal and external pair of primers used for this purpose (Table 1) was determined using online primer 1 software (http://primer1.soton.ac.uk/primer1.html) plus the relevant studies (15) followed by performance of appropriate bioinformatics analysis to ascertain the functional accuracy of the primers. In next steps, the PCR products were employed to investigate the -429T/C (rs1800625) and -374T/A (rs1800624) polymorphisms of RAGE gene. To confirm the accuracy of results, a number of PCR products were sequenced which are available on Gene Bank database by the Accession Number MN108261.

Table 1. The specifications of the primers used in TETRA-Primer ARMS-PCR to investigate the -429T/C and -374T/A polymorphisms of RAGE gene

SNP, rs ID of polymorphism	Chromosome	Primer sequence	Primer size
RAGE, −374T/A (rs1800624)	6:32184610	Forward inner primer (A allele): CCTTGCCTTCATGATGCAGGCCCTAA	155 bp
		Reverse inner primer (T allele): GCCAGACTGTTGTCTGCAAGGGTGGAA	
		Forward outer primer (5'-3') TCAGCCCCTGAACTAGCTACCATCTG	496 bp
		Reverse outer primer (5'-3') CTCAGAGCCCCCGATCCTATTTATT	
RAGE, −429T/C, (rs1800625)	6:32184665	Forward inner primer (C allele): AAAAAAAATGATTTTCTTTCACGACGC	411 bp
		Reverse inner primer (T allele): GGGAACAGGAGAGAAACCTGTTTGTTAA	
		Forward outer primer (5'-3') TCAGCCCCTGAACTAGCTACCATCTG	496 bp
		Reverse outer primer (5'-3') CTCAGAGCCCCCGATCCTATTTATT	

Statistical analysis

The demographic characteristics and the biochemical indices tested for the study population were introduced to SPSS version 21 software. To compare the qualitative variables with quantitative variables, t-student test was used in case of qualitative dichotomous variables and ANOVA for qualitative nominal variable. To evaluate the interaction effect of two or more qualitative categorical variables on quantitative

value of eGFR, two-way ANOVA was employed. The Hardy-Weinberg equilibrium (HWE) test and polymorphisms correlation, calculation of odds ratio (OR), and haplotype analysis were conducted by using the FAMHAP software (16).

Results

In the present study, the criterion for detecting diabetic nephropathy was the eGFR value less than 60 ml/min/1.73 m^2 calculated by MDRD (Modification of Diet in Renal Disease). Therefore, according to the laboratory test associated with creatinine assay and eGFR level, the patients were divided into the two groups marked as control group (patients with type 2 diabetes mellitus but without diabetic nephropathy with eGFR higher than 60 ml/min/1.73m^2) and case group (patients with type 2 diabetes mellitus with diabetic nephropathy and eGFR lower than 60 ml/min/1.73 m^2.

The demographic characteristics, clinical, and laboratory findings of patients were thoroughly examined and as shown in Table 2, there were significant differences in the sex, age, course of disease, BMI, creatinine, BUN, and eGFR levels between the two groups ($p<0.05$) but insignificant differences regarding the FBS, 2hpp glucose, HbA1C, cholesterol, HDL, and LDL levels between the two study groups.

Table 2. Demographic, clinical, and laboratory finding in the two study groups with or without diabetic nephropathy

variable	Without nephropathy	With nephropathy	p-value
FBS (mg/dl)	178.6 ±71.5	185.8 ±86.9	0.587
HbA1C	8.2 ±1.6	8.4 ±1.8	0.493
Creatinine (mg/dl)	1.07 ±0.13	1.40 ±0.77	0.001
BUN (mg/dl)	14.1 ±4.12	18.6 ±9.15	<0.001
eGFR	68.89 ±7.28	48.03 ±10.46	<0.001
HDL (mg/dl)	36.54 ±10.47	39.41 ±11.48	0.113
LDL (mg/dl)	87.42 ±25.99	85.42 ±33.98	0.688

The results of Hardy-Weinberg equilibrium (HWE) showed that the -429 T/C polymorphism of RAGE gene was in equilibrium in both case and control groups. The -374T/A polymorphism was in equilibrium in the control group but not in the case group. The distribution and frequency of -374T/A and 429T/C polymorphisms of the RAGE gene for both case and control groups are presented in table 3. Our results revealed no significant correlation between the two groups regarding the most alleles and genotypes of these two polymorphisms. However, it seemed that the CC genotype of -429T/C polymorphism has a protective effect against the development of nephropathy whereas, the AA genotype of -374T/A polymorphism is associated with a higher risk of the disease. Also, the findings obtained by the analysis of alleles demonstrated the C allele of -429T/C polymorphism may have a protective effect against the occurrence of nephropathy (OR=0.79, 95%; CI: 0.48-1.314) whereas, the A allele of -374T/A polymorphism may be related with growing risk of the disease (OR=1.452, 95%; CI: 0.783-2.695).

The results of haplotypes analysis showed (table 4) that there was no generally significant correlation between the SNP (-429T/C) and SNP (-374T/A) (χ^2=5.125, p-value: 0.135); although, it seemed that while the CA haplotype plays a protective role against the development of nephropathy (OR=0.48, 95%: CI: 0.14-1.64), the TA haplotype is associated with a higher risk of the disease (OR=2.06, 95%; CI: 1.01-4.23).

Table 3. Correlation analysis of -429T/C and -374T/A polymorphisms of RAGE gene in the two groups with or without diabetic nephropathy

Marker	Genotype	Without nephropathy (frequencies)	With nephropathy (frequencies)	OR (95% CI)	Genotype_2df (p-value)
	CC	3(4.2%)	6(7.6%)	0.586 (0.158-2.167)	
-429 T/C	CT	14(19.7%)	18(22.8%)	0.894 (0.467-1.709)	0.621
	TT	54(76.1%)	55(69.6%)	1.276 (0.67-2.43)	
	AA	6(8.5%)	4(5.1%)	1.889 (0.454-7.854)	
-374 T/A	AT	32(45.1%)	33(41.8%)	1.221 (0.556-2.683)	0.556
	TT	33(46.5%)	42(53.2%)	0.708 (0.342-1.465)	

Table 4. Haplotype analysis of -374T/A and -429T/C polymorphisms of RAGE gene

Haplotype [(-429T/C)/(-374T/A)]	Case Frequency	Control Frequency	OR (95% CI)
T T	0.571	0.602	0.88 (0.55-1.4)
C T	0.237	0.257	0.9 (0.53-1.52)
T A	0.166	0.088	2.06 (1.01-4.23)
C A	0.026	0.053	0.48 (0.14-1.64)
Chi-square (χ^2) = 5.125, p-value: 0.135			

Discussion

According to the previous studies, the interaction between AGE-RAGE has an important role in the development of type-2 diabetes mellitus (T2DM) microvascular complications such as diabetic nephropathy (17). This role has been particularly boosted by numerous studies on animal models and through pharmacological antagonists and deletion of RAGE gene (18, 19). In addition to microvascular disorders, different polymorphisms of RAGE gene have been the focus of attention associated with disorders including Alzheimer, systemic lupus erythematosus, prostate cancers, pulmonary adenocarcinoma, and non-diabetic renal diseases such as glomerulosclerosis (20-26). The results of studies concerning microvascular complications have, to some extent, demonstrated contradictory findings among different populations. Due to the significance of promoter region sequences in the expression of genes and also lack of adequate studies among the Iranian population, the present research aimed to investigate the correlation between the -374T/A and -429T/C polymorphisms at promoter region of RAGE gene and the occurrence of diabetic nephropathy. It has to be noticed that this was the first study in Iran in which the correlation of some polymorphisms with microvascular complications in type-2 diabetes mellitus patients in Qazvin was evaluated.

The results concerning the genotypes distribution and frequency of two -374T/A and -429T/C polymorphisms of RAGE gene in both diabetic groups with or without nephropathy showed no significant correlation when performed for most alleles and genotypes of these two polymorphisms; even though, it seems that the CC genotype of -429T/C polymorphism exerts a protective role against the development of nephropathy while the AA genotype of -374T/A polymorphism is associated with a higher risk of nephropathy. Also, the data regarding the alleles analysis indicated that the C allele may have a protective effect against the occurrence of nephropathy (OR=0.794) whereas, the A allele of -374T/A polymorphism may be linked with an increased risk of developing nephropathy (OR=1.452). Although our data were generally not significant statistically yet these findings, when considered in details, are in disagreement with other previous results reported so far and in the meantime it is interesting that these data could act as an incentive for further studies in finding solutions to solve the present barriers in achieving more solid data to confirm or reject our current findings.

In a study performed on Indian population, it was shown that the -374T/A polymorphism is significantly correlated with the development of type-2 diabetes mellitus but in the meantime it reduces the risk of developing macrovascular complications in patients with type-2 diabetes mellitus whereas, the -429T/C polymorphism demonstrated a significant association with expansion of macrovascular complications in these patients. This report has also described that these two polymorphisms have no significant correlation with microvascular complications in patients with type-2 diabetes (27), a finding not in harmony with the data observed in the present study. Prasad et al. reported a significant association between the -429T/C polymorphism and renal failure among a group of Asian Indian population (28). Another study on Malaysian people showed no significant relationship between the -429T/C, -374T/A, 2184A/G and 1704G/T polymorphisms and the deletion of 63-bp. Also, no association with diabetic patients with CKD (chronic kidney disease) and non-diabetic CKD patients was observed (21), a finding, to a large extent, in parallel with the results of our present study.

A previous meta-analysis study on the -429T/C and -374T/A polymorphisms of the RAGE gene was performed on only four and six patients available and covered a limited number

of population (29). As this meta-analysis faced some limitations including the inclusion of only type-2 diabetes mellitus, the Caucasian population, and lack of other ethnic groups such as Asian and African populations, the results of this meta-analysis could not get concrete approval. Our current study demonstrated that it is possible that the genetic and environmental shares, influencing the risk factors of a disease in a particular geographical and racial category, may produce findings different from those in other studies, a fact that could confirm the idea that the diabetic nephropathy is a disease of multifactorial etiology and that the polymorphisms of a gene cannot influence the development of diabetic nephropathy or protection against the disease alone and maybe accompanied with contradictory results.

Another study on Swedish population revealed a significant correlation between the -374T/A polymorphism and the risk of diabetic nephropathy (30). Also, in a prospective study, it was claimed that the -374T/A polymorphism in Italian population was associated with rapid reduction of renal function in patients with CKD which is somehow consistent with our results (31). However, the -374T/A polymorphism was found to have protective effect against the progression of renal disorders and albumin excretion among Finnish patients (32), a finding relatively inconsistent with the results of our study. A study on Dutch patients demonstrated that, in addition to diabetic nephropathy, there was an association between the 2184A/G, -374T/A, and -429T/C polymorphisms and reduction of renal function (24). The results of studies on two groups of white and dark skin color Brazilians (33), as well as Malaysian population (15), indicated that there was no significant correlation between the -429T/C polymorphism and diabetic nephropathy among Brazilian patients and chronic renal disorders in patients from Malaysia which showed more analogy with our study. The criterion of the study on Malaysian patients in determining chronic renal disorders was, similar to the present study, an eGFR value less than 60 ml/min/1.73 m^2 (15) which could be considered as a favorable parameter to make the two studies with more comparable results. Determination of microalbuminuria is one of the diagnostic methods for detecting early-stage diabetic nephropathy in patients with type-2 diabetes (12); however, considering a large number of patients with developed kidney disorder but with normoalbuminuria (34, 35), strict caution must be taken to avoid such difficulties in appropriate diagnosis, classification, and stage detection of the disease which could eventually lead to different results in genotyping between the various studies, resulting in misinterpretation of data. This is the reason why the authors of the present study used the eGFR level criterion, instead of AER value, to identify diabetic nephropathy.

Since the haplotype-based analysis is regarded as one of the important techniques in identifying the genes with intermediate impact (36) therefore, we used the haplotype-based analysis of two -429T/C and -374T/A polymorphisms of RAGE gene in which all four haplotype combinations demonstrated a value more than 1% for haplotype frequency. The results of haplotype-based analysis in our study showed that there was generally no significant association between the SNPs of -429T/C and -374T/A polymorphisms, yet it seemed that the CA haplotype had a protective role against the development of nephropathy while the TA haplotype was associated with an increased risk of the disease. Also, the present study revealed that the frequency of the haplotype containing the minor allele at location -429, was higher than that observed in the diabetic patients without nephropathy whereas, the frequency of the haplotype containing the minor allele at location -374, was lower than that found in the diabetic group without nephropathy. These findings for both alleles were not statistically significant although the value found in our study was higher, compared to two studies on Chinese population for alleles C (22.7%) and A (13.9%) and Caucasian population for alleles C (18%) and A (20%) (9, 37).

In a linkage disequilibrium analysis performed on a group of Caucasian population, the frequency of a haplotype (GTGGGG) containing the 1704G/T, -429T/C, -374T/A, G82S, 2245G/A, and 2184A/G polymorphisms was higher in patients with type-2 diabetes mellitus nephropathy (38). Also, Tripathi *et al*., demonstrated that the CTG haplotype caused increased risk for the development of macrovascular complications in diabetic patients. Obviously, the results of these two recent studies were different from our present data; however, the TAG haplotype is shown to exert a protective effect against the macrovascular complications in patients with type-2 diabetes which is relatively in accordance

with our results although the protective effect was against the microvascular problems (27). A report by Zee *et al.*, covering the American population, produced similar results in which the TAG haplotype was demonstrated to be associated with reduced risk of ischemic stroke (39). On the contrary, Peng *et al.*, reported no haplotype relationship with the severity of CAD in a group of Chinese participants (40). In general, an acceptable explanation to justify this discrepancy in results may be regional and racial differences between Iran (Qazvin) and other populations, worldwide. Various racial groups live in Iran and a group of population of Pars race participated in the current study which may be different from the people of other countries, genetically. This research faced several limitations as follows: (a) all participants were only selected from one medical center which may not be considered as the representative of a particular population category, (b) lack of sRAGE assay, (c) the cross-sectional nature of the study and a relatively small sample size which could decrease the statistical power although, the allele frequency of the disease was not shown to be low. Considering the impact of 2184GG and -429CC genotypes within the RAGE gene in promoting the sRAGE level (41) and their role in preventing the attachment of the RAGE ligands to RAGE gene and inhibiting the activation of signaling pathways which follows by generation of oxidative stress and inflammation in vascular cells and also the role of genotype determinants in their expression level (25), it is possible to achieve more useful information over the effect of gene polymorphisms on progression or prevention of diabetic nephropathy.

Conclusion

The present study showed that the -374T/A and -429T/C polymorphisms of RAGE gene had no significant correlation with diabetic nephropathy in a group of population in Qazvin (Iran). Considering the contradictory results between the present study and those carried out elsewhere, it is not possible to confidently confirm or reject the idea of using these polymorphisms as suitable markers for detecting diabetic nephropathy as a disease of multifactorial etiology; therefor, more future researches focusing on status of disease progression, assaying other parameters such as RAGE protein serum level, and the use of large-scale sample sizes are needed to better clarify the genetic nature of diabetic nephropathy.

Acknowledgements

The authors of the present research project would like to appreciate the technical assistance offered by the staff of Biochemistry laboratories and Razi hospital in Qazvin. Also, our thanks go to the Deputy for the Research and Technology Division and the Cellular and Molecular Research Center of Qazvin University of Medical Sciences for supporting the project. This research project was part of an MSc dissertation carried out by Abbas Tavakoli during 2018 to 2020.

Authors' contributions

The study was supervised by HP. HP, AT and IS participated in the literature search, study design and data collection. HP and IS participated in the data analysis. HP, DI and IS participated in the data interpretation. HP, EH and IS participated in the writing. HHY participated in critical revision. All authors approved the final manuscript.

Funding

This research project was financially supported by Deputy for the Research and Technology Division and the Cellular and Molecular Research Center of Qazvin University of Medical Sciences.

Conflict of interests

The authors declare no competing interests.

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