RESEARCH ARTICLE

Association analysis of -429T/C receptor for advanced glycation end products (RAGE) gene polymorphism with type 2 diabetic retinopathy and serum soluble RAGE levels in Pakistani patients

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Abstract

Objective: To investigate the association of receptor for advanced glycation end products gene polymorphism 429T/C (rs1800625) with diabetic retinopathy and serum soluble receptor for advanced glycation end products levels in patients with type 2 diabetes.

Methods: The case-control study was conducted from January 2017 to December 2018 at Pakistan Railway Hospital, Rawalpindi, and the Multidisciplinary Laboratories of Islamic International Medical College, Riphah International University (RIU), Islamabad, Pakistan. Those included were healthy controls in group A, diabetics without retinopathy in group B and patients having diabetic retinopathy in group C. Genotyping for 429T/C was done by tetra-primer amplification refractory mutation system-polymerase chain reaction. Serum soluble receptor for advanced glycation end products levels were measured using enzyme-linked immunosorbent assay. Data was analysed using SPSS 22.

Results: Of the 450 subjects, 150(33.3%) were in each of the three groups. The frequency of TT, TC and CC genotypes of 429T/C polymorphism were 137(91.3%), 10(6.7%) and 3(2%) in group A; 133(88.6%), 13(8.7%) and 4(2.7%) in group B; and 127(84.7%), 18(12%) and 5(3.3%) in group C. No significant association of 429T/C genotypic and allelic frequencies were found with groups B and C ($p>0.05$). Serum soluble receptor for advanced glycation end products levels were significantly high in patients with proliferative diabetic retinopathy and were positively correlated with fasting plasma glucose in group C ($p<0.05$). TC and CC genotypes were significantly associated with raised serum soluble receptor for advanced glycation end products, and TC with raised fasting plasma glucose in group C.

Conclusion: The 429T/C receptor for advanced glycation end products gene polymorphism was found to be associated with severe non-proliferative diabetic retinopathy, and serum soluble receptor for advanced glycation end products levels had a positive correlation with severity of diabetic retinopathy.

Keywords: Type 2 diabetes mellitus, Diabetic retinopathy, Receptor for advanced glycation end products gene, RAGE, 429T/C polymorphism, Soluble RAGE. (JPMA 71: 1175; 2021)

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Introduction

Diabetes mellitus (DM) is a complex metabolic disease characterised by raised plasma glucose levels. According to the International Diabetes Federation, worldwide prevalence of diabetes will rise from 463 million in 2019 to 578 million by 2030 and may reach up to 700 million by 2045.[1] Pakistan has the 7th biggest population of diabetics in the world, with the prevalence of type 2 DM (T2DM) of 11.77%.[2] Long-term hyperglycaemia may lead to macrovascular and microvascular complications. Diabetic retinopathy (DR) is the most common microvascular complication and is a leading cause of vision-loss globally in adults aged 20-74 years. A meta-analysis showed that the worldwide prevalence of DR was 27% between 2015 and 2019.[3] According to Mumtaz et al., the pooled DR prevalence in Pakistan was 8.6% whereas vision-threatening DR (VTDR) was 28.2% from 1990 to 2017.[4]

Diabetes mellitus duration, poor glycaemic control, and hypertension (HTN) are the major risk factors for developing DR, but clinical studies in diabetics revealed a considerable variation in onset and severity of retinopathy, which are not fully explained by the known risk factors.[5] Genetic factors affecting several biochemical mechanisms play a vital role in the vulnerability to develop retinopathy[5] but the exact genetic mechanism is still not well-established. A variety of candidate genes, like receptor for advanced glycation end products (RAGE), vascular endothelial growth factor (VEGF), aldose reductase, nitric oxide synthase (NOS), and methylenetetrahydrofolate reductase (MTHFR), have been considered for association with DR, but have shown inconsistent reports in different populations. Amongst genes associated with DR, the RAGE encoding gene is important as it is positioned in the major histocompatibility complex (MHC) region on chromosome[6]. This is gene-dense region and holds several

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inflammatory genes possibly involved in diabetic complications. In chronic hyperglycaemia, advanced glycation end products (AGEs) are formed due to non-enzymatic glycation of proteins, lipids or nucleic acids[6] which cause damage by binding to RAGE. AGEs-RAGEs activated inflammatory pathways lead to the generation of matrix metalloproteinases (MMPs) which form soluble RAGE (sRAGE) by cleaving C-truncated RAGE which is a cellular form of RAGE. Soluble RAGE has cytoprotective effect against AGE-RAGE interaction by acting as a decoy for RAGE ligands.[7]

RAGE gene polymorphisms have been studied in DR patients in various populations, including Caucasian, Chinese and Indian[8-10] but not in Pakistani population. Limited literature is available on the genetic elements contributing to DM and DR in Pakistani patients.[11-13] It is, therefore, important to investigate genetic factors involved in pathogenesis of DR to provide adequate therapy through pharmacological or surgical treatment.

The current study was planned to investigate the association of RAGE gene single nucleotide polymorphism (SNP) 429T/C (rs1800625) in promoter region with DR in T2DM patients. To assess possible biological relevance of RAGE gene polymorphism, the relationship of serum sRAGE levels with 429T/C (rs1800625) gene polymorphism was also planned to be investigated.

Patients and Methods

The case-control study was conducted from January 2017 to December 2018 at Pakistan Railway Hospital (PRH), Rawalpindi, and the multidisciplinary laboratories of Islamic International Medical College, Riphah International University (RIU), Islamabad, Pakistan. Subjects were enrolled from Al-Shifa Eye Hospital, Rawalpindi, PRH, and Divisional Headquarter (DHQ) Hospital, Mirpur, Azad Jammu and Kashmir (AJK).

After approval from the RIU ethics review committee, the sample size was calculated using Cochran's formula:[14]

$n= Z^2 (pq) \div e^2$

where n was the required sample size, Z was the value of Z at 95% confidence interval (CI) which was 1.96, p was the prevalence of disease, q was the 1-p, and e was the accepted margin of error which was 0.05.

The sample was raised using non-probability convenience sampling technique. Those included were patients having T2DM for duration of $\geq$5 years, aged $\geq$30 years, taking medical treatment for DM either oral hypoglycaemics or insulin, and belonging to Punjabi, Kashmiri and Pathan ethnicities, which are the three main ethnic groups in Pakistan. A group of non-diabetic subjects matched for age and gender were also enrolled as the healthy control (HC) group.

Those excluded were patients with T1DM, malignancy, haematological disorder, acute and chronic cardiovascular, renal or hepatic disease, history of any other chronic endocrine or inflammatory disorder, history of any acute ophthalmic infection like uveitis, thrombotic event, or other ocular disorders like glaucoma.

The enrolled patients were further categorised into diabetics without retinopathy (DWR) group, and patients with diabetic retinopathy (DR)

After taking written informed consent from all the subjects, demographic and clinical data was recorded. All diabetic patients underwent a complete ophthalmological examination by certified ophthalmologists for diagnosis of retinopathy by grading colour fundus photography.

Fasting venous blood samples were collected from all the subjects for genomic deoxyribonucleic acid (DNA) extraction, biochemical analysis of serum sRAGE, fasting plasma glucose (FPG), serum total cholesterol (TC), low-density lipoprotein-cholesterol (LD-C), triglycerides (TG), high-density lipoprotein-cholesterol (HDL-C), urea and creatinine.

Genomic DNA was extracted from blood by 5-7% chelex (Bio-Rad) and stored at -20°C till PCR analysis. Its concentration was measured by Nano drop spectrophotometer (Nano drop 2000c, Thermo Fischer Scientific) at 260/280 nm for PCR amplification. Serum sRAGE was measured by enzyme-linked immunosorbent assay (ELISA) using kit supplied by Elabscience, United States of America (USA). Other biochemical tests were performed on Cobas c III (Mannheim, Germany) using kits supplied by Roche Diagnostics. The genotyping of 429T/C (rs1800625) gene polymorphism was conducted by simple and cost-effective allele specific tetra-primer amplification refractory mutation system-polymerase chain reaction (T-ARMS-PCR) using common and allele-specific primers. Primer sequences were taken from literature.[15] Four primer sets used for the amplification of 429T/C were: two forward and reverse outer, (FO-5'- GGGGCAGTTCTCTCCTCACTTGTAAA) and (RO-5'-CCTTTGGGACAAGAGTCCTTCAGG); two forward and reverse inner allele-specific primers, C-allele (FI-5'-AAAAAAAATGATTTTCTTTCACGACGC) and T-allele (RI -5'-GGGAACAGGAGAGAAACCTGTTTGTAA). PCR reactions were performed in thermal cycler (Major Sciences, USA), using a commercially available DreamTaq Green PCR master mix (2x) by thermoscientific, in accordance with company's instructions. To detect RAGE polymorphism 429T/C, a total volume of 21μl of following components was added into a

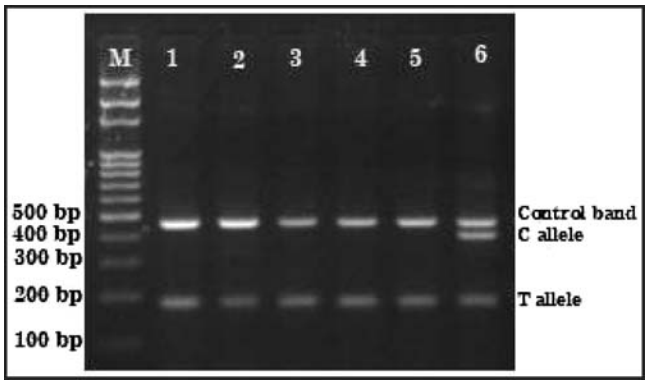

Figure: Electrophoretogram on 2% agarose gel showing amplified polymerase chain reaction (PCR) products of 429T/C receptor for advanced glycation end products (RAGE) gene polymorphism.
Lanes 1-5 represent TT homozygous and lane 6 represents TC heterozygous genotypes. Molecular size for T and C alleles are indicated as 187bp and 336bp respectively and control band of 470bp. M is molecular size markers of 100 bp.

PCR tube of 0.2 mL: 2μL template DNA (100 ng/μL); 1μL of 429T/C primer mix (FI, RI, FO and RO primers), each primer of 10 pmol/μL; 8μL master mix and 10μL deoxyribonuclease (DNase)-free water. PCR cycling conditions were optimised. DNA was denatured at 95°C for 5min, followed by seven cycles of 30secs at 95°C, 30secs at 60°C and 30secs at 72°C, 10 cycles of 40secs at 95°C, 40secs at 52°C and 30secs at 72°C; 10 cycles of 40secs at 95°C, 40secs at 51°C and 30secs at 72°C and a final extension for 10min at 72°C.

The amplified PCR products were resolved onto 2% agarose gel containing 5ul ethidium bromide solution (0.5 ug/ul) per 100ml agarose solution to visualise the DNA bands under ultraviolet (UV) light in gel documentation system (G-Box, syngene USA). The PCR amplicons of 429T/C were as 187bp for T-allele, 336 bp for C-allele and 470bp for internal control bands (Figure). Ten percent of samples were re-assayed by using primer sequences from another company.

Data was analysed using SPSS 22. Chi-square / Fisher's exact test and odds ratio (OR) were used to compare allelic and genotype frequencies and to see association of genotypes with DM, DR and ethnicity. To evaluate the differences in biochemical variables among the groups, analysis of variance (ANOVA) with post-hoc Tukey's test was used. Pearson correlation, univariate and multiple logistic regression analysis were done to find relations between variables and for risk estimation among the study groups.

Results

Table-1: Comparison of demographic and biochemical characteristics of HC, DWR and DR groups.

Parameters	HC (n = 150) N (%)	DWR (n = 150) N (%)	DR (n = 150) N (%)	p value
Age (years)	55.90 ±10.90	58.16 ±9.42	56.25 ± 8.56	0.094
Gender (male/female) (%)	67/83 (44.7/55.3)	65/85 (47/56)	63/87 (42/58)	0.897
Ethnicity (%)				
Punjabi	59 (39.3)	60 (40)	86 (57.3)	0.779[a]
Kashmiri	52 (34.7)	56 (37.3)	34 (22.7)	0.007[b]
Pathan	39 (26.0)	34 (22.7)	30 (20)	0.006[c]
Duration of DM (years)	---	9.31 ± 4.01	13.02 ± 5.71	<0.001[b]
Serum sRAGE (pg/ml)	164.05± 70.53	582.04 ± 206.04	600.12 ± 238.54	0.001[a,b]
				0.679[c]
FPG (mg/dl)	88.06 ± 8.58	165.34 ± 51.93	190.87 ± 55.33	<0.001[a,b]
				<0.001[c]
Total cholesterol (mg/dl)	148.86± 21.535	206.54 ± 42.89	220.67± 42	<0.001[a,b]
				0.003[c]
Triglyceride (mg/dl)	137.09 ± 28.25	180.71± 62.53	175.15± 43.53	<0.001[a,b]
				0.560[c]
HDL-C (mg/dl)	47.66 ± 7.12	44.0± 8.31	45.0± 8.02	<0.001[a,b]
				0.509[c]
LDL-C (mg/dl)	90.85± 16.95	128.36± 42.13	143.31± 37.65	<0.001[a,b]
				<0.001[c]
Urea (mg/dl)	25.91± 9.34	30.89± 9.38	33.22± 9.57	<0.001[a,b]
				0.083[c]
Creatinine (mg/dl)	0.91± 0.33	1.09± 0.38	1.19± 0.38	<0.001[a,b]
				0.066[c]

HC: Healthy controls; DWR: Diabetic without retinopathy; DR: Diabetic retinopathy; a: HC compared with DWR; b: HC compared with DR, c: DWR compared with DR; p< 0.05 was significant.
DM: Diabetes mellitus; sRAGE: Soluble receptor for advanced glycation end products; FPG: Fasting plasma glucose; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol.

Table-2: Genotype and allele distribution of RAGE gene polymorphism 429T/C for HC, DWR and DR groups.

429T/C	HC N=150	DWR N=150	DR N=150	Odds ratio (95% CI)	P Value
Genotypes	N (%)	N (%)	N (%)		
TT	137 (91.3)	133 (88.6)	127 (84.7)	Ref 1	
TC	10 (6.7)	13 (8.7)	18 (12)	1.33 (0.56-3.15)[a]	0.502[a]
				1.94 (0.86- 4.36)[b]	0.103[b]
				1.45 (0.68-3.08)[c]	0.332[c]
CC	3 (2)	4 (2.7)	5(3.3)	1.37(0.30- 6.25)[a]	0.720[a]
				1.79(0.42-7.67)[b]	0.490[b]
				1.30(0.34-4.98)[c]	0.745[c]
Allele					
T	284 (94.7)	279 (93)	272 (90.6)		
				1.33 (0.68-2.61)[a]	0.396[a]
				1.82 (0.96-3.45)[b]	0.06[b]
C	16 (5.3)	21 (7)	28 (9.3)	1.36 (0.75-2.46)[c]	0.296[c]
Dominant Model					
TT	137 (91.3)	133 (88.6)	127 (84.6)	1.34 (0.62-2.88)[a]	0.442[a]
TC+CC	13 (8.7)	17 (11.4)	23 (15.3)	1.90 (0.92- 3.92)[b]	0.07[b]
				1.41 (0.72-2.77)[c]	0.307[c]
Recessive Model					
TT+TC	147 (98)	146(97.3)	145(96.7)	1.34(0.29- 6.10)[a]	0.999[a]
CC	3 (2)	4 (2.7)	5(3.3)	1.68 (0.396-7.20)[b]	0.722[b]
				1.25 (0.33 - 4.78)[c]	0.999[c]

HC: Healthy controls; DWR: Diabetic without retinopathy; DR: Diabetic retinopathy; [a]: HC compared with DWR; [b]: HC compared with DR, [c]: DWR compared with DR; p< 0.05 was significant.
sRAGE: Soluble receptor for advanced glycation end products; FPG: Fasting plasma glucose; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; SNP: Single nucleotide polymorphism.

Table-3: Association of mean serum sRAGE levels and other biochemical parameters with 429T/C genotypes in DR group.

	SNP429T/C genotypes			
Parameters	TT (n=127)	TC (n=18)	CC (n=5)	p-value
Serum sRAGE (pg/ml)	571.5 ± 231.7	740.7 ± 225.6	818.5 ± 191.4	0.01# 0.05##
FPG (mg/dl)	185.2 ± 53.8	223.2 ± 54.2	217.4 ± 72.1	0.01#
Total cholesterol (mg/dl)	219.1 ± 42.9	231.7 ± 37.3	218.8 ± 31.8	0.49
HDL-C (mg/dl)	44.6 ± 8.1	46.7 ± 7.7	47.0 ± 6.9	0.51
LDL-C (mg/dl)	141.9 ± 37.9	153.6 ± 36.9	141.2 ± 33.9	0.46
Triglyceride (mg/dl)	176.3 ± 45.3	167.7 ± 34.0	144.0 ± 14.4	0.50
Urea (mg/dl)	32.7 ± 9.4	34.6 ± 10.7	39.4 ± 4.3	0.25
Creatinine (mg/dl)	1.17 ± 0.36	1.28 ± 0.52	1.34 ± 0.19	0.35

sRAGE: Soluble receptor for advanced glycation end products; DR: Diabetic retinopathy; p<0.05 significant; # TT vs TC, ## TT vs CC; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol.

Of the 450 subjects, 150(33.3%) were in each of the three groups. The mean age in HC group was 55.90±10.90, DWR 58.16 ±9.42 and DR 56.25 ± 8.5 years; and the proportion of male-to-female was 67(44.7%) vs 83(55.3%), 70(47%) vs 80(53%), and 63(42%) vs 87(58%) in the three groups, respectively. Proportion of Punjabi ethnicity was significantly higher in DR group compared to DWR (p=0.006) and HC (p=0.007). The duration of diabetes was significantly longer in DR patients 13.02±5.71 years compared to DWR group 9.31±4.01 years (p<0.05).

Serum sRAGE, FPG, TC, TG, LDL-C, urea and creatinine were raised in patient groups compared to HC, and a significant increase in TC and LDL-C was found in DR compared to DWR (p<0.05). DR subjects were sub-divided into non-proliferative (NPDR) and proliferative (PDR) groups, and serum sRAGE was significantly higher in PDR compared to NPDR and DWR (p<0.001) respectively. A positive correlation was found between serum sRAGE and FPG in DR group (p=0.004) (Table-1).

No significant association was found when wild type TT genotype was compared with heterozygous TC and mutant homozygous CC genotypes among the three groups (p>0.05). No significant difference with C allele variants was observed among the three groups (p>0.05). Frequency distribution of combined TC + CC genotypes in dominant model and of CC genotype in recessive model were also non-significant (p>0.05) (Table-2).

Association of genotypic and allelic frequencies of SNP 429T/C remained statistically non-significant when DR subjects were divided into NPDR and PDR groups and compared with DWR group. Upon further stratification of NPDR group into mild, moderate and severe, a moderate association of heterozygous TC genotype with severe

NPDR was observed in comparison to DM group in both univariate and multinomial regression analysis (OR: 4.09; 95% CI: 1.35-12.35; p=0.01). No significant association was found in terms of ethnicity (p>0.05).

In DR group, TC and CC types were significantly associated with raised serum sRAGE and TC genotype with raised FPG at univariate level (Table-3). After multiple regression analysis, the association of these genotypes with raised sRAGE and FPG remained significant (p<0.05).

Discussion

The study found no significant association of 429T/C genotypic and allelic frequencies with DR. However, a moderate association with severe NPDR was seen in comparison to DWR.

Several studies have been done to explore the association of genetic polymorphism 429T/C with DM and DR in diverse populations, but the results have remained conflicting. Hudson et al. found a significant association of C allele with DR and an increased protein expression of RAGE gene in vitro.[8] Erna et al. found homozygous TT genotype of 429T/C to be protective, while mutant C allele was associated with retinopathy in Indonesian population.[16]

In contrast, various studies carried out in Asians and Caucasian populations found no association of DR with -429T/C polymorphism.[10,17,18]

Several meta-analyses performed in Chinese, Asian and Caucasian populations to evaluate the associations of RAGE gene polymorphisms with DM and DR showed no significant association for 429T/C variant.[19,20] The reason for inconsistent results of -429T/C RAGE gene polymorphism in different populations may reflect the presence of variances in the pathogenesis of DR among populations. Gene variations in the same region may be involved in pathogenesis of DR as SNP 429T/C is positioned upstream to 374T/A (rs1800624) in the RAGE gene promoter region.

Although the current study found a moderate association of heterozygous TC genotype of 429T/C polymorphism, with severe NPDR at univariate and multivariate level, this association was not evident when mild, moderate and severe sub-groups of NPDR were studied together, and this needs to be interpreted very carefully.

The current study found significantly raised serum sRAGE levels in PDR patients compared to NPDR and DWR. A significant association of heterozygous TC and mutant CC genotypes with raised serum sRAGE and heterozygous TC with FPG was also seen at univariate and multivariate levels in DR group. These findings suggest that raised serum sRAGE and 429T/C polymorphism may be associated with pathogenesis of DR in Pakistani population. Circulating sRAGE protein and RAGE gene SNP may be a useful biomarker to predict vascular disease. In diabetic vascular complication, down-regulation of RAGE expression might be a favourable target for therapeutic intervention.

Consistent with our findings, Kerkeni et al. found significantly raised serum sRAGE, AGEs and pentosidine levels in PDR compared to NPDR in Tunisian patients.[21] Hamid et al. found significantly elevated plasma sRAGE levels with decreased total antioxidant status in PDR subjects compared to DWR in Egyptian population.[22] Increased vitreous sRAGE was also found in PDR patients, indicating a role of RAGE-axis in the pathogenesis of proliferative retinal diseases.[23] The current study found a positive correlation between serum sRAGE and FPG in DR group. Generally, it is considered that AGEs and sRAGE both become elevated in hyperglycaemia, but the increase in AGEs is more, leading to high AGE/sRAGE ratio.[24] Up-regulation of serum sRAGE in chronic hyperglycaemia acts as a counter-system against endothelial cell damage and vasotoxic influence of AGE-RAGE axis.[25]

In contrast to these findings, several studies show that sRAGE levels are decreased in T2DM and DR patients[26,27] possibly due to increased binding with circulating AGEs and consumption of all endogenous mechanisms that release sRAGE.

Further studies on a larger scale are needed to assess whether this RAGE gene polymorphism is related to DR and to clarify the relationship between DR and serum sRAGE levels in Pakistani population.

Conclusion

There was a moderate association of SNP rs1800625 with severe NPDR. The TC and CC genotypes of SNP rs1800625 were strongly associated with higher sRAGE levels in DR patients. Also, sRAGE was found to be a potential substitute marker of RAGE expression and the severity of target organ damage.

Disclaimer: The text is based on a PhD thesis.

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