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Nanopore Sequencing of *RAGE* Gene Polymorphisms and Their Association with Type 2 Diabetes

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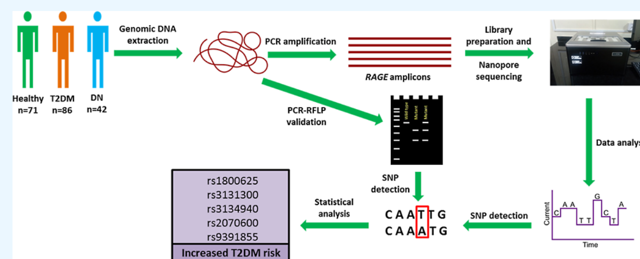
ABSTRACT: The receptor for advanced glycation end products (RAGE) is a transmembrane protein that interacts with its ligands, advanced glycation end products (AGEs). AGEs are elevated in diabetes and diabetic complications, leading to increased oxidative stress and activation of pro-inflammatory pathways facilitated by AGE−RAGE signaling. Polymorphisms in the *RAGE* gene can potentially affect AGE−RAGE interaction and its downstream signaling, which plays a crucial role in the progression of diabetes and its complications. In this study, we used nanopore sequencing for genotyping of *RAGE* polymorphism and identified a maximum number of 33 polymorphisms, including two previously unreported novel mutations in a cohort of healthy, type 2 diabetics without nephropathy and type 2 diabetics with nephropathy in order to identify associations. Two novel *RAGE* polymorphisms in the intron 8 and 3′UTR region at genomic locations 32181834 and 32181132, respectively, were detected with a low frequency. For four previously reported polymorphisms, cross-validation by PCR-RFLP showed 99.75% concordance with nanopore sequencing. Analysis of genotype distribution and allele frequencies revealed that five single nucleotide polymorphisms, i.e., rs1800625, rs3131300, rs3134940, rs2070600, and rs9391855, were associated with an increased risk for type 2 diabetes.



1. INTRODUCTION

Diabetes is a complex metabolic disorder caused by insufficient insulin secretion, as in type 1 diabetes mellitus, or insulin resistance, as in type 2 diabetes mellitus. According to the International Diabetes Federation, 537 million adults worldwide had diabetes in 2021, which is projected to increase to 783 million by 2045. Chronic hyperglycemic condition in diabetics promotes the development of several microvascular and macrovascular complications, such as diabetic retinopathy, diabetic nephropathy, diabetic neuropathy, and cardiovascular diseases.[1] Among these, diabetic nephropathy is a major complication of diabetes, and 20−40% of diabetic patients develop diabetic nephropathy in their lifetime.[2,3] It is a leading cause of end-stage renal disease.[4]

Several etiological factors, including lifestyle, genetics, environment, and ethnicity, are among the major factors responsible for the development of diabetes and its complications.[5−7] Among these, genetic factors are responsible for differential susceptibility to the progression of diabetes and its complications. Differential expression and polymorphism in several genes have been reported to be associated with diabetes and its complications, such as *PPARG*, *IRS-1*, *IRS-2*, *IL-1β*, *SLC2A2*, *PI3KR1*, *VEGF*, *ELMO1*, *ADIPOQ*, and *RAGE*.[8−12] The *RAGE* gene is highly polymorphic, and *RAGE* polymorphisms are among the most investigated for their association with numerous diseases.[11] The *RAGE* gene encodes for a protein called receptor for advanced glycation end products. It is a membrane-bound multiligand receptor belonging to the immunoglobulin superfamily. It consists of an extracellular variable domain, two extracellular constant domains, one transmembrane domain, and one cytosolic domain. RAGE binds to AGEs, and this AGE−RAGE interaction activates the nuclear transcription factor NF-κB, which increases cytokine production, oxidative stress, and inflammation and contributes to the pathogenesis of diabetes and its complications.[1,13,14]

The involvement of RAGE in diabetes and diabetic nephropathy has been well established.[11] The single nucleotide polymorphisms (SNPs) present in the exonic regions of the *RAGE* gene can cause non-synonymous replacement of the amino acids that can alter the RAGE structure.[15] Alteration in the RAGE structure can potentially affect its binding with its ligands and downstream signaling. Also, SNPs present in the promoter region of *RAGE* can affect the transcriptional activity of *RAGE*, hence affecting its expression.[16] Although SNPs in

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the intron region may not directly affect the RAGE structure, they might affect the splicing of RAGE mRNA, leading to the expression of different RAGE isoforms.[11] The sequences for 13 different splice variants of RAGE have been reported and are available in GenBank (ncbi.nlm.nih.gov/genbank, Oct 2022).

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) is routinely used for SNP detection. However, it is not a high-throughput method as it detects only one SNP at a time and is time-consuming.[17] Moreover, by PCR-RFLP, one can interrogate only previously reported mutations. On the other hand, DNA sequencing facilitates the identification of all variations associated with the locus of interest. Oxford Nanopore Technology (ONT) (nanoporetech.com) based DNA sequencing is a third-generation nucleic acid sequencing technology that has recently gained attention for rapid and high-throughput sequencing of DNA and RNA. The ONT approach generates long-read sequences with high accuracy and is increasingly used for high-throughput *de novo* sequencing and SNP detection.[18−20]

Several studies have reported the association of *RAGE* polymorphisms with the risk of diabetes and its complications;[11] however, the reported findings are often contradictory and may have missed many mutations due to reliance on techniques such as PCR-RFLP. In this study, nanopore sequencing of the *RAGE* gene was performed to detect all *RAGE* polymorphisms (known and novel) in a cohort population from Pune, India, and to evaluate the possibility of establishing this technique as a robust and routine diagnostic method for SNP detection. To compare and cross-validate the findings from nanopore sequencing, we used the PCR-RFLP method to interrogate the four most commonly reported SNPs of the *RAGE* gene, i.e., rs3134940, rs1800624, rs1800625, and rs2070600 from the samples. Statistical analyses were performed to check for the association of detected *RAGE* SNPs with type 2 diabetes and diabetic nephropathy. Further, we quantified diabetes-associated biochemical markers, such as fasting blood glucose (FBG) and HbA_{1c} (glycated hemoglobin). We also measured plasma fructosamine, glycated albumin, an inflammatory cytokine, i.e., tumor necrosis factor-α (TNF-α), malondialdehyde (MDA), fasting insulin, homeostatic model assessment for insulin resistance (HOMA-IR), and C-reactive protein (CRP). Finally, we assessed these clinical and biochemical parameters for their association with type 2 diabetes-associated *RAGE* SNPs. Since diabetes and its complications remain asymptomatic until the appearance of the first symptoms or diagnosis,[2,21] it is crucial to detect them early to reverse, stop, or regress the progression of diabetes and its complications. Any association of SNPs with the risk of diabetes or diabetic nephropathy development can serve as a disease risk biomarker, enabling the screening of individuals vulnerable to disease development at a much earlier stage for early preventive measures.

2. MATERIALS AND METHODS

2.1. Study Design. We designed a case−control study to check for the association of *RAGE* SNPs with type 2 diabetes. A few of the diabetic subjects had diabetic nephropathy, and hence *RAGE* SNPs were also evaluated for their association with diabetic nephropathy. DNA was extracted from the blood samples of healthy, type 2 diabetics without nephropathy (DM) and type 2 diabetics with nephropathy (DN). The full-length *RAGE* gene was PCR amplified from each sample, and the amplicons were sequenced by ONT for the purpose of identifying all genetic variations associated with the gene (such as SNPs and in-dels). This approach enabled us to determine the genotype and allele frequencies of the detected *RAGE* SNPs in each subject group and the possibility of detecting any novel polymorphisms in the *RAGE* gene in our cohort group. Appropriate statistical analyses were performed to determine the association of specific genotypes and alleles with type 2 diabetes and diabetic nephropathy. Four previously reported SNPs (rs3134940, rs1800624, rs1800625, and rs2070600), which were also detected by nanopore sequencing in the samples used in this study, were cross-validated by the PCR-RFLP method in all of the samples. To assess the effect of type 2 diabetes-associated SNPs, the levels of diabetes-associated biochemical markers such as FBG, HbA_{1c}, fructosamine, glycated albumin, TNF-α, MDA, fasting insulin, HOMA-IR, and CRP were compared between the wild-type and carrier genotype subjects.

2.2. Subjects. A total of 128 subjects who were confirmed as type 2 diabetic, with or without diabetic nephropathy, and 71 unrelated healthy subjects were recruited in this cross-sectional study at Bharati Vidyapeeth (DTU) Medical College, Pune, India. Subjects having FBG ≥126 mg/dL were diagnosed as type 2 diabetic based on the 2003 American Diabetes Association diagnostic criteria for diabetes.[22] Type 2 diabetic subjects were further divided into two subgroups, i.e., without diabetic nephropathy (n = 86), having a urinary-albumin-to-creatinine ratio <30 mg/g, and with diabetic nephropathy (n = 42), having a urinary-albumin-to-creatinine ratio ≥30 mg/g. Type 2 diabetic subjects with diabetic complications other than diabetic nephropathy, type 1 diabetic subjects, pregnant women, subjects with angina or heart failure, and patients suffering from severe concurrent illness were excluded from the study. This study was approved by the ethics committee of Bharati Vidyapeeth (DTU) Medical College, Pune, and each participant gave written informed consent before participating.

2.3. Biochemical Analysis. Clinical and biochemical parameters such as age, sex, FBG, HbA_{1c}, blood urea, serum creatinine, cholesterol, triglyceride, high-density lipoprotein (HDL), fasting insulin, and CRP were measured for each participating subject at Bharati Vidyapeeth (DTU) Medical College, Pune. Besides these parameters, the plasma concentration of total protein (estimated by Bradford's method), fructosamine (using the fructosamine assay kit from Abbexa Ltd, Cambridge, UK), albumin (using bromocresol green albumin assay kit MAK124 from Sigma-Aldrich), glycated albumin (using the Human glycated albumin ELISA kit, CSB-E09599h, Cusabio, China), and MDA (using lipid peroxidation assay kit from Sigma-Aldrich), were determined. The plasma fructosamine concentration was normalized to total plasma protein concentration and was expressed as μM/g of plasma protein. To determine the plasma glycated albumin concentration, a four-parameter logistic regression curve was plotted using the web tool GainData ELISA data calculator (Arigo Biolaboratories, Hsinchu City 300, Taiwan). Glycated albumin concentration was normalized with respective plasma albumin concentrations and expressed in μM/g of albumin. HOMA-IR was calculated based on the following formula.[23]

$$\text{HOMA-IR} = [\text{fasting glucose (mmol/L)} \times \text{fasting insulin (mIU/L)}]/\ 22.5$$

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2.4. Nanopore DNA Sequencing of *RAGE* Gene. *2.4.1. DNA Isolation from Blood and PCR Amplification of RAGE Gene.* The genomic DNA was extracted from whole blood collected from subjects enrolled in the study using a DNA isolation kit (Qiagen, Hilden, Germany) and used as template DNA to amplify the *RAGE* gene. The nucleotide sequence of the *RAGE* gene was retrieved from NCBI with gene ID-177 (Oct 2022). Forward and reverse primers were designed such that the complete *RAGE* gene could be amplified along with the promoter region and 5′UTR and 3′UTR regions. The nucleotide sequence of the forward primer was 5′-GGGCAGTTCTCTCCTCACTT-3′ and that of the reverse primer was 5′-GCAAAGTTCCCTCT-GACTCTTCC-3′. All PCR amplifications were performed in 25 μL reaction volume using the LongAmp Taq 2X master mix PCR reactions (New England BioLabs Inc, Ipswich, MA, USA) with the addition of 25 ng of genomic DNA as a template and 0.2 μmol/L of each primer. The PCR reactions were run on a Veriti 96-well thermal cycler (Applied Biosystems, Foster City, California, USA) using the following protocol: initial heating of the reaction mixture at 95 °C for 10 min, followed by 35 cycles of denaturation at 95 °C for 30 s, primer annealing at 61 °C for 30 s, and extension at 65 °C for 5 min. The final extension was done at 65 °C for 10 min, after which the reaction mixture was cooled to 4 °C.

2.4.2. Oxford Nanopore DNA Sequencing Library Preparation. For sequencing the DNA amplicons, the sequencing library was prepared using the Oxford Nanopore Technologies Ligation Sequencing (SQK-LSK109) and Native Barcoding (EXP-NBD196) Kits. The *RAGE* amplicons were first purified using 1X AMPure XP beads (Beckman Coulter, USA), and the DNA concentration was measured using Qubit dsDNA BR Assay Kit (Invitrogen, USA) on a Qubit 2.0 fluorometer (Invitrogen, USA). A total of 200 fmol of each amplicon, i.e., 517 ng, was taken forward for the end-repair step, where the 3′ end was A-tailed using NEBNext Ultra II End Prep Enzyme Mix (New England BioLabs Inc, USA). The reaction mixture was incubated at 20 °C for 10 min, followed by heating at 65 °C for 10 min. Following the end-repair step, the amplicons were attached with barcodes available from the EXP-NBD196 kit (Oxford Nanopore Technologies, UK) using the 1X Blunt/TA ligase reaction (New England BioLabs Inc, USA). The reaction mixture was incubated at RT for 20 min, followed by heating at 65 °C for 10 min to denature the enzyme and stop the ligation reaction. The barcoded samples were kept in ice for 1 min and pooled, and the pooled barcoded library was purified by using the AMPure XP beads (0.4X of sample volume) from the SQK-LSK109 kit (Oxford Nanopore Technologies, UK). The washed library was eluted in 35 μL nuclease-free water and quantified by the Qubit 2.0 fluorometer. Next, the sequencing adapters were added to the ends of the barcoded DNA fragments by combining 200 fmol of the purified library with Adapter Mix II (Oxford Nanopore Technologies, UK) and Quick T4 ligase (New England BioLabs Inc, USA) and incubating this reaction mixture for 30 min at room temperature. The adapter-ligated library was purified using 1X AMPure XP beads according to the manufacturer's instructions and eluted in 15 μL elution buffer available from the SQK-LSK109 kit (Oxford Nanopore Technologies, UK). The final adapter-ligated purified library (50 fmol) was loaded onto the FLO-MIN106D flow cell, and sequencing was done on the GridION MK1 sequencer (Oxford Nanopore Technologies, UK) until at least 200 sequence reads were collected for each barcode.

2.4.3. Analysis of Oxford Nanopore Sequence Reads and Variant Calling. While sequencing the samples, real-time base calling and demultiplexing were carried out using the Guppy v.5.0.14 program, which is part of the MinKNOW v21.05.12 operating software (Oxford Nanopore Technologies, UK), which runs the GridION sequencer. The super-accurate base calling model was selected for base calling, and demultiplexing was performed using the "require barcodes at both ends" option. A bash script was written to perform all the steps required for further analysis, such as length filtering, variant calling, and generation of consensus assembly. The bash script can be accessed through the GitHub link https://github.com/ajinkyakhilari/ampvar. After the demultiplexing step, the read length and quality filtering was carried out using the NanoFilt software v2.5.0.[24] Minimap2 v2.24[25] was used to align the length and quality-filtered nanopore reads to the sequence of full-length human chromosome 6 (where the *RAGE* gene is located) downloaded from GenBank (ncbi.nlm.nih.gov/genome/gdv/browser/gene/?id=7124, Oct 2022). The individual aligned reads were sorted according to genomic coordinates using the SAMtools v1.14[26] sort command, and variant calling was performed on the aligned and sorted reads using the Nanopolish v0.13[27] variant caller with the ploidy set to 2. Thereafter, *de novo* assembly and read polish were carried out using Canu v2.2,[28] Racon v1.4.3,[29] and Medaka v1.5[30] programs using their default parameter settings. Variant calling and annotation were performed using the SnpEff v4.3[31] from the Galaxy ToolShed,[32] and all *RAGE* SNPs detected were analyzed for linkage disequilibrium using the web tool SNPStats.[33]

2.5. Genotyping of the *RAGE* Gene by PCR-RFLP. Four previously reported SNPs of the *RAGE* gene (rs3134940, rs1800624, rs1800625, and rs2070600) were detected by nanopore sequencing in the samples analyzed in this study, and these were verified using the PCR-RFLP method. Previously reported primers were used to amplify the regions of the *RAGE* gene containing these SNPs,[34−36] except the reverse primer for rs1800624. The primer sequences used for PCR amplification and the length of PCR products are summarized in Table 1.

Table 1. PCR-RFLP Primer Pair Used for the Amplification of a Region Spanning the Polymorphic Site of the *RAGE* Gene and the Size of PCR-Amplified Product

SNP	primer sequence	PCR product size (bp)
rs3134940	forward primer: 5′-TAATTTCCTGCCCCATTCTG-3′	396
	reverse primer: 5′-CATCGCAATCTATGCCTCCT-3′	
rs1800624	forward primer: 5′-GGGGCAGTTCTCTCCTCACT-3′	502
	reverse primer: 5′-CGTCTTGTCACAGGGAATGC-3′	
rs1800625	forward primer: 5′-GGGGCAGTTCTCTCCTCACT-3′	249
	reverse primer: 5′-GGTTCAGGCCAGACTGTTGT-3′	
rs2070600	forward primer: 5′-GTAAGCGGGGCTCCTGTTGCA-3′	397
	reverse primer: 5′-GGCCAAGGCTGGGGTTGAAGG-3′	

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Table 2. Restriction Enzymes Used for SNP Detection by PCR-RFLP, Restriction Sites, and the Size of Restriction Digestion Products

SNP	restriction enzyme	restriction site	sequence in wild-type allele	sequence in mutant allele	restriction digestion product size (bp)	
					wild-type	mutant
rs3134940	BsmF1	GGGAC (10/14)[a]	GG**A**AC	GG**G**AC	396	160 + 236 (RS creation)
rs1800624	Mfe1	C/AATTG	CA**A**TTG	CA**A**ATG	215 + 287	502 (RS deletion)
rs1800625	Alu1	AG/CT	AG**T**T	AG**C**T	249	89 + 160 (RS creation)
rs2070600	Alu1	AG/CT	**G**GCT	**A**GCT	248 + 149	181 + 67 + 149 (RS creation)

[a]BsmFI cuts 10 nucleotides away from the restriction site in the same DNA strand and 14 nucleotides away in the complementary DNA strand. The nucleotide at the polymorphic site is represented by bold font. RS: restriction site.

Table 3. Clinical Characteristics of Healthy Control, DM, DN, and DM + DN Subjects[a]

clinical characteristics	healthy	DM	DN	DM + DN
age (years)	55.77 ± 10.16	56.4 ± 9.58	58.71 ± 11.27	57.16 ± 10.18
sex (male/female)	37/34	43/43	27/15	70/58
FBG (mg/dL)	99.73 ± 8.28	218.9 ± 79.48[†]	251.5 ± 78.06[†,‡]	229.6 ± 80.19[†]
HbA_{1c} (%)	5.54 ± 0.59	9.15 ± 2.16[†]	8.52 ± 1.91[†]	8.94 ± 2.1[†]
blood urea (mg/dL)	22.93 ± 6.83	24.52 ± 7.85	87.71 ± 28.63[†,‡]	45.26 ± 34.54[†]
serum creatinine (mg/dL)	0.82 ± 0.14	0.88 ± 0.19	4.8 ± 1.99[†,‡]	2.17 ± 2.17[†]
cholesterol (mg/dL)	149.3 ± 35.28	159.7 ± 43.71	177.8 ± 44.24[†]	165.7 ± 44.54[†]
triglyceride (mg/dL)	106.3 ± 34.92	138.9 ± 75.99[†]	187.1 ± 85.05[†,‡]	154.7 ± 81.95[†]
HDL (mg/dL)	42.27 ± 9.18	38.81 ± 12.01	34.68 ± 11.18[†]	37.45 ± 11.86[†]
normalized fructosamine (μM/g)	25.86 ± 10.06 (n = 51)	38.26 ± 13.61[†] (n = 64)	40.66 ± 15.00[†] (n = 40)	39.19 ± 14.14[†] (n = 104)
normalized glycated albumin (μM/g)	0.77 ± 0.31 (n = 51)	1.02 ± 0.39[†] (n = 64)	1.18 ± 0.44[†] (n = 40)	1.08 ± 0.41[†] (n = 104)
TNF-α (pg/mL)	16.97 ± 22.00 (n = 26)	18.97 ± 19.83 (n = 22)	37.76 ± 54.28 (n = 28)	29.50 ± 43.36 (n = 50)
MDA (nM/mL)	39.18 ± 28.22 (n = 29)	51.81 ± 33.8 (n = 24)	46.18 ± 31.72 (n = 31)	48.64 ± 32.46 (n = 55)
insulin (μM/mL)	17.38 ± 16.66 (59)	17.69 ± 17.3 (68)	20.19 ± 48.04 (36)	18.55 ± 31.31 (104)
HOMA-IR	4.23 ± 4.12 (59)	8.96 ± 7.68 (68)	14.01 ± 38.27 (36)	10.71 ± 23.28 (104)
CRP (mg/L)	16.33 ± 39.97 (53)	35.37 ± 60.31 (66)	53.15 ± 78.37 (36)	41.65 ± 67.39 (102)

[a]Data are presented as mean ± SD or number of subjects. Number of subjects: healthy, n = 71; DM, n = 86; DN, n = 42; unless otherwise mentioned, [†]$p < 0.05$ vs healthy; [‡]$p < 0.05$ vs DM. HDL: high-density lipoprotein; TNF-α: tumor necrosis factor-α.

All PCR amplifications were performed in 15 μL reaction volume by adding 10 ng of genomic DNA as a template and 0.3 μmol/L of each primer of primer pair in the master mix for PCR reactions (New England BioLabs Inc, USA). The PCR amplification protocol was the same as that used for amplifying the *RAGE* gene, except that the primer annealing temperature was set at 62 °C. The restriction digestion of the PCR amplicons was carried out using specific restriction enzymes (New England BioLabs Inc, USA) for each SNP, the details of which are shown in Table 2. The digested PCR products were resolved on a 2.5% (w/v) agarose gel, and the DNA bands were visualized under UV light. The number and size of DNA bands enabled differentiation between homozygous wild-type, heterozygous, and homozygous mutant subjects for the particular SNP, as shown in Table 2.

2.6. Statistical Analysis. All statistical analyses were performed using GraphPad Prism for Windows version 8.0.2 (GraphPad Software Inc, California, USA) or SPSS version 17.0 (SPSS Inc. Chicago, IL, USA). The clinical characteristics of the subjects were expressed as mean ± standard deviation (SD). The clinical characteristics and biochemical parameters across the groups were compared by one-way analysis of variance (ANOVA) followed by a *post hoc* Tukey's test. An unpaired Student's t-test was used to compare the biochemical parameters between wild-type and carriers (heterozygous and homozygous mutants). Intergroup genotype and allele distributions were compared by the Chi-square test, but Fisher's exact test was used for comparing genotype and allele distribution in cases where the number of subjects in any of the groups being compared was less than five. Logistic regression analysis was performed to check the association of the *RAGE* SNPs with clinical parameters. A p-value <0.05 was considered statistically significant. The odds ratio (OR) and 95% confidence interval (CI) were calculated.

3. RESULTS

3.1. Clinical Characteristics of Healthy Control, DM, DN, and DM + DN Subjects. The clinical characteristics of all the participating subjects are summarized in Table 3. There was no significant difference in the age of the participants in the different groups. The DM, DN, and DM + DN groups had significantly higher FBG, HbA_{1c}, and triglyceride levels than the healthy control group. FBG and triglycerides in the DN group were higher than in the DM group. Cholesterol levels were higher in the DN and DM + DN groups than in the healthy group. Blood urea and serum creatinine, which reflect the nitrogenous waste content in the blood, were significantly higher in the DN group than in the DM and healthy control groups. Blood urea and serum creatinine were also higher in the DM + DN group than in the healthy control group. The DN and DM + DN groups had higher cholesterol and lower HDL levels than the healthy group.

Normalized fructosamine concentration, which reflects a measure of glycation of all plasma proteins, was significantly elevated in the DM, DN, and DM + DN groups compared to the healthy control group. Similarly, the normalized glycated

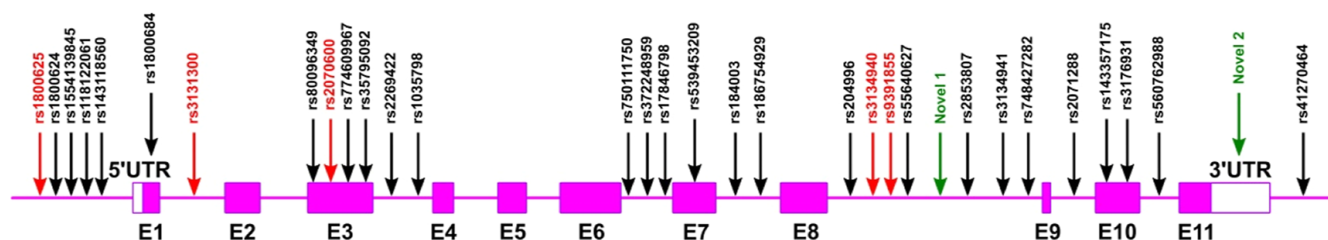

Figure 1. Schematic representation of the *RAGE* gene depicting the location of the polymorphisms detected. The locations of all the polymorphisms detected in this study are shown by arrows. Previously reported polymorphisms are shown by black arrows, whereas the two novel polymorphisms are indicated by green arrows. SNPs associated with type 2 diabetes are shown by red arrows.

Table 4. SNPs Detected in the *RAGE* Gene, Their Position on Chromosome 6, Location on the Gene, Nucleotide Change, and Genotype Distribution Among Healthy, DM, DN, and DM + DN Groups

				healthy		DM		DN		DM + DN	
polymorphism	genomic position	location	wild-type allele/mutated allele	wild-type	mutant	wild-type	mutant	wild-type	mutant	wild-type	mutant
rs1800625	32184665	upstream	T/C	56	15	55	31	31	11	86	42
rs1800624	32184610	upstream	T/A	50	16	64	20	28	11	92	31
rs1554139845	32184580	upstream	63 bp deletion[a]	66	5	84	2	39	3	123	5
rs118122061	32184479	upstream	G/A	70	1	83	3	41	1	124	4
rs143118560	32184478	upstream	A/T	71	0	85	1	42	0	127	1
rs1800684	32184217	exon 1	T/A	68	3	83	3	39	3	122	6
rs3131300	32184157	intron 1	T/C	56	15	55	31	31	11	86	42
rs80096349	32183681	exon 3	C/T	70	1	86	0	42	0	128	0
rs2070600	32183666	exon 3	G/A	60	11	61	25	31	11	92	36
rs774609967	32183650	exon 3	C/T	70	1	86	0	42	0	128	0
rs35795092	32183643	exon 3	C/G	68	3	86	0	42	0	128	0
rs2269422	32183517	intron 3	A/G	64	7	78	8	37	5	115	13
rs1035798	32183445	intron 3	C/T	52	19	66	20	31	11	97	31
rs750111750	32182808	intron 6	G/A	71	0	86	0	41	1	127	1
rs372248959	32182728	intron 6	T/C	71	0	84	2	42	0	126	2
rs17846798	32182721	intron 6	C/T	66	5	84	2	39	3	123	5
rs539453209	32182697	exon 7	G/C	70	1	86	0	42	0	128	0
rs184003	32182519	intron 7	G/T	43	28	51	35	25	17	76	52
rs186754929	32182494	intron 7	G/A	70	1	86	0	40	2	126	2
rs204996	32182106	intron 8	G/A	58	13	76	10	36	6	112	16
rs3134940	32182039	intron 8	A/G	56	15	55	31	31	11	86	42
rs9391855	32182024	intron 8	G/A	60	11	61	25	31	11	92	36
rs55640627	32181979	intron 8	G/A	67	4	81	5	41	1	122	6
novel 1	32181834	intron 8	G/A	70	1	86	0	42	0	128	0
rs2853807	32181795	intron 8	C/T	58	13	76	10	36	6	112	16
rs3134941	32181760	intron 8	G/C	56	15	58	28	31	11	89	39
rs748427282	32181673	intron 8	C/T	71	0	85	1	42	0	127	1
rs2071288	32181483	intron 9	G/A	66	5	84	2	39	3	123	5
rs143357175	32181442	exon 10	C/T	66	5	81	5	36	6	117	11
rs3176931	32181363	exon 10	G/A	66	5	84	2	41	1	125	3
rs560762988	32181266	intron 10	C/T	71	0	86	0	41	1	127	1
novel 2	32181132	3′ UTR	CAG/G	70	1	83	3	41	1	124	4
rs41270464	32180947	downstream	G/A	66	5	79	7	38	4	117	11

[a]63 bp deletion: TTCCCCAGCCTTGCCTTCATGATGCAGGCCCAATTGCACCCTTGCAGACAACAGTCTGGCCTGA/A.

albumin concentration was higher in the DM, DN, and DM + DN groups than in the healthy control group. However, there was no significant difference between the DM and DN groups in normalized fructosamine and normalized glycated albumin levels. The plasma levels of inflammatory cytokine TNF-α, MDA, insulin, HOMA-IR, and CRP were not significantly different between the subject groups.

3.2. PCR Amplification and SNP Detection by Nanopore Sequencing of the *RAGE* Gene.

PCR amplification of the *RAGE* gene was verified by agarose gel electrophoresis and confirmed to be in the expected size of ~4 kb (Figure S1). From nanopore sequencing of the *RAGE* gene amplicons, a total of 33 polymorphisms, including 8 exon variants, 18 intron variants, 5 upstream variants, 1 3′UTR variant, and 1 downstream variant of the *RAGE* gene, were detected. During validation by PCR-RFLP analysis, we observed that there was a discrepancy in the detection of rs3134940 by nanopore sequencing for one healthy and one diabetic sample. To resolve this discrepancy, Sanger sequencing of the amplicons spanning the mutation was carried out for these two samples

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Table 5. Association of Polymorphisms Detected in the *RAGE* Gene with Type 2 Diabetes and Diabetic Nephropathy[a]

	healthy vs DM			healthy vs DN			DM vs DN			healthy vs DM + DN		
polymorphism	χ^2	p	OR (95% CI)	χ^2	p	OR (95% CI)	χ^2	p	OR (95% CI)	χ^2	p	OR (95% CI)
rs1800625	**4.179**	**0.041**	**2.104 (1.039–4.396)**	0.382	0.537	1.325 (0.545–3.351)	1.243	0.265	0.63 (0.292–1.415)	3.051	0.081	1.823 (0.933–3.546)
rs1800624	0.004	0.951	0.977 (0.467–2.124)	0.202	0.654	1.228 (0.501–3.104)	0.273	0.601	1.257 (0.545–2.89)	0.021	0.884	1.053 (0.543–2.073)
rs1554139845		0.246	0.314 (0.061–1.547)		>0.999	1.015 (0.258–4.174)		0.33	3.231 (0.633–18.59)	0.941	0.332	0.537 (0.168–1.723)
rs118122061		0.627	2.53 (0.369–33.28)		>0.999	1.707 (0.088–32.84)		>0.999	0.675 (0.051–4.656)		0.657	2.258 (0.362–27.99)
rs143118560												
rs1800684		>0.999	0.819 (0.187–3.602)		0.669	1.744 (0.39–7.713)		0.393	2.128 (0.477–9.38)		>0.999	1.115 (0.294–4.17)
rs3131300	**4.179**	**0.041**	**2.104 (1.039–4.396)**	0.382	0.537	1.325 (0.545–3.351)	1.243	0.265	0.63 (0.292–1.415)	3.051	0.081	1.823 (0.933–3.546)
rs80096349												
rs2070600	**4.057**	**0.044**	**2.235 (1.042–4.773)**	1.926	0.165	1.935 (0.741–5.059)	0.116	0.734	0.866 (0.393–2.019)	**4.039**	**0.044**	**2.134 (1.035–4.667)**
rs774609967												
rs35795092												
rs2269422	0.014	0.906	0.938 (0.313–2.497)	0.116	0.733	1.236 (0.408–3.937)	0.209	0.647	1.318 (0.454–3.984)	0.004	0.947	1.034 (0.414–2.648)
rs1035798	0.256	0.613	0.829 (0.415–1.667)	0.004	0.947	0.971 (0.417–2.264)	0.132	0.716	1.171 (0.515–2.64)	0.157	0.692	0.875 (0.448–1.704)
rs750111750												
rs372248959												
rs17846798		0.246	0.314 (0.061–1.547)		>0.999	1.015 (0.258–4.174)		0.33	3.231 (0.633–18.59)	0.941	0.332	0.537 (0.168–1.723)
rs539453209												
rs184003	0.026	0.873	1.054 (0.555–2.025)	0.012	0.913	1.044 (0.48–2.282)	0.001	0.981	0.991 (0.46–2.043)	0.027	0.87	1.051 (0.585–1.938)
rs186754929					0.554	3.5 (0.393–51.33)					>0.999	1.111 (0.127–16.31)
rs204996	1.389	0.239	0.587 (0.236–1.409)	0.306	0.58	0.744 (0.256–2.142)	0.182	0.669	1.267 (0.416–3.55)	1.238	0.266	0.637 (0.295–1.411)
rs3134940	**4.179**	**0.041**	**2.104 (1.039–4.396)**	0.382	0.537	1.325 (0.545–3.351)	1.243	0.265	0.63 (0.292–1.415)	3.051	0.081	1.823 (0.933–3.546)
rs9391855	**4.057**	**0.044**	**2.235 (1.042–4.773)**	1.926	0.165	1.935 (0.741–5.059)	0.116	0.734	0.866 (0.393–2.019)	**4.039**	**0.044**	**2.134 (1.035–4.667)**
rs55640627		>0.999	1.034 (0.294–3.478)		0.649	0.409 (0.033–2.632)		0.663	0.395 (0.033–3.084)		0.747	0.824 (0.211–2.663)
Novel 1												
rs2853807	1.389	0.239	0.587 (0.236–1.409)	0.306	0.58	0.744 (0.256–2.142)	0.182	0.669	1.267 (0.416–3.55)	1.238	0.266	0.637 (0.295–1.411)
rs3134941	2.556	0.11	1.802 (0.874–3.805)	0.382	0.537	1.325 (0.545–3.351)	0.54	0.462	0.735 (0.338–1.677)	2.016	0.156	1.636 (0.829–3.203)
rs748427282												
rs2071288		0.246	0.314 (0.061–1.547)		>0.999	1.015 (0.258–4.174)		0.33	3.231 (0.633–18.59)	0.941	0.332	0.537 (0.168–1.723)
rs143357175	0.098	0.754	0.815 (0.252–2.639)	1.576	0.209	2.2 (0.579–6.928)	2.578	0.108	2.7 (0.715–8.434)	0.149	0.7	1.241 (0.435–3.328)
rs3176931		0.246	0.314 (0.061–1.547)		0.409	0.322 (0.027–2.533)		>0.999	1.024 (0.069–9.004)		0.137	0.317 (0.082–1.255)
rs560762988												
Novel 2		0.627	2.53 (0.369–33.28)		>0.999	1.707 (0.088–32.84)		>0.999	0.675 (0.051–4.656)		0.657	2.258 (0.362–27.99)
rs41270464	0.066	0.797	1.17 (0.383–3.405)		0.725	1.389 (0.405–5.045)		0.75	1.188 (0.37–4.1)	0.149	0.7	1.241 (0.435–3.328)

[a] χ^2: Chi-square value; OR: odds ratio; CI: confidence interval, the text in the bold indicates significant association.

(Table S1). In this case, the results from Sanger sequencing agreed with the PCR-RFLP results, and based on this, the result of the two discrepant cases was rectified in the SNP distribution detected by nanopore sequencing. The details of the *RAGE* polymorphisms and their distribution in healthy, DM, DN, and DM + DN groups are presented in Figure 1 and Table 4, respectively.

Out of the 33 *RAGE* polymorphisms detected, two SNPs are reported here for the first time, and these were not found in dbSNP (https://www.ncbi.nlm.nih.gov/snp, Oct 2022). One of these two novel variants is located on intron 8, causing a nucleotide change from G to A at position 32181834 of chromosome 6, and was detected in 0.5% of the subjects. The other novel variant is a deletion mutation, causing the nucleotide change from CAG to G in the 3′UTR of the

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RAGE gene at position 32181132 of chromosome 6 and was detected in 2.6% of the subjects.

3.3. Association of the Mutations with Cohort Groups. Due to the low frequency of homozygous mutant genotypes of the *RAGE* polymorphisms, we performed association analyses using the dominant inheritance model. For this, the subjects were grouped into homozygous wild-type and carrier populations (including both the heterozygous and homozygous mutant genotypes). The result of the association study is summarized in Table 5. The SNP rs1554139845 causes insertion/deletion of the 63 bp stretch in the upstream region of the *RAGE* gene. Since rs1800624 is located within this 63 bp stretch, deletion of this 63 bp also causes the absence of the location at which the SNP rs1800624 occurs. Hence, 10 subjects in the present study carrying rs1554139845 were not considered while analyzing the genotype distribution and allele frequency of rs1800624. Among the 33 *RAGE* polymorphisms detected, five SNPs were associated with an increased risk of type 2 diabetes. The SNP rs1800625 was found at a significantly higher frequency in the DM group (OR = 2.104, p = 0.041) as compared to the healthy group. However, this SNP was not associated with the DN group. Similarly, when DM and DN groups were merged as a diabetic group (DM + DN) and compared to the healthy group, the extent of the association of rs1800625 with the merged diabetic group was reduced (OR = 1.823, p = 0.081). The SNPs rs3134940 and rs3131300 were found to be in perfect linkage disequilibrium with rs1800625 (Table S2). Hence, their association with the DM and DN groups was the same as that of rs1800625.

The SNP rs2070600 was significantly associated with the DM group compared to the healthy group (OR = 2.235, p = 0.044). However, rs2070600 was not associated with the DN group compared to the healthy or DM group. When DM and DN groups were merged as a diabetic group, rs2070600 was associated with the merged diabetic group (DM + DN) compared to the healthy group (OR = 2.134, p = 0.044). Thus, this SNP is more strongly associated with type 2 diabetes than the other three SNPs discussed earlier. The SNP rs9391855 was found in perfect linkage disequilibrium with rs2070600 (Table S2), and hence this SNP, too, was associated with DM and DM + DN group as compared to the healthy group. The rest of the polymorphisms detected did not show any association with the DM or DM + DN group. When the DN group was compared with the DM group as a control, none of the 33 polymorphisms detected were associated with the DN group. Association analysis couldn't be performed for many low-frequency polymorphisms due to the absence of carrier genotypes in one or more subject groups. A few other SNPs were also found to be in perfect linkage disequilibrium with each other (Table S2 and Figure S2). Wild-type major allele and mutated minor allele frequencies for *RAGE* polymorphisms were calculated and are presented in Table S3. The result of allele association with type 2 diabetes or diabetic nephropathy is shown in Table S4. We conclude that there was no association of any alleles detected from the *RAGE* gene with type 2 diabetes or diabetic nephropathy.

3.4. Validation of Mutations Identified in Nanopore Sequencing of the *RAGE* Gene by PCR-RFLP. PCR-RFLP was used to detect the SNPs rs3134940, rs1800624, rs1800625, and rs2070600 to compare and validate the nanopore sequencing results. These four SNPs were chosen for the comparison as they have a relatively high frequency than most of the other SNPs in the *RAGE* gene and have been most investigated for their association with diabetes and diabetic complications.[11] The restriction enzymes used for the digestion of PCR-amplified products for detecting these SNPs, restriction sites and the size of restriction-digested products for wild-type and mutant allele are summarized in Table 2. The nucleotide change caused by rs3134940, rs1800625, and rs2070600 results in the creation of a restriction site, whereas rs1800624 causes the deletion of the restriction site for the restriction enzymes, as mentioned in Table 2. A representative agarose gel image depicting DNA bands corresponding to undigested PCR-amplified products and restriction-digested products of different genotypes for the four SNPs is shown in Figure 2. The results of genotype distribution, allele frequencies, and their association with type 2 diabetes and diabetic nephropathy were the same, as shown in Tables 4−5 and Tables S3 and S4. There was 99.75% agreement between nanopore and PCR-RFLP genotyping results, with only two discrepant cases out of 199 samples for the four *RAGE* SNPs detected by the two methods (Table S1).

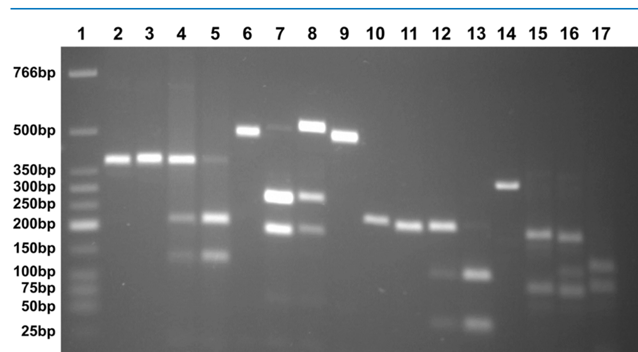

Figure 2. PCR-amplified and restriction-digested products for SNPs rs3134940, rs1800624, rs1800625, and rs2070600. Lane 1: Low-molecular-weight DNA ladder. Lane 2: PCR-amplified product for rs3134940. Lanes 3−5: Restriction digested products of wild-type (AA), heterozygous (AG), and homozygous mutant (GG) genotypes of rs3134940, respectively. Lane 6: PCR-amplified product for rs1800624. Lanes 7−9: Restriction digested products of wild-type (TT), heterozygous (TA), and homozygous mutant (AA) genotypes of rs1800624, respectively. Lane 10: PCR-amplified product of rs1800625. Lanes 11−13: Restriction digested products of wild-type (TT), heterozygous (TC), and homozygous mutant (CC) genotypes of rs1800625, respectively. Lane 14: PCR-amplified product of rs2070600. Lanes 15−17: Restriction digested products of wild-type (GG), heterozygous (GA), and homozygous mutant (AA) genotypes of rs2070600, respectively.

3.5. Association of Diabetes-Risk-Associated SNPs with Various Clinical Parameters. *RAGE* SNPs showing significant association with type 2 diabetes were investigated for the association of carrier genotypes of these SNPs with AGEs (fructosamine and glycated albumin), inflammatory cytokine (TNF-α), MDA, insulin, HOMA-IR, CRP, and diabetes diagnosis markers (FBG and HbA_{1c}). Carrier genotypes of none of these SNPs were associated with any of these clinical and biochemical parameters by unpaired Student's t-test (Table S5). The result of logistic regression analysis for the association of type 2 diabetes-associated *RAGE* SNPs with clinical parameters is shown in Table S6. While blood urea, insulin, and CRP were positively associated with rs1800625, rs3134940, and rs3131300, MDA and HOMA-IR

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Table 6. Comparison of the Results of the Present Study with Previous Reports

SNP	findings of this study	previous supportive reports	previous contradictory reports
rs1800625	increased risk of type 2 diabetes	increased type 1 diabetes risk[45]	no association with diabetes mellitus[39] and type 2 diabetes[37,38,40]
	no association with diabetic nephropathy	no association with diabetic nephropathy (in dominant model)[38,41]	increased risk of diabetic nephropathy[46]
rs3134940	increased risk of type 2 diabetes		no association with diabetes[47] or type 2 diabetes[40]
	no association with diabetic nephropathy		increased risk of diabetic nephropathy[48] and decreased risk of diabetic nephropathy[34]
rs2070600	increased risk of type 2 diabetes	increased risk of diabetes[47] and type 1 diabetes[49]	no association with type 2 diabetes[37,50]
	no association with diabetic nephropathy	no association with diabetic nephropathy[46,48,51−53]	

were negatively associated. Similarly, HDL showed a negative association with rs2070600 and rs9391855.

4. DISCUSSION

Diabetes is a multifactorial disease, and genetics plays a role in developing diabetes and its complications. Among various genes associated with diabetes and its complications, the *RAGE* gene is a potential candidate known to be involved in the pathogenesis of diabetes and diabetic complications. AGE−RAGE signaling increases the expression of inflammatory cytokines and reactive oxygen species, which exacerbate the progression of diabetes and its complications. As a result, polymorphism in the *RAGE* gene may influence the outcomes of the AGE−RAGE interaction. In this study, we first detected all *RAGE* polymorphisms from the subjects by nanopore sequencing of the respective *RAGE* gene amplicons and studied their association with type 2 diabetes and diabetic nephropathy. PCR-RFLP was performed for four *RAGE* SNPs, i.e., rs3134940, rs1800624, rs1800625, and rs2070600, to assess the accuracy and utility of nanopore sequencing as a high-throughput method of SNP detection over the conventional PCR-RFLP method.

An overall comparison of the findings of this study with the previous reports of *RAGE* SNP association with diabetes and diabetic nephropathy is summarized in Table 6. Previous studies have reported contradictory results on the association of *RAGE* SNPs with diabetes and its complications.[11] In this study, the SNP rs1800625 was associated with an increased risk of type 2 diabetes but not diabetic nephropathy. While some reports conclude no association of rs1800625 with type 1 and type 2 diabetes,[37−40] diabetic nephropathy,[38,41] and diabetic retinopathy,[42−44] other studies report an increased risk of type 1 diabetes[45] and diabetic nephropathy.[46] The SNP rs1800625 is present in the promoter region of the *RAGE* gene. One in vitro study has illustrated a two-fold increase in the expression of the reporter gene caused by the mutant allele of rs1800625.[16] Thus, the increased expression of RAGE may lead to increased AGE−RAGE interaction and downstream signaling, which may promote the pathogenesis of diabetes and its complications.

The intronic SNP rs3134940 was in perfect linkage disequilibrium with rs1800625. Hence, it was associated with an increased risk of type 2 diabetes but not diabetic nephropathy. However, contradictory to our finding, previous studies have reported no association of rs3134940 with type 2 diabetes[40,47] and an increased[48] or decreased risk[34] of diabetic nephropathy. This SNP was also not associated with chronic kidney disease[54] and diabetic retinopathy.[42,55,56] During mRNA processing, introns are spliced from pre-mRNA; therefore, any SNPs in the intron region are least likely to affect the protein. The mechanism by which rs3134940 can increase the risk of diabetes or diabetic complications is unknown. It has been proposed by various groups that rs3134940 may affect the alternative splicing of RAGE mRNA involving the region between intron 7 and 9, which could affect the expression of endogenous secretory RAGE (esRAGE), one of the RAGE isoforms.[34,55−58] Unlike RAGE, esRAGE is not involved in AGE−RAGE signaling as it lacks intramembrane and cytoplasmic domains. Thus, esRAGE acts as a decoy receptor for AGEs, and the physiological level of esRAGE can affect the extent of AGE−RAGE signaling and the associated pathogenesis. However, no experimental evidence has shown the effect of rs3134940 on esRAGE production.

Compared to the SNPs rs1800625, a stronger association of rs2070600 with type 2 diabetes was observed, even though it was not associated with diabetic nephropathy. These results agreed with those of the previous studies, which reported that rs2070600 increases the risk of diabetes[47,49] but not diabetic nephropathy.[46,51−53] However, there are reports contradicting these results, which claim no association between rs2070600 and type 2 diabetes.[37,50] No literature has reported a clear association between rs2070600 and diabetic nephropathy. The non-synonymous replacement of glycine by serine at the 82nd position of RAGE caused by rs2070600 induces changes in the structure of the variable domain of RAGE,[15] which can potentially affect AGE−RAGE signaling. In vitro studies have elucidated that RAGE with rs2070600 has a higher binding affinity for amyloid-beta peptides (Aβ42) and prototypic S100/cangrulin, which upregulated inflammatory mediators.[59,60] Substitution of glycine with serine at the 82nd position of RAGE promotes N-linked glycosylation of adjacent asparagine residue at the 81st position, which may also affect ligand binding and further downstream signaling initiated by AGE−RAGE interaction.[61] Several studies have also found a significantly lower plasma sRAGE level in the subjects carrying rs2070600,[42,53,62,63] and the same has been elucidated using a cell culture model.[64] Decreased plasma sRAGE levels might fail to sufficiently clear the already elevated plasma AGEs in diabetics, resulting in increased AGE−RAGE signaling. In this study, sRAGE levels were not measured; hence, the role of this SNP in type 2 diabetes development via sRAGE remains obscure. The discrepancies in the results of *RAGE* SNP association with type 2 diabetes and diabetic nephropathy in this study and previous studies can be attributed to the different environments, lifestyles, genetics, ethnicity, and geography of the study population.

In this study, nanopore sequencing showed a high accuracy of 99.75% compared to the result of PCR-RFLP. In fact,

several studies have reported even up to 100% concordance between the results of nanopore and Sanger sequencing, a gold standard approach.[65−68] The high accuracy of the current nanopore sequencing approaches makes it a rapid, cost-effective, and high-throughput approach that can be used for SNP detection, including in clinical diagnostics.[65]

This study reports two novel polymorphisms in our population at positions 32181834 and 32181132 of chromosome 6, which are located in intron 8 and 3′ UTR of the *RAGE* gene, respectively. These two SNPs were present at a very low frequency in our study population, and hence a large sample size would be required to sufficiently detect these novel polymorphisms and confidently check for their association with type 2 diabetes and diabetic nephropathy. We also found that none of the SNPs detected were associated with diabetic nephropathy, which could be due to the limited sample size of the diabetic nephropathy group; hence, a study involving a large population size is required.

Fructosamine and glycated albumin levels represent the extent of glycation, and a higher physiological level of AGEs is expected to promote both diabetes and diabetic complications.[1,13] In this study, both type 2 diabetes and diabetic nephropathy subjects had significantly higher levels of fructosamine, glycated albumin, FBG, and, HbA_{1c}. Yet, these parameters were not associated with the carrier genotypes of the SNPs associated with type 2 diabetes. Hence, the carrier genotypes of type 2 diabetes-associated SNPs may not be involved in the observed disease-specific elevation of these biochemical parameters. These findings are similar to a previous report that found no association between rs1800625 or rs2070600 and AGEs.[69] The level of AGEs can be reasonably expected to be associated with glycemic status, which is elevated in diabetes, rather than gene polymorphisms. The TNF-α levels increase upon AGE−RAGE signaling, followed by NF-κB activation.[1] No association between rs1800625 or rs2070600 and the TNF-α level was observed in this study. Thus, the carrier genotypes of rs1800625 and rs2070600 may not affect the TNF-α levels despite these SNPs being associated with type 2 diabetes. The TNF-α level was not associated with type 2 diabetes and diabetic nephropathy in this study. This result is the same as previously found[70] but contradictory to several other reports.[71−73] Further, the carrier genotypes of rs1800625 and rs2070600 were not associated with FBG and HbA_{1c} despite being associated with type 2 diabetes. Also, MDA (a marker for oxidative stress), insulin, HOMA-IR, and CRP were neither elevated in any disease groups nor associated with any type 2 diabetes-associated SNPs as analyzed by one-way ANOVA and unpaired Student's *t*-test, respectively. Logistic regression analysis revealed that rs1800625 might increase the fasting insulin level, and a higher fasting insulin level is linked to insulin resistance and type 2 diabetes.[74] In this study, HOMA-IR was found to be negatively associated with rs1800625, which could be due to the maintenance of blood glucose level in subjects with high insulin levels undergoing oral hypoglycemic drug treatment. However, recent studies suggest that hyperinsulinemia is a causal factor for glucose intolerance.[74] Therefore, hyperinsulinemia may predict the future risk of type 2 diabetes. CRP, an inflammatory marker, showed a weak positive association with rs1800625. Elevated CRP level is positively associated with the risk of type 2 diabetes development.[75−77] While MDA showed a weak negative association and blood urea showed a weak positive association with rs1800625 in logistic regression analysis, these results must be verified in a larger cohort to check if the association is due to polymorphism or by chance. Also, HDL was found to be negatively associated with rs2070600 in logistic regression analysis. The association results for rs3134940, rs3131300, and rs9391855 are the same as the corresponding SNPs in complete linkage disequilibrium (Table S6). Although we have found a weak association of SNPs with insulin and CRP in this study, SNPs are generally not used as diagnostic markers to detect insulin resistance in clinical settings compared to measuring insulin or CRP. In fact, the measurement of blood glucose is sufficient to diagnose diabetes. However, SNPs or other markers such as insulin or CRP could be helpful in predicting the risk of development of type 2 diabetes, and both of these parameters are associated with SNP rs1800625.

5. CONCLUSIONS

In this study, we used the Oxford Nanopore Technology-based DNA sequencing to detect the SNPs in the *RAGE* gene, which play a significant role in the progression of diabetes and its complications. The nanopore sequencing method detected 33 *RAGE* polymorphisms with high accuracy for SNP detection, as validated by PCR-RFLP. Therefore, the application of nanopore sequencing can be extended to identify disease-specific genetic variations in clinical settings. Five of the *RAGE* gene polymorphisms detected, i.e., rs1800625, rs3131300, rs3134940, rs2070600, and rs9391855, were associated with an increased type 2 diabetes risk. The usefulness of these five *RAGE* SNPs as type 2 diabetes risk prediction markers requires to be studied in a larger cohort, which would also facilitate the study of the less frequent *RAGE* SNPs for their association with type 2 diabetes and its complications.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c00297.

PCR amplification of the *RAGE* gene; linkage disequilibrium map of *RAGE* SNPs; result of the nanopore, PCR-RFLP, and Sanger sequencing for verification of the discrepant results between nanopore and PCR-RFLP; *RAGE* SNPs in perfect linkage disequilibrium; allele frequency of *RAGE* polymorphisms in healthy, DM and DN, and DM + DN groups; association of mutated alleles of *RAGE* polymorphisms with type 2 diabetes and diabetic nephropathy; association of type 2 diabetes-associated *RAGE* SNPs with AGEs, TNF-α, diabetes diagnosis markers, MDA, insulin, HOMA IR, and CRP; logistic regression analysis results for clinical parameters and genetic variants (SNPs) (PDF)

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Notes

The authors declare no competing financial interest.

The nanopore sequencing data have been deposited in the Sequence Read Archive at NCBI (http://www.ncbi.nlm.nih.gov/sra) under the BioProject accession number PRJNA904121. The bash script used to analyze raw data generated by nanopore sequencing can be accessed through the GitHub link https://github.com/ajinkyakhilari/ampvar.

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