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Genetic variants of renin on the prevalence of diabetic nephropathy

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Abstract

Aim: Renin, a component of the Renin-Angiotensin-Aldosterone System (RAAS), is produced in the juxtaglomerular cells of the kidney. It is an important factor for the regulation of blood pressure and electrolyte balance and encoded by the REN gene. Recent studies suggest that the RAAS is a regulator of kidney functions. Individuals with REN variants have been associated with high blood pressure. We substantiated the hypothesis that genetic variants of REN gene have significant association with prevalence of nephropathy and in the development of nephropathy in type 2 diabetes mellitus (T2DM).

Methods: We enrolled to the study 718 consecutive subjects who were registered patients in two individual hospitals in Kolkata city, India. They consisted of 246 (34.26%) T2DM patients without nephropathy cases, 168 (23.40%) type 2 diabetes with nephropathy cases (T2DNH) and 304 (42.34%) healthy controls. Genotypes were assayed with genomic DNA for two known variants of the REN gene, i.e., rs16853055 and rs41317140 using sequencing methods.

Results: Association between the REN gene variants and prevalence of T2DM and T2DNH was tested. A significant association of T2DNH and variant rs41317140 was obtained and it was evident that the rs41317140 (C>T) shows a significant difference between T2DM and T2DNH (x^2 = 4.92; P = 0.03; OR = 0.6162; 95% CI: 0.4006-0.948). The results from the multiple model test that additive model predicted the association at genotype level and shows a significant difference between T2DM and T2DNH (OR = 0.6067; P = 0.03). There was no significant association between T2DNH or T2DM and variant rs16853055.

Conclusion: Thus, it is concluded that a genetic variant of the REN gene should have a significant impact on the onset of type 2 diabetic nephropathy.

Keywords: Single nucleotide polymorphism, type 2 diabetes mellitus, nephropathy, Indian population

INTRODUCTION

Nephropathy is related to damage or disease of the kidney. Diabetic nephropathy is impairment to the kidney caused by hyperglycemia. In severe cases the kidney can fail. The kidneys filter waste from blood through its capillaries. Diabetes resulting in high blood sugar can destroy these tiny blood vessels (American Diabetes Association). Renal failure or kidney disease in diabetes is intervened by various biochemical pathways such as renin-angiotensin-aldosterone system (RAAS)[1,2], aldose reductase-polyol[3], di-acyl glycerol-protein kinase C[4], advanced glycosylation-end products (AGE)[5,6] and hexosamine pathway[7]. The RAAS regulates blood pressure and water balance. Renin is secreted by the kidneys when blood pressure is low and it stimulates the production of angiotensin (Ang). Ang causes blood vessels to constrict, which results in high blood pressure. Experimental and clinical evidence recommend that the RAAS is a controller of kidney functions and is proposed to play an important role in the progression of nephropathy in type 2 diabetes mellitus[8-10].

Renin is a component of the RAAS and it is a protein containing 406 amino acids together with a pre segment carrying 20-23 and a pro segment of 43-47 amino acids[11-14]. In a number of steps, pro-renin is generated in the juxtaglomerular cells of the kidney by the elimination of 23 amino acids from C-terminus of prepro-renin, and is later converted into mature renin by removal of N-terminal fragment of pro-renin[12,14]. Renin, an aspartyl protease, is encoded by the REN gene and is mapped to 1q25-q32 by in situ hybridization[15]. It spans 12.5 kb in length of DNA and contains 8 introns[16] and encodes 10 exons[17]. It cleaves angiotensinogen and it converts it to Ang I after which, Ang I-converting enzyme (ACE) transforms it into Ang II, a potent vasoconstrictor. The concentrations of angiotensinogen circulating in the blood is abundant and perhaps more than 1000 times in excess of plasma Ang I and Ang II concentrations[18]. Although with exception of some species, activity of renin thus is a key factor for the determination of the rate of Ang I formation in the plasma from enormous supplies of circulating angiotensinogen[19,20]. Therefore, even small relative changes in the rates of Ang I formation perhaps lead to a large absolute difference in the circulating concentrations of Ang II. It is well known that renin is synthesized and stored in substantial quantities in the granules of juxtaglomerular cells and is released in response to various stimuli[20,21]. Thus, large changes in plasma renin levels can rapidly changes the generation of Ang I. Therefore the polymorphism in the promoter region of REN gene may be of great significance in the changes Ang I. Ang I is easily converted to Ang II because the widespread presence of Ang converting enzyme on endothelial cells of many vascular beds including lung[19,20,22]. The resultant increases in plasma Ang II exert powerful actions throughout the body through activation of AGTR1 receptors[20,23]. In this context renin is also an important regulator of blood pressure and electrolyte balance. Individuals with REN polymorphisms have been associated with high blood pressure[24,25], susceptibility to hypertension[26-28] and end-stage renal disease[29].

Keeping the existing body of knowledge in view, the aim of the present study was to investigate the distribution of genotype, allele frequency of REN gene polymorphism and its relationship with type 2 diabetic nephropathy patients in an Eastern Indian population.

METHODS
Subjects
Patients were recruited from registered patients list of two participating medical institutions of Kolkata, West Bengal. A standardized protocol was implemented to obtain data from each of the study participants.

Ethical committee clearance was obtained from the medical institutions prior to the recruitment of subjects in this study. An informed consent was obtained from all the participants prior to their recruitment for the study.

The study included 168 type 2 diabetic patients with nephropathy cases on hemodialysis (T2DNH), 246 type 2 diabetes patients without nephropathy cases (T2DM) and 304 controls (CON). The identification of type 2 diabetic and nephropathy patients was based on physician's recommendation or registered patient for dialysis. A detailed medical history of each patient was recorded. The unrelated controls were randomly selected and recruited from local community centers. Participants were born into Bengali families in Kolkata and the surrounding area.

Genotyping

Genomic DNA was prepared from fresh whole blood by using the conventional phenol-chloroform extraction method followed by ethanol precipitation[30]. In this study, previously published primers 5'GCTGTCTTCTG GTGGTACTGCC3'(sense) and 5'TGCTGGCCATGAACTGGTTCTAGC3' (antisense) were used for the PCR based detections of single nucleotide polymorphisms (SNPs). PCR amplification was performed in a final volume of 10 µL reaction mixture containing 50 ng of genomic DNA, 20 pmol of each primer, 10X Taq PCR buffer, 25 mM $MgCl_2$, 100 mM of each dNTPs and 0.5 U/uL of Red Taq polymerase. PCR amplification was performed in a DNA thermo cycler (Bio-Rad). PCR was carried out with an initial denaturing time at 95 °C for 5 min. Then the DNA was amplified for 35 cycles with denaturation at 94 °C for 1 min, annealing at 69 °C for 1:30 min and extension at 72 °C for 1:30 min and final extension 72 °C for 10 min. The PCR products were checked by 1% agarose gel electrophoresis with ethidium bromide staining and directly visualized in UV light. Only those PCR products that had a single amplification product with no evidence of non-specific amplification were used for DNA sequencing. The samples were analyzed on ABI 3730 genetic analyzer with a 48 capillary (Applied Biosystems, USA) to generate DNA sequences. Details described in our previous article[31,32].

Statistical analysis

Allele frequencies were calculated for all the SNPs and were tested for Hardy-Weinberg equilibrium (HWE) and allelic association with the disease (Chi-Square test/Fisher exact test). Allelic and genotype association with the phenotypes was tested under different genetic models for both quantitative and qualitative traits by regression analysis and Fisher model test. Allele frequencies were calculated for the SNPs and tested for HWE and allelic association with disease (Fisher exact test, logistic regression and Fisher model tests) using PLINK software[33]. For comparing the allelic distributions between study groups, the odds ratio (OR) with 95% confidence interval (CI) were also calculated. A level of $P < 0.05$ was assumed statistically significant.

Linkage disequilibrium (LD) between all the SNPs and also for associated SNP's was estimated using Haploview 4.2 software[34]. The pair wise LD statistics D' and r2 was calculated for all markers and also for associated SNP's. The Haploview 4.2 with default program or settings (Gabriel *et al.*[35], 2002) was used to assess the linkage disequilibrium (D' and r2) between each pair of SNPs.

RESULTS

The present study has focused on the 5' region with a special attention to rs41317140 (C to T) mutation. Through DNA sequencing seven SNPs were identified, out of which five new mutations were observed (data are not presented here) and two SNPs have been described previously as rs16853055 and rs41317140[36-39]. The SNP rs16853055 is located at 3879 upstream from the start codon consisting of a C to A mutation. The rs41317140 (TaqI RFLP) is located at 4063 upstream from the start codon in 5' region with a substitution of C by T mutation. The sequence electropherogram of REN gene presented in Figure 1 for the SNP rs16853055indicating C → A mutation in heterozygote CA condition and the SNP rs41317140 depicted in Figure 2 is indicated with the red arrow, i.e., in heterozygote condition (CT).
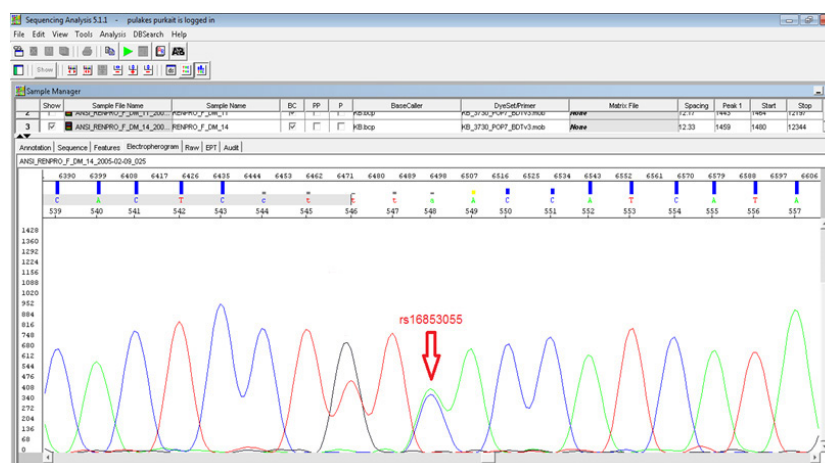
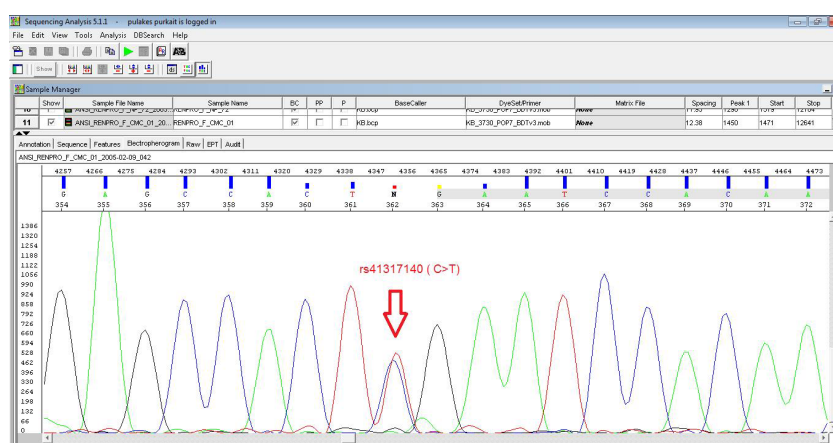**Figure 1.** Sequence electropherogram of Renin gene for the SNP rs16853055

Figure 2. Sequence electropherogram of Renin gene for the SNP rs41317140

The genotype data of the Renin gene SNPs are presented in Table 1. The SNP rs16853055 was found among 80 (26.32%) control, 66 (26.83%) T2DM and 34 (20.24%) T2DNH patients, whereas the SNP rs41317140 was observed among 74 (24.34%) control, 64 (26.6%) T2DM and 34 (20.24%) T2DNH patients while homozygote "TT" was found among 2.44% of T2DM patients and 0.66% control.

The results of HWE test are presented in Table 2. From the HWE test it was found that the SNPs rs16853055 and rs41317140 were in HWE, indicating maintenance of allele frequency for control group of the study population. The Fisher exact test for allelic association of rs16853055 and rs41317140 of REN gene is presented in Table 3. From the Fisher exact test, no significant differences were evident in the allele frequencies of the SNP rs16853055 between different combination of study groups that is case and control subjects. However, the rs41317140 (C>T) shows a significant difference between T2DM and T2DNH (x^2 = 4.92; P = 0.03; OR = 0.62; 95% CI: 0.4006-0.948) groups, indicating that a person with the SNP rs41317140 (C>T) will develop resistance for T2DM. Logistic regression analysis was performed to confirm the association at different genetic model and the results are presented in Table 4 and exhibit that additive model predicted the association at genotype level and shows significant difference between T2DM and T2DNH groups (OR = 0.61; P = 0.03).

It is observed that all three models predicted the association but not to a significant extent for the SNP rs16853055. To the best of our knowledge still there is no literature with respect to any possible role of this polymorphic change with any health problem.

Table 1. Genotype distribution of RENIN gene variants among the study groups

SNP	Genotype	Control		T2DM		T2DNH	
		n = 304	%	*n* = 246	%	*n* = 168	%
rs16853055	A A	2	0.66	6	2.44	2	1.19
	C A	80	26.32	66	26.83	34	20.24
	C C	222	73.03	174	70.73	132	78.57
rs41317140	C C	228	75.00	176	71.54	134	79.76
	C T	74	24.34	64	26.02	34	20.24
	T T	2	0.66	6	2.44	0	0.00

Table 2. Hardy-Weinberg Equilibrium test for rs16853055 (C>A) and rs41317140 (C>T) of RENIN gene among the study groups

SNP	Alleles	Study groups	ObsHET	PredHET	HWpval	MAF
rs16853055	C:A	CON	0.263	0.238	0.0969	0.138
		T2D	0.242	0.241	1	0.14
		T2DM	0.268	0.267	1	0.159
		T2DNH	0.202	0.201	1	0.113
rs41317140	C:T	CON	0.243	0.224	0.1929	0.128
		T2D	0.237	0.23	0.7763	0.133
		T2DM	0.26	0.261	1	0.154
		T2DNH	0.202	0.182	0.3119	0.101

Alleles: major and minor alleles for this marker; CON: Control group; T2D: over all type 2 diabetes with and without nephropathy group; T2DM: type 2 Diabetes without nephropathy group; T2DNH: type 2 diabetes with nephropathy who are on hemodialysis group; ObsHET: marker's observed heterozygosity; PredHET: marker's predicted heterozygosity (2*MAF*(1-MAF)); HWpval: Hardy-Weinberg equilibrium *P* value; MAF: minor allele frequency

Table 3. Fisher exact test for allelic association of SNPs rs16853055 (C>A) and rs41317140 (C >T) of RENIN gene among the study groups

SNP	A1	A2	Groups	F_A	F_U	CHISQ	*P* value	OR	L95	U95
rs16853055	A	C	CON *vs*.T2DM	0.1585	0.1382	0.899	0.34	1.175	0.8415	1.641
			CON *vs*. T2DNH	0.1131	0.1382	1.208	0.27	0.7955	0.5286	1.197
			T2DM *vs*. T2DNH	0.1131	0.1585	3.422	0.06	0.6768	0.4468	1.025
rs41317140	T	C	CON *vs*. T2DM	0.1545	0.1283	1.548	0.21	1.241	0.8827	1.746
			CON *vs*. T2DNH	0.1012	0.1283	1.52	0.21	0.765	0.4992	1.172
			T2DM *vs*. T2DNH	0.1012	0.1545	4.92	0.03*	0.6162	0.4006	0.948

SNP: single nucleotide polymorphism; A1: code for allele 1 (the more rare or "minor" allele based on the entire sample frequencies); A2: code for allele 2 (the more common or "major" allele); CON: Control group; T2D: over all type 2 diabetes with and without nephropathy group; T2DM: type 2 Diabetes without nephropathy group ; T2DNH: type 2 diabetes with nephropathy who are on hemodialysis group; F_A: frequency of minor allele in affected individuals (case); F_U: frequency of minor allele in unaffected individuals (control); CHISQ: Chi-squared value for allelic association (with 1 df); *P*: the asymptotic *P*-value for chi-square test; OR: odds ratio; L95: lower bound of the 95% confidence; U95: upper bound of the 95% confidence; *Significant

DISCUSSION

The development of diabetic nephropathy is multifactorial[40-43] and genetic predisposition has been anticipated to be an important factor in the development and progression of the disease. Apart from that, hypertension is presumed to be the single most important factor that accelerates the development of diabetic renal disease[25,44,45].

Although the RAAS system has an important function in the controlling blood pressure, maintaining the stable equilibrium of Na^+ ion and extracellular fluid volume[46,47], more and more evidence also point out an influence towards the development of diabetic nephropathy. Particular RAAS gene polymorphisms were recognized as risk factors for type 2 diabetes mellitus complications, including hypertension[31], coronary heart disease[48], nephropathy[48-50] and retinopathy[51].

Many researchers have established the importance of tissue RAAS in the heart, vasculature, adrenal glands and brain as well as in the kidney[52,53]. Though each organ system in the body has components of the RAAS,

Table 4. Logistic regression analysis of SNPsrs16853055 (C >A) and rs41317140 (C >T) of RENIN gene among the study groups

SNP	Study groups	Additive model				Dominant model				Recessive model			
		OR	L95	U95	*P* value	OR	L95	U95	*P* value	OR	L95	U95	*P* value
rs16853055	CON *vs*. T2DM	1.18	0.84	1.67	0.32	1.12	0.77	1.62	0.55	3.77	0.75	18.87	0.11
	CON *vs*. T2DNH	0.78	0.51	1.19	0.25	0.73	0.47	1.15	0.18	1.81	0.25	13.03	0.55
	T2DM *vs*. T2DNH	0.67	0.44	1.02	0.06	0.65	0.41	1.04	0.07	0.48	0.09	2.41	0.37
rs41317140	CON *vs*. T2DM	1.25	0.88	1.77	0.20	1.19	0.81	1.74	0.36	3.77	0.75	18.87	0.11
	CON *vs*. T2DNH	0.74	0.47	1.16	0.19	0.76	0.48	1.20	0.24	0.00	0.00	inf	0.99
	T2DM *vs*. T2DNH	0.61	0.39	0.94	0.03*	0.63	0.39	1.02	0.05	0.00	0.00	inf	0.99

SNP: single nucleotide polymorphism; A1: code for allele 1 (the more rare or "minor" allele based on the entire sample frequencies); A2: code for allele 2 (the more common or "major" allele); CON: control group; T2D: over all type 2 diabetes with and without nephropathy group; T2DM: type 2 Diabetes without nephropathy group ; T2DNH: type 2 diabetes with nephropathy who are on hemodialysis group; F_A: frequency of minor allele in affected individuals (case); F_U: frequency of minor allele in unaffected individuals (control); CHISQ: Chi-squared value for allelic association (with 1 df); *P*: the asymptotic *P*-value for chi-square test; OR: odds ratio; L95: lower bound of the 95% confidence; U95: upper bound of the 95% confidence; *Significant

the kidney is unique, because it contains all the elements of the RAAS with compartmentalization in the interstitial networks and tubules as well as intracellular accumulation[54]. In this regard, the adrenal glands along with the kidneys are distinctive because of their tissue concentrations of Ang II, which are much higher than can be explained by the concentrations transported by the arterial blood flow[55]. There is considerable evidence that the major fraction of Ang II present in renal tissues is greater locally from Ang delivered to the kidney as well as from angiotensinogen locally produced by proximal tubule cells[56,57]. Renin secreted by the juxtaglomerular apparatus cells and delivered to the renal interstitium and vascular compartment appear to be a most powerful controller for producing Ang I from Ang[58,59]. To regulate the production of Ang II, renin is most important, because once Ang I is formed, conversion readily occurs as there are abundant amounts of Ang converting enzyme[54] in circulation. In this regard the genetic variants of REN gene may have a vital role in the regulation of the gene expression as well as the regulation of Ang II production.

Previous findings show that renin gene polymorphism have been associated with diabetic nephropathy[39,60-62], increased risk of vascular complications[25,63], plasma renin activity (PRA)[44], susceptibility to hypertension in a variety of ethnic groups[24,25,27,64,65], T2DM[66,67], and CRI[68] although often with inconsistent results[69,70]. A few studies have reported that the renin rs4131714 (-4063C/T) a promoter variant has no association with DN[39,68,71], whereas the present study found a positive association between rs4131714 (-4063C/T) and T2DNH (P = 0.02655), which shows high linkage disequilibrium (LD) with rs16853055 (D' = 0.936; LOD = 82.03; r2 = 0.823), [Figure 3]. However, the rs16853055 did not show any significant association with diabetes and diabetic nephropathy diseases. Deinum *et al.*[39] also reported weak association of REN gene (Bgl I RFLP) polymorphism in the first intron with diabetic nephropathy along with increased level of plasma renin. The first intron is involved in the renin gene transcription regulation[72] and therefore Bgl I RFLP may have some contribution in this regard. Likewise the renin rs4131714 (-4063C/T) promoter variant may also have some regulatory function in the expression of prorenin or renin which is subject to further study. Though we have not measured the level of plasma prorenin or renin which is beyond our scope, but one can illustrate that area. In any future study one can also investigate the precise role of rs16853055 (-3879C/A) polymorphism in connection with diabetic nephropathy, as it shows high linkage disequilibrium with rs4131714 (-4063C/T).

Genetic sketching of the functional genes helps to explore a particular disease as well as helps to recognize the tendency of that disease within a particular population that may eventually be of support to the doctors for recommending personalized medicine.

The present study has to be taken under consideration within its limitations; that it was limited to a specific ethnic group (Eastern Indian Bengali population). A larger study from different ethnic groups will be needed to confirm for any contribution of renin gene polymor-phism to T2DM complications for development of
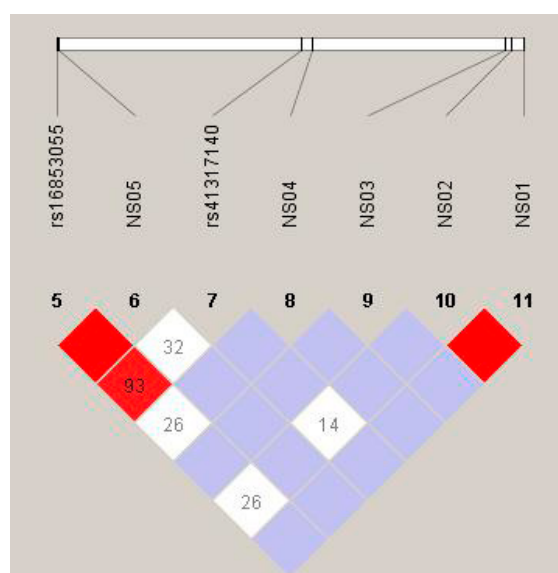
Figure 3. Linkage disequilibrium plot of Renin genes SNPs

renal problem or nephropathy. It is also worth mentioning here that this research work only deals with the association study (irrespective of gender) between diabetic nephropathy and the genetic variation of renin gene within its promoter sequence.

DECLARATIONS

Acknowledgments

Authors' contributions

Involved in the sequencing experiments, screening for gene mutations, performed the statistical analysis as well as participating in the write up of the manuscript: Purkait P
Contributed to preparation of the manuscript: Halder K
Supervised the project: Naidu JM
Supervised the project and compliance with Institutional ethical procedures: Sarkar B
Read and approved the final manuscript: all authors

Availability of data and materials

Reader can ask or mail to corresponding author for materials.

Financial support and sponsorship

Conflicts of interest

All authors declare that there are no conflicts of interest.

Ethical approval and consent to participate
Ethical committee clearance was obtained from the respective medical institutions and Ethical committee of the Anthropological Survey of India, Government of India. Verbal and written well informed consent was obtained from all participants before they were eligible for recruitment into the study.

Consent for publication
Although the manuscript does not involve the use of live photographs of any of the participants, consent was obtained from them for the data to be published as at the time recruitment into the study.

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