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Original Article

Renin- Angiotensin- Aldosterone System gene polymorphisms in Type 2 Diabetic patients among the Mewari population of Rajasthan.

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ABSTRACT

Aim: Diabetes is the one of the most serious health problems of modern times and India is the top most country with the highest number of individuals affected with type 2diabetes and an extremely limited data on the genetics of diabetes in the Indian populations is available. In this context the aim of the present study was to investigate the distribution of RAAS pathway genes polymorphism and its relationship with Type 2 Diabetes patients in Mewari population. Methods: The present study examined the prevalence of RAAS gene polymorphism among 111 Mewari individuals from Udaipur, Rajasthan, India. Among them 50 individuals belong to type 2 diabetic (T2DM) and 61 healthy controls. Isolated DNA samples from the studied subjects were genotyped using PCR-RFLP methods. Result: There was a significant difference in age, gender, SBP, BMI, glucose, GGT, urea, creatinine, LDL and total protein between T2DM and Control subjects ($p < 0.05$). The minor allele frequency of RAAS pathway gene polymorphism AGT (rs4762), REN (rs41317140), ACE (rs4646994), AGTR1 (rs5186), CYP11B2 (rs1799998) in T2DM patients (T2DM) are found to be 0.18, 0.20, 0.41, 0.05 and 0.30 respectively. The allele frequencies of the same polymorphisms among the normal controls are found to be 0.13, 0.16, 0.48, 0.10 and 0.40 respectively. The general associations were not found in significant result ($p > 0.05$). Conclusion: The study found that no association of RAAS gene polymorphisms with increased risk of type 2 diabetes among the Mewari population except rs4762. The AGT (rs4762) was associated at recessive model ($p = 0.038$).

1. Introduction

Diabetes is a common and complex metabolic disorder in modern time and caused by a combination of genes and environmental factors. Multiple genetic factors including those not underlying diabetes can contribute to the development of complications such as nephropathy, neuropathy, and retinopathy and most common complication of diabetes is hypertension. Hypertension increases with decreasing renal function [1] and is a risk factor for the development of ESRD [2-3]. This results from an activation of the renin-angiotensin-aldosterone system (RAAS) and the RAAS may [4] predispose to the development of renal failure and [5] promote a more rapid loss of glomerular filtration rate in patients suffering from renal diseases. The renin -angiotensin system (RAS) plays major roles in blood pressure regulation and electrolyte metabolism [6] and pivotal roles in the pathophysiology of cardiovascular, renal, and metabolic conditions [7-8]. Genetic variants of this system have been developed to test their association with cardiovascular and renal conditions. Clinical evidence suggests that the renin- angiotensin-aldosterone system (RAAS) is associated with the etiology of type 2 diabetes [9 – 11]. Among the candidate genes of RAAS the Renin (REN), Angiotensinogen (AGT), Angiotensin- converting enzyme (ACE), Angiotensin II type1 receptor (AGT1R) and aldosterone synthase gene (CYP11B2) are of particular interest.

Therefore, the aim of the present study was to investigate the distribution of RAAS pathway genes polymorphism and its relationship with Type 2 Diabetes patients in Mewari population. In this work, we aimed at investigation the association of REN

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(rs41317140), AGT (rs4762), AGTR1 (rs5186), ACE (rs4646994), CYP11B2 (rs1799998) genetic markers and common anthropometric and clinical factors with type 2 diabetes among the Mewari population of Udaipur city of Rajasthan.

2. Materials and Methods

2.1. Subjects

The present study on RAAS pathway genes polymorphisms were carried out among 111 Mewari populations both sex, consisting of 50 Type 2 Diabetes patients (T2DM) and 61 healthy controls in and around Udaipur city of Rajasthan. Registered patients were recruited from Bhupalpura Govt. Dispensary, Udaipur and a detailed medical history of each patient was recorded accordingly. The detection of Type 2 diabetes was based on physician's recommendation. The healthy unrelated controls were randomly selected and recruited from local community centers. 10 ml of venous blood (6 ml was drawn into EDTA tube for genetic analyses and 4 ml was drawn into serum tube for biochemistry analysis) was collected from the participants by using sterilized disposable blood collection kits. Prior to the recruitment of subjects the ethical committee clearance was obtained from the respective medical institutions and accordingly informed consent was obtained from all the participants.

2.2. Anthropometric and Physical measurements

Anthropometric measurements and indices were taken in each subject, using standard methodology. Clinical data included information of duration of diabetes, presence of any complication, history of other disorders, systolic blood pressure, and diastolic blood pressure. Anthropometric data, weight (kg), and Height (cm), were obtained to calculate body mass index (BMI) using the formula, weight (kg)/ [height (m2)]. The blood pressure was measured on the right arm of the subjects using an automated blood pressure monitor (Omron, Japan) by seated and rested for 15 minutes. Blood glucose was measure using the Breez 2 glucometer (blood glucose monitor).

2.3. Biochemical analysis

4 ml of venous blood samples were drawn in the morning. Thereafter, the blood samples were centrifuged, serum were separated, collected and stored at -86°C until further analysis. All laboratory measurements were conducted at the DNA Laboratory in the Anthropological Survey of India, Western Regional Centre, Udaipur. The serum levels of GPT, GOT, GGT, Urea, Creatinine, Uric Acid, triglycerides(TG), total cholesterol , high-density lipoprotein cholesterol (HDL), low density lipoprotein cholesterol (LDL), Chloride, albumin and total Protein (TP) were measured enzymatically on a Fully auto analyzer EM360 (TRANSASIA with kits supplied by TRANSASIA BIO MEDICAL LTD).

2.4. Genotype assessment

Approximately 6 ml of venous blood was drawn from each of the subjects in EDTA vials and genomic DNA was extracted from whole fresh blood using standard salting out method using phenol-chloroform [12]. Five polymorphisms from five genes namely ACE (I/D; rs4646994) , AGT (T174M; rs4762) , AGTR1 (A1166C; rs5186) , CYP11B2 (T -344C; rs1799998) , REN (C -4063 T; rs41317140) were chosen for genotyping using the polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis using the primers and details of which are presented in Table 1. All reaction were performed with 20 pmol of each primer in a final volume of 10µl containing 50ng of genomic DNA, 10X Taq PCR buffer, 25 mM MgCl2 ,100 mM of each dNTPs and 1 U/uL of Taq polymerase. Electrophoreses of PCR products and digested PCR products were resolved on 2- 3% agarose gel stained with ethidium bromide and directly visualized in UV light.

2.5. Statistical analysis

Data were analyzed using statistical package for Social Sciences statistical software (SPSS Version 16, Chicago, Illinois, USA). Data were presented as mean ± SD and comparison between T2DM and Control and among group were analyzed by the independent-sample T tests and one way ANOVA and cross tab analysis respectively.

Genotype and Allele frequencies were calculated for the SNPs and tested for Hardy-Weinberg equilibrium and allelic association with disease (χ2-test, exact tests, and Fisher- model tests) using PLINK (http://pngu.mgh.harvard.edu/~purcell/plink/) [18]. 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 statistical significance.

3.Results

3.1.Baseline characteristics of the subjects

The anthropometric measurement and clinical characteristics of the subjects are presented in the Table 2. Significant differences were found between T2DM and Control SBP ($p = 0.000$) , BMI ($p = 0.043$), glucose ($p = 0.000$), GGT ($p = 0.022$), urea ($p = 0.001$), creatinine ($p = 0.000$), LDL ($p = 0.045$), TP ($p = 0.044$) , while the age, height, weight, DBP, GPT, GOT, uric acid, triglyceride, cholesterol, HDL, chloride and albumin did not reach statistical significance.

3.2. Primary information, distribution and different model tests of selected SNPs of the Renin - Angiotensin – Aldosterone system pathway genes

Primary information and allele frequencies of the selected SNPs of the renin - angiotensin – aldosterone system pathway genes are presented in Table 3. All the genotype distributions in these study groups were consistent with Hardy – Weinberg equilibrium expectation ($p > 0.05$). Allelic frequencies of all polymorphisms did not show any difference between T2DM and Control subjects of Mewari population of Udaipur city. The associations were further verified through different Fisher models test to confirm which of these 5 model best predict the associations at micro level (Table 4).

Table 1: RAAS pathway genes SNPs, their location, primer sequence, PCR annealing temperature, product size and restriction enzyme with allele product sizes.

Gene & Polymorphism	rs number	Primer sequence	Product Size (bp)	Annealing temp./ Restriction enzyme/ allele sizes
AGT (Thr 174 Met) [C3889T]	rs4762	F: 5'–AGGCTGTACAGGGCCTGCTAGT– 3' R: 5' –GCCTTACCTTGGAAGTGGACGTAG – 3' Ref: Nakajima T et al., 2002 [13]	310	Nco I C = 310; T = 64, 246
RENIN (C -4063 T)	rs41317140	F: 5'–GCTGTCTTCTGGTGGTACTGCC– 3' R: 5' –TGCTGGCCATGAACTGGTTCTAGC– 3' Ref: Deinum J et al., 1999 [14]	964	Taq I T = 964 bp C = 394, 570
ACE (Ins/Del)	rs4646994	F: 5'–CTGGAGACCACTCCCATCCTTTCT – 3' R: 5' –GATGTGGCCATCACATTCGTCAGAT– 3' Ref. Rigat B et al., 1992 [15]	490,190	I = 490 D = 190
AGTR1 (A 1166 C)	rs5186	F: 5'– AAGAAGCCTGCACCATGTTT – 3' R: 5' – CCATCTTACGGGCATTGTTT– 3' Ref: Prasad P et al., 2006 [16]	626	HpyF3I (DdeI) A = 536, 90 C = 417, 119, 90
CYP11B2 (T -344 C)	rs1799998	F: 5'– CAGGAGGAGACCCCATGTGAC – 3' R: 5' – CTCCACCCTGTTCAGCCCAAAT – 3' Ref: Xiao J et al.,2007 [17]	536	Hae III C = 202,138,125,71 T = 278,138,125

Table 2: Anthropometric and Clinical Characteristics of the subjects

Variables	Control (N = 61) Mean ± SD	T2DM (N = 50) Mean ± SD	P
Age (Years)	53.26 ± 5.53	55.70 ± 9.98	0.107
SBP (mmHg)	123.16 ± 18.77	138.80 ± 21.37	0.000*
DBP (mmHg)	84.39 ± 8.78	87.68 ± 11.22	0.086
Height (cm)	164.19 ± 8.69	162.27 ± 8.31	0.24
Weight (kg)	164.19 ± 8.69	162.27 ± 8.31	0.308
BMI (kg/ m2)	25.13 ± 3.13	26.55 ± 4.14	0.043*
Glucose (mg/dl)	117.25 ± 20.37	184.66 ± 92.16	0.000*
GPT (U/L)	32.11 ± 17.39	32.97 ± 22.05	0.819
GOT (U/L)	23.55 ± 12.36	25.00 ± 24.14	0.683
GGT (U/L)	30.54 ± 19.89	41.10 ± 27.92	0.022*
UREA (mg/dl)	17.00 ± 4.93	23.46 ± 13.75	0.001*
Creatinine (mg/dl)	0.99 ± 0.14	1.10 ± 0.19	0.000*
Uric Acid (mg/dl)	5.86 ± 1.35	5.87 ± 1.56	0.966
Triglyceride (mg/dl)	157.69 ± 72.33	182.52 ± 101.85	0.137
Cholesterol (mg/dl)	162.31 ± 38.90	177.50 ± 48.52	0.07
HDL (mg/dl)	41.40 ± 10.19	43.79 ± 9.56	0.208
LDL (mg/dl)	98.166 ± 24.79	109.21 ± 32.63	0.045*
Chloride (mmol/L)	101.00 ± 3.47	100.60 ± 3.16	0.523
Albumin (g/dl)	3.91 ± 0.24	3.93 ± 0.27	0.682
Total Protein (g/dl)	7.11 ± 0.53	7.33 ± 0.58	0.044*

*** Significant at the P<0.05 level**

Table 3: Summary statistical analysis of RAAS genes by General association and HWE

CHR	rs number	Genomic location	Minor allele	MAF of T2DM	MAF of CON	Major allele	X^2	P value	OR	95% CI L	95% CI U	HWE p value
1	rs4762	3889	T	0.18	0.13	C	1.011	0.3146	1.454	0.6991	3.025	0.2795
1	rs41317140	(T174M)-4063	T	0.20	0.16	C	0.7435	0.3886	1.355	0.6781	2.709	0.5174
17	rs4646994	Intron 16	D	0.41	0.48	I	0.9516	0.3293	0.7668	0.4496	1.308	0.8475
3	rs5186	1166	C	0.05	0.10	A	1.818	0.1776	0.4825	0.164	1.419	1
8	rs1799998	-344	C	0.30	0.40	T	2.477	0.1155	0.6385	0.3646	1.118	0.413

(CHR= Chromosome, MAF = Minor allele frequency, * Significant at the P <0.05 level)

Table 4: Summary statistical analysis of RAAS pathway genes by Fisher – Model Tests

CHR	rs number	Minor allele	Major allele	TEST	T2DM	CON	P
1	rs4762	T	C	GENO	4/10/36	0/16/45	0.075
				TREND	18/82	16/106	0.337
				ALLELIC	18/82	16/106	0.352
				DOM	14/36	16/45	0.834
				REC	4/46	0/61	0.038*
1	rs41317140	T	C	GENO	2/16/32	0/19/42	0.335
				TREND	20/80	19/103	0.366
				ALLELIC	20/80	19/103	0.479
				DOM	18/32	19/42	0.687
				REC	2/48	0/61	0.201
3	rs5186	C	A	GENO	0/5/45	0/12/49	0.192
				TREND	5/95	12/110	0.159
				ALLELIC	5/95	12/110	0.211
				DOM	5/45	12/49	0.192
				REC	0/50	0/61	1
8	rs1799998	C	T	GENO	4/22/24	12/25/24	0.229
				TREND	30/70	49/73	0.129
				ALLELIC	30/70	49/73	0.124
				DOM	26/24	37/24	0.442
				REC	4/46	12/49	0.106
17	rs4646994	D	I	GENO	9/23/18	12/34/15	0.421
				TREND	41/59	58/64	0.319
				ALLELIC	41/59	58/64	0.345
				DOM	32/18	46/15	0.215
				REC	9/41	12/49	1

*** Significant at the P<0.05 level**

4. Discussion

Diabetes is the one of the most serious health problems of modern times and India is the top most country with the highest number of individuals affected with type 2 diabetes. Regarding the epidemiological studies in India, an extremely limited data on the genetics of diabetes in the Indian populations is available. Numerous diabetes genetic studies that have been conducted in India thus far are population-based candidate gene association studies [16, 19-24].

In this report on RAAS pathway genes, we tested association of a total five polymorphism from promoter, exonic and intronic regions of five genes and 20 variables are tested for association between type 2 diabetic and controls in Mewari population.

The AGT T174M polymorphism is located at the exon 2 of the AGT gene on Chromosome 1. Genetic linkage study showed that AGT gene T174M and M235T variants were strongly associated with high blood pressure in diabetic patients [25]. In this study we observed a higher frequency of AGT 174 M (T) allele in the T2DM subjects (0.18) that in control subjects (0.13) in Mewari population. Estimated OR of T2dm with carrier M (T) allele as compared with control subjects was (1.454) with 95% CI (0.6991 – 3.025). We found T174 genotype frequency almost pattern to other populations and found no homozygous MM genotype in Control Subject like [26–29].

The Renin (– 4063 C >T) polymorphism is located at the promoter region of the gene. I has been showed that the frequency of the T allele is increased in T2DM subjects (0.20%) that control (0.16%) and estimated OR of T2DM with carrier T allele as compared with control subjects was 1.355 with 95% CI (0.678 – 2.709) and found no association with T2DM and rennin gene promoter polymorphism in Mewari population Our study showed same result reported by Mtiraoui N et al. [30].

The ACE gene is located on chromosome 17q23 and the ACE gene polymorphism results from the insertion (I) or deletion (D) of a 287 bp Alu sequence near the 3′ end of intron 16 (Rigat et al., 1992). In our study the frequency of the ACE deletion (D) allele has been found to be lower in T2DM patients (0.41) than control (0.48) subjects, found null association with T2DM and the result was totally different than the earlier reports [31-34]. At this stage we can presume that ACE gene can be used as a suitable marker for studying genetic variation among different human populations.

The AGTR1 gene A1166C polymorphism is located at the 5' end of the 3' UTR of the gene [35]. In our study showed that no association between the AGTR1 gene polymorphism and Mewari T2DM patients. It has been showed that the frequency of the AGTR1 C allele is increased in control (0.10) than T2DM patients (0.05).

The -344 (C >T; rs1799998) variant is a commonly reported polymorphism of the CYP11B2 gene, which is located at the promoter region [36]. The -344 (C >T) polymorphism is associated with serum aldosterone level and production [37-39], blood pressure [40-43]. Its association was reported with progression of renal function [44-46] and ESRD [47]. However, there were few studies from India have been found on the association of CYP11B2 -344C/T polymorphism and CRI [16], healthy volunteers [48]; hypertension [49-50]. In our study, The C allele frequency was the highest among Control subjects (0.40) than T2DM subject (0.30) and expected OR was 0.6385 with 95% CI (0.3646 – 1.118) but we could not observe significant differences in allele frequency (X2 = 2.477, p = 0.1155) of the CYP11B2 gene (-344 T > C) polymorphism between T2DM patients and Controls subjects and the result follow our previous study [51].

5. Conclusion

This is the first report on genetics of type 2 diabetes among the Mewari population of Udaipur city, Rajasthan. On the whole it was observed through the analysis that there was no significant association at micro level also between REN, AGTR1, ACE and CYP11B2 gene polymorphism and T2DM in Mewari population. The AGT (T174M, rs4762) was statistically significant at micro level recessive model (p = 0.038). This inconsistence result may be for our small sample size .Therefore while studying the susceptibility of RAAS gene with the etiology of type 2 diabetes we have to take care of the ethnic background of the population. Further studies and more samples size will definitely add more information to this issue.

6. Conflict of interest

The authors declare no conflict of interest for the present research outcome.

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