Association of Mbo I-RFLP at the Renin Locus (rs2368564) with Essential Hypertension

Deepak N. Parchwani¹ · Digisha D. Patel² · Jairam Rawtani³ · Nirupama Dikshit⁴

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Abstract Several lines of experimental and clinical evidence have alluded a pivotal role of renin in blood pressure homeostasis and therefore a relevance of molecular variants of the renin gene and essential hypertension have been speculated. This study was designed to evaluate the pattern, alliance and risk of renin Mbo I (10631A>G; rs2368564) polymorphism at the locus intron 9 for a possible role in modulating essential hypertension in adult population from Gujarat (India). A total of 257 consecutively enrolled essential hypertensive patients and 270 controls were genotyped using polymerase chain reaction-restriction fragment length polymorphism method for the selected marker. Suitable descriptive statistics was used for different variables. Genotypic (χ^2 10.43, p 0.0054) and allelic (χ^2 11.46, p 0.0007) distribution of this SNP displayed significant differences between cases and controls with an increased frequency of the A allele (χ^2 6.275; p 0.0122) and A/A geno-type (χ^2 8.247; p 0.0041) in hypertensive individuals. However, it showed no deviation from Hardy–Weinberg equilibrium in either affected or control group. A significant association was found in the A/A variant of rs2368564 with essential hypertension (p 0.0032), along with a statistically significant increase in odds of hypertension (OR 1.69; CI 1.46–2.28; p 0.02), even after confounding factors were adjusted in multiple logistic regression analysis

and is substantiated by inter-genotypic variations in the mean systolic and diastolic blood pressure in patients. In conclusion, renin 10631A>G gene mutation at the ninth intron play critical roles in BP (dys)regulation and can be implicated in an individual's susceptibility for hypertension.

Keywords Essential hypertension · Mbo I restriction endonuclease · Polymerase chain reaction · Renin · Restriction fragment length polymorphism

Introduction

Essential hypertension (EHT), a global escalating public health problem, is a complex, pleomorphic, multifactorial, polygenic trait that often leads to a series of pathologies which are detrimental to human health condition. In India, hypertension alone is responsible for 24 % of all coronary heart disease deaths and 57 % of all the stroke deaths [1]. This is a warning sign for Indian health care system, and to optimize patient care, not only adequate management is required but it is of importance to identify the molecular factors that influence the susceptibility of an individual to EHT, as more and more, population based studies; adoption studies; segregation analysis and twin studies have suggested that about 30–60 % of human blood pressure (BP) variance is genetically determined. Despite copious reports suggesting a ample genetic contribution, enigma is, no major susceptibility genes have been identified hitherto, and a genomic scan have recognized over 100 quantitative trait loci (in chromosomes 1, 2, 3, 17 and 18) for the phenotypic expression of BP [2, 3]. Among these, genes encoding for the components of the renin-angiotensin-aldosterone system (RAAS) are deemed the most plausible, logical susceptibility determinant. This has occurred

✉ Deepak N. Parchwani
drdeepakparchwani@yahoo.com; drdparchwani@gmail.com

¹ GMERS Medical College, Himmatnagar, Gujarat, India

² Gujarat Adani Institute of Medical Sciences, Bhuj, Gujarat, India

³ SN Medical College, Jodhpur, Rajasthan, India

⁴ GCS Medical College, Ahmedabad, Gujarat, India

because of its physiological magnitude in cardiovascular homeostasis i.e. as a circulating or hormonal system, components of this system plays an elementary role in the regulation of BP through its effect on vascular tone, renal hemodynamics, electrolyte and volume homeostasis [4] and is substantiated by the findings that dysfunction of this system leads to hypertensive phenotypes.

The effects of RAAS are predominantly mediated by angiotensin II, which is produced by the cleavage of angiotensin I (AGT I) by angiotensin converting enzyme and AGT itself being synthesized by the cleavage of the precursor angiotensinogen by renin [5]. Thus, all the genes or genetic loci responsible for excess angiotensin II production or availability are potential candidates for development of hypertension. However, because of the biochemical or physiological role of the renin (first and rate-limiting enzyme of the RAAS), the gene that encodes this molecule is an excellent rational candidate quantitative trait locus involved in the etiology of EHT. Thus, it is reasonable to hypothesis that the renin gene (REN) polymorphisms have a measurable influence on the hypertension mechanism and contribute to, or reduce, this disease.

Renin gene spans 12.5 kb (10 exons and 9 introns), located at chromosome 1q32, encodes a 340 amino acids protein with a mass of 37 kDa [6, 7]. Due to variations in the amount of linkage disequilibrium observed at the human REN locus, studies have focused attention on assorted REN single nucleotide polymorphisms (SNP) such as Hind III in the 3' region, Taq I in 5' region, Hinf I and Bgl I in the first intron and Mbo I in intron 9. However, no study have been published or carried out on REN Mbo I two-allele polymorphism (10631A>G; SNP ID: rs2368564) in a Gujarat population to refute or confirm the potential existence. Hence, we opted to evaluate the pattern, alliance and risk of REN Mbo I (10631A>G; rs2368564) polymorphism at the locus intron 9 (chromosome 1) for a possible role in modulating EHT in adult population from Gujarat (India), as it is a region characteristically having high consanguinity rates plus relatively high aggregation rates for familial diseases such as hypertension. The present study is a step towards understanding the molecular basis of essential hypertension by examining mutations (or polymorphisms) in candidate genes, which may facilitate the development of new targeted forms of pharmacological therapy that can be tailored to the needs of individual patients and thereby minimize the risk of morbidity and mortality.

Material and Method

Ethical Consideration

The study protocol complied with the Declaration of Helsinki [8] on biomedical research on humans, and was

approved by the Institutional Human Research Ethical Committee. Written informed consent was obtained (after providing a detailed study overview) from all participants prior to their recruitment for the study.

Participants and Study Protocol

This case-control study was conducted from January 2013 to November 2014. Patient selected for this study included unrelated essential hypertensive adult Gujarati patients. The history of EHT was operationally based on patient self report of a prior physician diagnosis and use of prescribed antihypertensive medication for a minimum of 1 year and only patients with positive family history of hypertension, defined by at least one parent being hypertensive were selected. Patients showing clinical signs, symptoms, or laboratory findings suggestive of secondary hypertension were excluded. Other criteria for exclusion were, patients with a history of diabetes mellitus (DM) or renal disease; showing impaired glucose tolerance, obesity (body mass index $> 30 \text{ kg/m}^2$), current or history of tobacco or alcohol intake; patients with, any severe illness, impairment of speech, hearing, vision, or cognition, continuous or periodic use of corticosteroids; pregnant females or who had given birth within the preceding 6 weeks, or any significant medical illnesses that prevented participants from adhering to the protocol, lack of approval by physician and patients showing disinterest or refusal to sign the consent form. A total of 527 participants (257 hypertensive patients and 270 healthy controls) were recruited in the present study. It was based on consecutive sampling technique i.e. all the patients who fulfilled the criteria in the period January 2013 to June 2014 were included and it was ensured that 1:1 matching was done. Healthy controls were selected at random from outpatients, who were on routine health check-ups and was defined as participants not suffering from hypertension (as per the criteria formulated by Seventh Report of the Joint National Committee, JNC 7) [9], nor having any family history of hypertension in first-degree relatives, not suffering from DM or from any acute or chronic disease, nor taking any drugs believed to affect blood pressure. All participants (patients and control) included in the study were ethnically matched, and we included only those individuals who had been born in and residents (without migration history) of Gujarat for at least three generations, including their own.

Participant's Examination and Measurements

All participants were studied as outpatient. A detailed present and past history of each case was recorded including name, age, sex, address, religion, occupation, economic status, nutritional and personal habits, education,

medication and history suggestive of any systemic illness. Each patient was then examined for various anthropometric parameters: Weight (Kg) and height (meters) were measured (using Omron digital body weight scale HN-286 and SECA 206 wall mounted metal tapes respectively). Body Mass Index (BMI) was calculated by Weight (Kg)/height squared (m^2). All anthropometric measures reflect the average of 3 measurements (measured by same person on same instrument to avoid inter-instrument and inter personal variation). BP was measured three times (on different days) in the seated position after 10 min of rest with a standard manual mercury sphygmomanometer (Diamond Deluxe Industrial Electronics and Products, Pune, India) and stethoscope (3 M Littmann, 3 M India Ltd, Bangalore, India) by auscultatory method. The recorded pressure of the three measurements was averaged so as to exclude false values due to variability that may be caused by various factors at any one point in time. Age was defined as the age at the time of interview (though no documentary proof had been entertained) and the date of diagnosis of EHT was obtained from the patient.

Sampling and Biochemical Analysis

After an overnight fast of 12 h, venous sampling was done for biochemical determinations and for isolation of DNA. Serum and plasma was separated by centrifugation of blood sample and were subjected for analytical procedures. Glucose (Glucose oxidase–peroxidase method, CV % 3.4), cholesterol (Cholesterol oxidase–peroxidase method, CV % 3.9), triglycerides (Enzymatic method, CV % 3.6), HDL-C (Phosphotungstic method, CV % 4.7), LDL-C (CV % 3.6) and creatinine (modified Jaffe's method, CV % 2.5) were measured in fully automated analyzer.

DNA Analysis: Determination of Genotypes of REN Mbo I (10631A>G) Polymorphism

DNA Extraction

Genomic DNA was extracted from peripheral blood leukocytes using commercially available DNA extraction and purification kit from GeNei Diagnostics (Bangalore, India) based on standard proteinase K technique. Eluted DNA was either stored at $-20\text{ }^{\circ}\text{C}$ or amplified immediately. All DNA samples were amplified within 3 days following extraction. Before amplification, quantity of DNA in each sample was assessed by measuring the absorption at 260 nm (using molar extinction coefficient of double stranded DNA: $0.020\text{ }\mu\text{g/ml/cm}$) [10] in a standard spectrophotometer (UV–VIS double beam–2205, Systronics, Ahmedabad, India).

DNA Amplification

Two oligonucleotide primers, forward: 5'-TGA GGT TCG AGT CGG CCC CCT-3' and reverse: 5'-TGC CCA AAC ATG GCC ACA CAT-3' based on the flanking sequences of intron 9 of the renin gene [11] were used to amplify the corresponding DNA fragments by polymerase chain reaction (PCR). Amplification was carried out in a DNA Thermal Cycler (2720, Applied Biosystems) in a final reaction volume of 50 μl containing 100 ng genomic DNA, 10 pM of each primer, 200 μM of each deoxyribonucleotides triphosphate, 0.25 U of thermus aquaticus DNA polymerase, 2 mM magnesium chloride, 20 mM Tris-hydrochloric acid and 50 mM KCl. The thermocycling profile consisted of 5 min of initial denaturation at $94\text{ }^{\circ}\text{C}$ followed by 30 cycles of amplification of denaturation at $94\text{ }^{\circ}\text{C}$ for 30 s, annealing at $68\text{ }^{\circ}\text{C}$ for 30 s and extension at $72\text{ }^{\circ}\text{C}$ for 30 s, followed by a final extension at $72\text{ }^{\circ}\text{C}$ for 5 min.

Restriction Endonuclease Digestion of PCR Products

The PCR amplified 250-bp products were analyzed for genotyping by restriction fragment length polymorphism (RFLP) using the restriction enzyme Mbo I (*Moraxella bovis* I), whose recognition site is $\downarrow\text{GATC}$ [12]. The 250-bp PCR fragments {10 μl (1 $\mu\text{g}/\mu\text{l}$)} were submitted to restriction endonuclease digestion by addition of 2 U of Mbo I endonucleases with restriction enzyme buffer, and incubated at $37\text{ }^{\circ}\text{C}$ for 2 h. The restriction fragments were separated and distinguished on agarose gel stained in ethidium bromide using DNA ladder as standard. After digestion, samples with no Mbo I restriction site {Mbo I (–); homozygous G/G} showed the single 250 bp band, those homozygous for the restriction site {Mbo I (+); homozygous A/A} resulted in two bands (171 + 79 bp) after digestion, while heterozygous samples showed all the three bands [12, 13].

Statistical Analysis

The statistical analyses were performed using a Statistical Package for Social Sciences for Windows version 15.0 (SPSS Inc, Chicago, Illinois, USA). Data were expressed as mean $\pm$ SD (continuous variables), or as percentages of total (categorical variables). Prior to hypothesis testing, data were examined for normality. Non-normally distributed variables were logarithmically transformed before analysis. Two-group comparisons were made using Chi square (χ^2) for categorical variables and Student's *t* tests or one-way ANOVA for continuous variables.

The distribution of alleles in studied groups was tested for fitting to the Hardy–Weinberg equilibrium (HWE) (using web base program: <http://www.oege.org/software/>

[hwe-mr-calc.shtml](#)) [14] through testing the difference between observed and expected frequencies of genetic variants using the χ^2 goodness-of-fit test. Odds ratios (ORs) with 95 % confidence intervals (CIs) were estimated to determine the risk levels for the associated genotypes. In addition, the strength of the association between EHT and the REN Mbo I (10631A>G) polymorphism was estimated using ORs (with the corresponding 95 % CIs). The ORs were also performed for a dominant model [(DD % + ID %) vs II %], a co-dominant model [ID % vs (II % + DD %)] and a recessive model [DD % vs (II % + ID %)]. Multivariate logistic regression analysis was also performed using different parameters as independent variables to find out their association with genotypic variations of renin gene. Associations were expressed as adjusted OR with 95 % CI. For all analyses, two-sided probability values < 0.05 were considered statistically significant.

Results

The subgroup and clinical characteristics of the study participants are presented in Table 1. Even though the number of male individuals was higher as compared to the number of female participants in both the study groups, the male to female ratio (sex distribution) (p 0.0847) and mean age (p 0.0541) was not significantly different between affected and control groups, demonstrating that the hypertensive and non-hypertensive groups were well-matched and are appropriate for the analyses in question.

A comparison of clinico-biochemical characteristics between hypertensive patients and control individuals revealed that patients had characteristic differences in their biochemical and hemodynamic profiles compared with normotensive participants i.e. hypertensive patients had

more pronounced evidence of atherogenic dyslipidemia (higher levels of total cholesterol, TG and lower levels of HDL-C) than non-hypertensive participants (Table 1), along with higher systolic blood pressure (SBP) readings (p < 0.0001) and diastolic pressure (p < 0.0001), in spite of under antihypertensive medication. Of the hypertensive patients, 154 patients (59.92 %) were taking diuretics, 118 patients (45.91 %) were taking ACE inhibitors, 92 (35.79 %), 74 (28.79 %), 66 (25.68 %) and 32 (12.45 %) patients were on angiotensin receptor blocker, calcium channel blockers, beta blockers and alpha blockers, respectively. Two or more antihypertensive drugs were being taken by 52.91 % of the patients, and 12.06 % were treated with 4 different blood-pressure lowering drugs. The difference in the distribution of epidemiological features were found to be significant between cases and controls with respect to BMI (p < 0.0001) and non-vegetarian dietary habit (p < 0.05), signifying an elevated risk of the condition when associated with these parameters. There were no significant differences in other studied indices {serum creatinine (p 0.0757), LDL-C (0.0769) and plasma glucose (0.0744); Table 1} between the two groups. Parameters that revealed significant differences between hypertensive patients and controls, along with age and sex as clinically significant variables were used for multiple logistic regression analysis as confounding factors.

Genotypic and allelic distribution of Mbo I dimorphism in hypertensive and non-hypertensive individuals is shown in Tables 2 and 3 respectively. The observed genotype frequencies are in agreement with frequencies predicted by Hardy–Weinberg equilibrium, both in affected and control groups (Table 2). Comparable results were obtained when the genotype and allele frequencies were analyzed according to sex and age. The overall frequencies of the genotypes A/A, G/A, and G/G were 43.19, 40.85, and 15.59 percent, respectively, in hypertensive patients and

Table 1 Characteristics of hypertensive (cases) and non-hypertensive (control) participants

Parameters	Non-hypertensive (n:270) Mean $\pm$ SD	Hypertensive (n:257) Mean $\pm$ SD
Age (years)	47.32 $\pm$ 8.4	48.8 $\pm$ 9.2
Sex, males; n (%)	159 (58.88 %)	171 (66.53 %)
Creatinine (mg/dl)	0.79 $\pm$ 0.09	0.81 $\pm$ 0.16
Body Mass Index (kg/m ²)	23.9 $\pm$ 2.6	25.4 $\pm$ 3.3*
Systolic blood pressure (mmHg)	119.8 $\pm$ 8.2	138.6 $\pm$ 10.4*
Diastolic blood pressure (mmHg)	77.4 $\pm$ 3.8	90.2 $\pm$ 4.8*
Fasting plasma glucose (mg/dl)	87.1 $\pm$ 8.6	88.8 $\pm$ 12.9
Triglyceride (mg/dl)	122.45 $\pm$ 15.11	141.12 $\pm$ 14.60*
Total Cholesterol (mg/dl)	182.46 $\pm$ 11.36	206.22 $\pm$ 13.59*
LDL-C (mg/dl)	110.56 $\pm$ 13.24	112.87 $\pm$ 16.57
HDL-C (mg/dl)	45.2 $\pm$ 3.1	40.4 $\pm$ 3.4*

* p < 0.0001

Table 2 Genotype frequencies of rs2368564 polymorphism (renin Mbo I 10631A>G) in hypertensive (cases) and non-hypertensive (control) participants

Genotype	Hypertensive (n:257)		Non-hypertensive (n:270)	
	Expected H W frequency	Observed	Expected H W frequency	Observed
G/G	34.02 (13.24 %)	41 (15.95 %)	58.8 (21.78 %)	65 (24.07 %)
G/A	118.97 (46.29 %)	105 (40.85 %)	134.4 (49.78 %)	122 (45.18 %)
A/A	104.02 (40.47 %)	111 (43.19 %)	76.8 (28.44)	83 (30.74 %)
χ^2	3.54		2.3	
<i>p</i>	0.0598		0.1295	

Table 3 Allele frequencies of rs2368564 polymorphism (renin Mbo I 10631A>G) in hypertensive (cases) and non-hypertensive (control) participants

Allele	Non-hypertensive {n:540 (%)}	Hypertensive {n:514 (%)}	95 % CI	<i>p</i>	OR
A	288 (53.33)	327 (63.62)	1.1955–1.9582	0.0007	1.5301
G	252 (46.67)	187 (36.38)	0.5107–0.8364	0.0007	0.6536

30.74, 45.18 and 24.07 percent, respectively, in controls which meant that this SNP displayed significant differences in the allele (χ^2 11.46, 2 *df*, *p* 0.0007) and genotype (χ^2 10.43, 2 *df*, *p* 0.0054) frequencies between cases and controls. The heterozygous genotypic pattern (G/A; 45.18 %) was most frequent as compared to A/A (30.74 %) and G/G (24.07 %) genotypes in the control group, while amongst patients there was a preponderance of A/A (43.19 %) genotype. In addition, cases showed significantly lower frequency of the wild type G/G genotype (15.59 vs 24.07 %; *p* 0.02). However, no significant difference could be observed in the sex-stratified groups for genotypic and allelic distributions (*p* > 0.05).

The A allele of Mbo I polymorphism of renin gene was much higher in the cases of hypertension as compared to controls (*p* 0.0276), that resulted in a significant association in the A/A genotype with EHT (*p* 0.0032), indicating that the individuals with homozygous for the restriction site may be at substantially higher risk of developing EHT than those with the other two genotypes. This association withstand in multiple logistic regression analysis (Table 4) with an estimated odds ratio of 1.69 with 95 % CI 1.46–2.28 after multiple correction of effect of confounding variables and is substantiated by inter-genotypic variations in the mean systolic and diastolic blood pressure in patients with essential hypertension (Tables 5, 6) i.e. there was statistically significant results found between the genotypes and SBP when compared with A/A versus G/A (*p* 0.0013) and A/A versus G/G (*p* 0.0001), and in the diastolic blood pressure the *p* values for A/A versus G/A and A/A versus G/G was found to be <0.0001. However, heterozygous genotype did not show statistically significant difference with wild homozygous pattern either for SBP (*p* 0.2419; Table 5) or DBP (*p* 0.6179; Table 5).

Table 4 Multivariate regression analysis

Covariates	<i>p</i>	OR	95 % CI
Age	0.65	0.89	0.69–1.1
Sex	0.54	0.72	0.62–1.01
Total cholesterol	0.07	0.91	0.89–1.38
Triglyceride	0.09	0.87	0.76–1.23
HDL-C	0.26	0.47	0.36–0.85
BMI	0.04	1.26	1.06–1.35
A/A versus other genotype	0.02	1.69	1.46–2.28
G/A versus other genotype	0.59	0.88	0.76–1.04

The inheritance hypothesis for this polymorphism was tested according to three models: co-dominant, dominant and recessive and it revealed that REN Mbo I polymorphic variant contribute to EHT predominantly through dominant mode. According to the dominant model, individuals with mutant A allele carriage (A/A and G/A genotype) had a 1.67-fold increased risk of developing hypertension {OR: 1.67 (95 % CI 1.081–2.581); *p* 0.0208} than for those homozygous for the G allele.

Discussion

Although transgenic animal model studies have provided strong evidence that development of hypertension is harboring the renin gene [15–17], there are ambiguous results from preceding molecular genetic studies of humans in linking REN Mbo I dimorphism with EHT. We therefore considered it worthwhile to perform an association study on candidate gene loci (rs2368564 located in intron 9 of

Table 5 Intergenotypic (A10631G variant of the renin gene) variations in clinico-biochemical characteristics of hypertensive patient

Parameters	A/A (n:111) Mean $\pm$ SD	G/A (n:105) Mean $\pm$ SD	G/G (n:41) Mean $\pm$ SD
Age (years)	47.4 $\pm$ 8.6	49.6 $\pm$ 9.1	48.6 $\pm$ 10.2
Sex, males; n (%)	76 (68.46)	66 (62.85 %)	29 (70.73 %)
Creatinine (mg/dl)	0.8 $\pm$ 0.15	0.79 $\pm$ 0.18	0.84 $\pm$ 0.09
Body mass index (kg/m ²)	26.0 $\pm$ 2.8	25.2 $\pm$ 3.4	24.9 $\pm$ 3.7
Systolic blood pressure (mmHg)	141.2 $\pm$ 8.6*†	136.8 $\pm$ 11.2*	134.4 $\pm$ 10.8†
Diastolic blood pressure (mmHg)	92.4 $\pm$ 4.2‡	88.6 $\pm$ 4.4‡	88.2 $\pm$ 4.2‡
Fasting plasma glucose (mg/dl)	91.2 $\pm$ 13.4	87.6 $\pm$ 14.2	88.2 $\pm$ 12.6
Triglyceride (mg/dl)	138.96 $\pm$ 16.26	143.05 $\pm$ 14.44	139.92 $\pm$ 10.35
Total cholesterol (mg/dl)	205.28 $\pm$ 14.26	209.17 $\pm$ 15.12	207.34 $\pm$ 10.9
LDL-C (mg/dl)	110.98 $\pm$ 14.97	115.14 $\pm$ 16.13	113.22 $\pm$ 17.68
HDL-C (mg/dl)	40.1 $\pm$ 2.8	39.8 $\pm$ 3.5	41.1 $\pm$ 3.8

* $p < 0.01$ (AA vs AG); † $p < 0.0001$ (AA vs GG); ‡ $p < 0.0001$ (AA vs AG and AA vs GG)

Table 6 Difference in systolic blood pressure and diastolic blood pressure associated with genotype of hypertensive patients

Genotype	Systolic blood pressure			Diastolic blood pressure		
	Difference	95 % CI	p	Difference	95 % CI	p
G/G	Reference group			Reference group		
G/A	−2.4	−6.44 to 1.64	0.2419	−0.4	−1.98 to 1.18	0.6179
A/A	−6.8	−10.14 to −3.46	0.0001	−4.2	−5.72 to −2.68	<0.0001

Data are shown as differences relative to the respective reference group expressed in mm Hg (95 % confidence intervals)

REN) and indeed found a strong positive correlation between blood pressure and renin 10631A>G gene mutation and identified that this SNP may play critical roles in BP (dys)regulation and susceptibility for hypertension in the studied population.

The genotype frequencies occurred in Hardy–Weinberg proportions in hypertensive as well as in non-hypertensive participants (Table 2), which not only ensured the choice of appropriate controls but even in patient group it suggested that for the locus in question, genotype frequencies will remain stable throughout life, thus rules out the possibility of survival bias. Since, any decrease observed for a particular genotype may suggest that alleles for that genotype carry an increased risk of mortality past the age of reproduction. Although in clinical phenotype such as hypertension deviation from HWE might have been expected, but considering that Mbo I polymorphism itself is in intron, it is unlikely to have produced such a strong effect of its own. As for allelic distribution, the frequency of the A allele is 63.62 % (hypertensive participants; Table 3), which is higher than the published frequency of 22 % in South Indian [18] Malaysian (18.6 %) [19], Bangladeshi (21 %) [20] US Caucasians (51 %) [21] and the UAE hypertensive population (51 %) [13].

Result presented here indicates that gene polymorphism in renin with transition of an adenine (A) to guanine (G) at

10631 independently and significantly increased the risk of hypertension 1.69-fold, which is lower than the 3.8-fold reported in a study on a United Arab Emirates (UAE) population [22], but differs markedly from studies done from a South India [18] Japanese population [23] and a Malaysian population [19], which failed to show an association between Mbo I-RFLP of the renin gene and predisposition to essential hypertension. We found that Mbo I A/A genotypes were significantly associated with increased EHT risk (p 0.0032), exemplified by the fact that there was significant difference between hypertensive and normotensive groups across genotypes (χ^2 10.43, 2 df , p 0.0054), and A allele was more frequent in HTN patients than normotensive controls (63.62 vs 53.33 %; p 0.0122). In the subgroup analysis by gender, no significant difference ($p > 0.05$) was found between males and females related to rs2368564. Multivariate logistic regression analysis (Table 4) confirmed the association in which we adjusted several variables including age and sex (as clinically significant variables) in the covariate list of regression analysis i.e. even after adjusting the known covariates odds ratio observed in our study is 1.69, indicating that the individuals with A/A genotypes is at higher risk of developing EHT than those with the other two genotypes at the locus in question. This is further strengthened by the intergenotypic variations in the mean systolic and diastolic

blood pressure in patients (Tables 5, 6). Results of multiple regression analyses also verified that the other variable with significant, independent association with EHT was BMI (p 0.04; Table 4), but A/A variant had the strongest influence (p 0.02; OR 1.69; 95 % CI 1.46–2.28). Since BMI represents the internal metabolic and physiological environment that plays a key role in development of high BP [24], it's not surprising that BMI may influence the development of EHT.

Similar to this study, rs2368564 SNP also found to be significantly and uniformly associated with hypertensive traits in a range of ethnic groups [13, 21, 25], with Okura et al. [26] were the first to report the linkage of the Mbo I-RFLP at the renin locus with an individual's genetic susceptibility to hypertension. Conversely, it is at variance with previously published REN Mbo I-HTN linkage and association studies [18–20, 23]. This incongruity may be due to the study design, statistical power and the heterogeneous phenotypic characterization of cases and controls. Population differences in genetic makeup or confounding environmental exposures are also plausible explanations, as disease prevalence is a consequence of the inter-section of the genetic variation with different environmental exposures and each population is expected to have a different distribution of relative genotype frequencies and a different constellation of possible environmental histories [27]. Moreover, EHT being a polygenic trait, it has also possible that the main effects of the individual loci might be of small degree to have a strong influence or penetrance and that loci may contribute to the onset and progression of EHT only by their interaction with other genes. Therefore, the difference between the significant association among one population and null effects among other may not be surprising. To reconcile the existing discrepancy, not only further large-scale prospective cohort studies are needed but more DNA sequence analysis is required to identify the EHT causing polymorphism/mutations.

By demonstrating a statistically significant association, this study not only extends earlier studies but also documents the deleterious effect of rs2368564 in studied population. However, compared to preceding studies, the strengths of this study lies in the selection criteria of patient cohort as we excluded patients with features suggesting of secondary hypertension, thus confined our study to EHT; we excluded two main environmental influences (obesity and DM), thus likely most of the selected hypertensive case patients had a strong genetic predisposition to the disease; we carefully designed and performed this study in a in a population of similar socio-economical geographical background, thus minimizes the possibility of any biases that may lead to potential false-positive or false-negative results. Although it may be argued that chance findings, as opposed to ethnic differences or contribution of confounding variables, may explain the inconsistency between our findings and those of

others, the adoption of regression analysis rules out this possibility. In addition, our trial fulfils the criteria of a genetic association study suggested by Hattersley et al. [28].

To conclude, with available evidence the present study suggests that REN 10631A>G gene mutation at the ninth intron play critical roles in BP (dys)regulation and can be implicated in an individual's susceptibility for hypertension and thus implies that genotyping for this renin gene locus could in the future become an important part of the clinical process of risk identification for EHT. Lending further support to our findings, rs2368564 has been shown to be associated with a reduced risk of oedema caused by peroxisome proliferator-activated receptor agonist, with the proposed mechanism being modification of the levels of REN expression [29].

Nonetheless, our findings described here are only statistical observations and provides only the evidences of the association between the REN Mbo I dimorphism with EHT and do not imply causation. Other limitations include that, we did not measure psychiatric co-morbidities, the major risk factor for hypertension; we did not sufficiently controlled the dietary factors (salts, electrolytes) and physical activity in the covariate list of regression analysis, which seem to influence BP variance. Finally, this study was carried out in relatively small ethnically homogenous population, which may not be representative of the entire patient population. The generalizability of these findings will need to be confirmed in future prospective design studies after incorporating larger groups with inclusion of other polymorphisms of RAAS genes and should preferentially focus on gene–environment interactions as well as haplotype analyses which we envisage may lead to better insight and eventually help to more accurately define the susceptibility markers for hypertension. This may assist in identifying individuals at specific risk for developing hypertension, while also supporting possible gene-oriented targeted therapy, i.e. a personalized medicine approach. Future studies should explore whether these relatively common variants may respond preferentially to pharmacological manipulation of the RAAS. In the meantime, the current study underscore that rs2368564 within the renin gene could be involved in the risk of developing hypertension. However, it offers no convincing evidence for an incremental value of screening for renin genotype compared with blood pressure screening. Thus, study findings ought to be interpreted within the context of its precincts.

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