Original Article

Strong Association of a Renin Intronic Dimorphism with Essential Hypertension

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The objectives of this project were two-fold: to identify the genetic mutation that has been detected as an *Mbol* dimorphism in intron 9 of the human renin (*REN*) gene and to confirm a previously reported, putative association between the *REN Mbol* dimorphism and clinical diagnosis of essential hypertension (EHT) in a population of Gulf Arabs from the United Arab Emirates. Sequencing of the *Mbol* dimorphic site was carried out on DNA of randomly chosen cases and controls. A retrospective case-control study was carried out in 689 unrelated subjects (326 first-time, clinically diagnosed hypertensives and 363 age- and gender-matched normotensive subjects), selected from the resident population of the Abu Dhabi Emirate. A polymerase chain reaction/*Mbol*-RFLP based method was employed to compare genotype and allele distributions. Nucleotide sequences at the *Mbol* site of the cut and uncut alleles were determined to be GATC and GGTC, respectively. This A>G mutation is located 10,631 base pairs (bp) 3' to the start of the *REN* gene, and 79 bp 3' to the end of exon 9. The genotype distributions of the *REN* 10631A>G dimorphism were found to be significantly different between hypertensive and normotensive subjects ($\chi^2 = 42.29$, $df=2$, $p<0.001$). Frequencies of A alleles were 0.54 in EHT vs. 0.37 in normotensive subjects, which is even more demarcated than what was found previously. The frequency of AA genotypes was higher in the hypertensive group than in the normotensive group (34.7% vs. 14.0%). The quantification of the association of A alleles with increased risk of EHT was assessed with corresponding odds ratios (OR), which gave the following values: OR of GG vs. AG genotypes, 1.3 (95% confidence interval [CI]: 0.90–1.88); OR of GG vs. AA, 3.75 (95% CI: 2.41–5.86). In conclusion, *REN* 10631A alleles are significantly associated with EHT in the Emirati population. This has now been found in two different and therefore independent sample populations from the Abu Dhabi Emirate. Moreover, this genetic effect seems to be acting in a recessive fashion. Hence, either the *REN* gene itself, or another gene that is in linkage disequilibrium with *REN* 10631A>G, is implicated in the pathogenesis of EHT in Emirati. (*Hypertens Res* 2005; 28: 339–344)

Key Words: genetics, hypertension, polymerase chain reaction, renin

Introduction

Essential hypertension (EHT) is a complex group of disorders with a spectrum of manifestations ranging from those of rare monogenic hypertensive syndromes with Mendelian inheritance

patterns, to multifactorial disorders involving multiple organ systems (1, 2). The cellular pathways involved in its etiology still have several unexplained aspects. The most plausible explanation yet proposed is that environmental factors in conjunction with genetic predisposing factors and epigenetic changes lead to the development of EHT (3, 4).

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It is important to uncover the molecular architecture of blood pressure regulation. This will help in directing pharmacotherapy specific to the needs of the patient and will lay the groundwork for treatment based on gene therapy. The reverse genetic approach of genetic dissection, together with evidence from pharmacological interventions, has revealed the importance of the renin-angiotensin aldosterone system (RAAS) in blood pressure regulation (5–10). Renin is a key enzyme in this system, and variations in the renin gene have been associated with high blood pressure levels. Transgenic animal studies have also alluded to the involvement of the renin gene in blood pressure regulation (10, 11).

One of the several approaches to studying the effect of genes in humans is the association method using a candidate gene approach. A number of association studies between single nucleotide polymorphic (SNP) markers of renin and other candidate genes and clinical variables of EHT have shown inconsistent results (7, 12–33). Okura *et al.* were the first to show a positive association between renin gene (*REN*) restriction fragment length polymorphic (RFLP) alleles using *Mbo*I restriction endonuclease, and EHT (12). Positive associations were also shown in the US Caucasian and United Arab Emirates (UAE) population (18, 26, 28, 29). However, no association was seen in other studies (7, 13, 14, 21–23). Recently, Fu *et al.* were unable to find any association between renin alleles and hypertension in the Japanese population (27). One of the possible explanations for this variable pattern of association is that it may involve more than one gene, with none of the involved genes having a strong influence or penetrance, as is expected for a complex polygenic disorder. Previously, Frossard *et al.* (26) showed a strongly positive association between a renin gene *Mbo*I polymorphism and EHT in the UAE Gulf Arab population.

To confirm the strong genetic influence observed by Frossard *et al.* (26), we chose a different population in the UAE, and checked the association of *Mbo*I polymorphism with EHT. Although the *Mbo*I RFLP has been reported before, its sequence has not been determined. We here report the exact DNA sequence of the polymorphic locus in the wild type, and the mutated allele that has the enzymatic site of *Mbo*I restriction endonuclease.

Methods

We conducted a case-control study to observe the putative association between renin gene *Mbo*I alleles and the clinical diagnosis of EHT. The ethical approval for the study was obtained from the Ethics Review Committee of the university and hospital before undertaking subject recruitment.

Subjects

The study subjects were Gulf Arabs and UAE nationals. A total of 689 unrelated subjects were selected from the resident population of Abu Dhabi Emirate. Informed consent was

Fig. 1. Structure of the human renin gene and the DNA sequences of the *Mbo*I polymorphic alleles (figure not drawn to scale).

obtained from each patient and control subject. The subjects included 355 males and 334 females with a mean age of 49 years. The unique advantage of this study is that all cases were first-time detected hypertensives.

Selection of Cases and Controls

Hypertensive patients were selected from the outpatient clinics of the Mafraq Hospital, Abu Dhabi. Patients were considered to have primary hypertension if they met all three of the following criteria: systolic blood pressure (SBP) >160 mmHg and/or diastolic blood pressure (DBP) >95 mmHg on at least three separate occasions; family history of hypertension; and absence of clinical signs, symptoms or laboratory findings suggestive of secondary hypertension. All hypertensive patients who had seen a physician before or were taking any drugs were excluded from the study. Following selection and informed consent, a 5 ml venous blood sample was taken. After diagnosis and inclusion, the patients were advised on appropriate dietary modification and pharmacotherapy.

Control subjects were individuals with an SBP <140 mmHg and a DBP <90 mmHg and no family history of hypertension. None of the control subjects was on antihypertensive or other pharmacotherapy that could affect blood pressure. Control subjects were selected from the normal healthy population.

The serum cholesterol levels were obtained from medical records for all subjects. In cases in which an individual was using lipid-lowering medication, the cholesterol values before starting the medication were used. None of the subjects had a history of smoking and all subjects claimed not to drink alcohol at all. Body weight and height measurements were taken to calculate body mass index (BMI).

DNA Extraction and Amplification

DNA was extracted from 5 ml of venous blood by the usual methods (34). The oligonucleotide sequences and polymerase chain reaction (PCR) assay were used as described previously (26) to get a final volume of 10 µl.

DNA Sequencing of *REN Mbo*I Alleles

The sequencing of the polymorphic region was carried out by

Table 1. Distribution by Gender, Mean Age, Systolic and Diastolic Blood Pressures, Total Cholesterol (Chol) Levels and Body Mass Index (BMI) Values amongst the Two Groups of UAE Subjects (Normal Healthy Controls, and Patients with EHT)

	Sample size	Gender (M/F)	Age (years)	SBP (mmHg)	DBP (mmHg)	Total Chol (mmol/l)	BMI
Normotensives	363	185/178	48.3±13.7	119.5±12.8	77.2±7.7	5.2±0.7	28.7±4.1
Hypertensives	326	170/156	49.7±9.5	158.0±15.4	98.2±7.3	6.7±2.5*	28.9±3.3

*Indicates a significant difference at $p < 0.05$ from control values. SBP, systolic blood pressure; DBP, diastolic blood pressure; EHT, essential hypertension; UAE, United Arab Emirates; M, male; F, female.

using the DNA from the Emirati population. Sequencing was carried out on 10 samples (5 cases and 5 controls) only, since the purpose was to precisely identify the polymorphic site rather than to screen. The DNA samples of these 10 individuals were amplified using the same PCR conditions to get a final volume of 50 μ l. To purify the amplified product to get the specific target DNA, the PCR product was separated by agarose gel electrophoresis. The target bands were cut out of the gel and DNA was purified using a Wizard PCR Preps DNA Purification System (Promega Biosciences Inc., San Luis Obispo, USA). The purified DNA was subjected to dye terminator cycle sequencing (Applied Biosystems, Foster City, USA) using the forward primer. The sequencing reaction products were purified again. The reaction products were dissolved in the standard formamide containing gel-loading buffer and sequenced by an ABI Prism 377 DNA sequencer (Applied Biosystems). The results were duplicated. The sequence changes found were confirmed by sequencing using the reverse primer.

Restriction Using *MboI* or *Sau3A1* Enzymes

After confirming the polymorphic site, RFLP was used to screen the populations. Five μ l of the 10 μ l PCR product was subjected to restriction reaction for 2–5 h at 37°C in a total volume of 10 μ l. The reactions were carried out using either restriction enzyme *MboI* (New England Biolabs, Beverly, USA) or its isoschizomer *Sau3A1* (Pharmacia Biotech, Pharmacia Export S.A., Glyfada, Greece). The results of the restriction reaction were determined by subjecting the reaction mixture to 1.5% agarose gel electrophoresis (Gibco BRL, Life Technologies, Gaithersburg, USA) at 80 V for 40 min. DNA fragments were visualized by staining with ethidium bromide. The *MboI*-cut allele was visualized as two DNA fragments of 171 base pairs (bp) and 79 bp, and the *MboI*-uncut allele as a 250 bp fragment.

Data Analysis

The statistical analyses were carried out using an SPSS® version 10.0 for Windows® software package. χ^2 analysis was used to determine statistically significant differences in the genotype distributions in cases and controls. Contingency

tables were directly used to calculate the odds ratios. Hardy-Weinberg proportions were determined using the χ^2 procedure. Values of $p < 0.05$ were considered to indicate statistical significance for all analyses.

Results

Identification of the *MboI* Dimorphism

The ninth intron of the renin gene was shown to harbor this single nucleotide dimorphism, which creates a palindromic site for the restriction endonuclease *MboI* and its isoschizomer *Sau3A1* enzymes. The nucleotide sequences at the *MboI* site of the cut and uncut alleles are GATC and GGTC, respectively. The A>G mutation is located 10,631 bp 3' to the start of the *REN* gene (Fig. 1).

Association of the *REN* 10631 A>G mutation with EHT

The study population of 689 Emirati subjects underwent complete phenotypic and genotypic characterization. The study group comprised 355 males and 334 females with an overall mean age of 49 years. The mean SBP and DBP in hypertensives were 158.0±15.4 and 98.2±7.3, respectively, and those in normotensives were 119.5±12.8 and 77.2±7.7. The clinical phenotypes in both groups are described in Table 1. The mean age, total cholesterol level and BMI were higher in the hypertensive group than in the controls, but the difference was statistically significant only in the case of the mean total cholesterol level. Both groups had high mean BMI values, and as would be expected, BMI was not associated with hypertension or either of the two alleles.

The genotype and allelic distributions in the hypertensives and controls are given in Table 2. The *MboI* genotypes were in Hardy-Weinberg equilibrium in the control group ($\chi^2 = 0.4325$, degrees of freedom [df]=1, $p > 0.1$), but the distribution in the hypertensive group was significantly different from the expectations of Hardy-Weinberg equilibrium ($\chi^2 = 17.3$, df=1, $p < 0.001$).

The allelic distributions in hypertensive and normotensive subjects varied significantly ($\chi^2 = 42.29$, df=2, $p < 0.001$). The frequency of the *MboI* A/A genotype was higher in the hyper-

Table 2. Distributions of *REN MboI* 10631 Genotypes and Allele Frequencies in the Two Groups (Normotensive and Hypertensive) of UAE Subjects

<i>MboI</i> dimorphism	N (%)	
	Normotensives	Hypertensives
Genotypes		
<i>MboI</i> G/G	149 (41.0%)	88 (27.0%)
<i>MboI</i> G/A	163 (44.9%)	125 (38.3%)
<i>MboI</i> A/A	51 (14.0%)	113 (34.7%)
Allele frequencies		
P[G]	461 (63.5%)	301 (46.2%)
Q[A]	265 (36.5%)	351 (53.8%)

N: number of subjects and true allele frequency. UAE, United Arab Emirates.

tensive group than in the normotensive group (34.7% vs. 14%). The frequency of the *MboI* G/G genotype was 27% and 41% in the hypertensive and control groups, respectively. The odds ratio showed a strong association between the *MboI* A allele and increased risk of EHT. The odds ratio for the *MboI* G/G genotype vs. the *MboI* G/A genotype was 1.3 (95% confidence interval: 0.90–1.88), while that for *MboI* G/G vs. *MboI* A/A was 3.75 (95% confidence interval: 2.41–5.86). The presence of the *MboI* A allele was associated with a higher blood pressure in both the hypertensives and the controls.

Discussion

The effects of genetic variations in the renin gene have been studied in humans by using linkage and sib-pair linkage analysis and association studies, based on linkage disequilibrium. The results of various sib-pair analyses have been unsuccessful in clearly implicating renin gene variations as the etiological basis of hypertension (35–38). Linkage disequilibrium between candidate gene loci and their association with intermediate clinical phenotypes of hypertension has been a useful genetic model for observing the effects of genetic factors that have reduced penetrance. Unfortunately, the significance of this model is easily lost in the presence of confounding factors. Hence, the results of association studies have not been consistent, even in the same populations.

In the case of the renin gene, both positive and negative results regarding the association between *REN* RFLP and EHT have been reported (7, 12–29). Of the studies showing a positive association between the *MboI* A allele and EHT, very few have been confirmed in the same population (28, 29). Although this *MboI* polymorphism is in an intron, it has shown strongly positive association with EHT more than once and in different population groups, which rules out the suspicion of this being a spurious association. Being in an intron, the *MboI* polymorphism may not have a direct effect on renin production, but at the moment we are mostly unaware of the

functions of the large unexpressed part of the genome, *i.e.*, the intronic DNA. Recently, Hasimu *et al.* identified a variable number of tandem repeats (VNTR) polymorphism in intron 7 of the renin gene that was not associated with EHT (39). On the other hand, Jin *et al.* reported positive association of an exonic polymorphism in the angiotensin II type 2 receptor gene, that was in linkage disequilibrium with an intronic polymorphism (40). Moreover, because it has been hypothesized that introns are important for the stability of the DNA molecule, intronic mutations, though not as important as those in exons, cannot be neglected.

The Emirati population consists of Gulf Arabs of Bedouin descent in a Federation of seven Emirates (UAE). This population is known for its high level of consanguinity. The UAE population is also unique for its lack of confounding factors such as smoking and alcohol, and thus is very well suited for association studies of cardiovascular disorders. In a previous study on the same locus of the renin gene in this population, the *MboI* A allele was found to be concentrated in hypertensive individuals and was significantly associated with EHT (26). The varying results of associations between renin *MboI* alleles and EHT in different populations suggested that it would be worthwhile to test our results in the Emirati population. This analysis has revealed an even stronger association between the *MboI* A allele and clinical diagnosis of EHT in a different subset of the Emirati population, and with a larger number of control subjects.

Frossard *et al.* (26) reported frequencies of 0.64 and 0.36 for the *MboI* G and *MboI* A alleles in normotensive subjects, whereas the allele frequencies in the hypertensive group were 0.49 and 0.51, respectively. In the present study, we found an even greater difference in allele distributions between the two groups, with the normotensive group having a greater proportion of the *MboI* G allele and the *MboI* A allele being more prevalent in the hypertensive subjects (0.63 vs. 0.54, respectively). The *MboI* A allele is responsible for putting the carriers at a 3.8 times higher risk of developing hypertension as compared to the *MboI* G allele carriers, at or before the age of 50 years.

The subjects (both hypertensives and controls) included in this study were different from the study subjects of Frossard *et al.* (26), *i.e.*, the members of their population were unrelated to those of our population, and the two were selected separately and at different times. Given the similarity in study designs, the results of the two analyses can therefore be merged to show the significance of the observed effect in a larger proportion of the Emirati population. Multiplying the *p*-value observed by Frossard *et al.* (26), $p < 10^{-6}$, by the value observed in the present study, $p < 10^{-8}$, gives a combined value of $p < 10^{-14}$, which represents an extremely strong statistical difference in the differential proportions of *MboI* alleles in the Emirati population. These results suggest that the *REN* gene plays a major role in the pathogenesis of EHT in Emiratis.

This notion is further supported by the fact that early onset

of disease indicates a greater influence of causative genes. The hypertensive subjects in our study were, on average, 3 years younger than those included in the previous study (26), which points towards the importance of *REN* polymorphism in the early development of hypertension in the UAE population.

The genotype frequency distribution in the normotensive subjects was found to be in agreement with Hardy-Weinberg equilibrium, which signifies appropriate population selection, given that the population under study is highly consanguineous. Frossard *et al.* (26) also found that the genotype frequencies of their control subjects were in Hardy-Weinberg equilibrium. On the other hand, the genotype distribution in the hypertensive group of the present study was not in accord with Hardy-Weinberg equilibrium. We checked the accuracy of our RFLP screening method by sequencing randomly selected samples and found no difference in results between the two methods (data not shown). The Hardy-Weinberg disequilibrium in the hypertensive group can be explained by the presence of another locus that is close to the *MboI* polymorphic site, has a strong influence on blood pressure and is strongly linked to the *MboI* site. The fact that the *MboI* polymorphism itself is in an intron is unlikely to have produced such a strong effect by itself.

The results suggest that the genetic background of the Emirati population is highly conserved in itself, but at the same time is different from that of other populations that have been studied so far. The effect of genetic polymorphisms has been found to be much higher in the UAE population, due possibly to the very high consanguinity in this population. Studying the difference in the genetic profiles of different geographic populations will help not only in dissecting the molecular pathways involved in hypertension but also in understanding the ancestral relationships and human evolution. In a recent key review, Katsuya *et al.* (41) showed that the distributions of allele frequencies of candidate genes for salt-sensitive hypertension differed between Japanese and Caucasians. Therefore, it is important to study the effect in populations that differ not only with respect to ethnicity but also geographically.

Although other polymorphisms of the renin gene and their haplotypes have been studied for their possible association with hypertension (28, 42), haplotype analysis could not be carried out in this study. However, we consider that our present results would be better followed-up by functional studies rather than haplotype analysis, as functional studies have been shown to provide stronger evidence of the involvement of a gene in a phenotype (43, 44).

We conclude that the *REN* 10631 (*MboI*) A allele is significantly associated with EHT in the Emirati population. Hence, either this renin gene locus itself, or another locus in that genomic region, has a role to play in the pathogenesis of EHT. The UAE population is suitable for carrying out genetic studies for complex disorders, due to its joint family system and high degree of consanguinity. Future sib-pair analyses would

further help in identifying the genetic effects underlying hypertension in this population.

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