Malaysian Journal of Medicine and Health Sciences Vol. 6 (2) June 2010: 19-24

Associations Between Primary Hypertension and Genes in the Renin Angiotensin System: A Prospective Two-center Study in University Kebangsaan Malaysia Medical Center and International Medical University Cardiology Clinic

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ABSTRACT

This study targeted two candidate genes from the best known regulator of blood pressure; the rennin angiotensin system; the ACE gene I/D polymorphism and the angiotensinogen M235T polymorphism. The study aimed to determine the genotypes trend between two different populations; the primary hypertensive patients, and the normal populations. 126 subjects were involved in this study (86 primary hypertensive patients and 40 normal individuals). All demographic factors were considered and analyzed. Insertion/deletion polymorphisms of the ACE gene were determined by an assay based on the polymerase chain reaction (PCR). Polymorphism analysis using PCR-RFLP procedure was used to identify the missense mutation M235T of the AGT gene. All significant data was collected using standardized case report form. The association of the different genotypes and the subjects' condition was analyzed using the chi squared and odds ratio analyses. In the pooled analysis of both groups, it was shown that the polymorphisms in these genes were significantly associated with the incidence of primary hypertension, $p<0.05$. Results also showed that the D allele of the ACE gene may be associated with increased risk of primary hypertension ($p<0.05$, O.R: 3.0 [C.I: 1.25 – 5.35]). The angiotensinogen M235T polymorphism also showed a significant result; the T allele is associated with increased risk of primary hypertension ($p<0.05$, O.R: 2.56[C.I: 1.55 – 5.28]). This knowledge of the candidate genes of rennin angiotensin system has rendered it possible to show that gene polymorphism in symphony leads to the individual risk of primary hypertension.

Keywords: ACE, M235T, rennin, hypertension

INTRODUCTION

Primary hypertension is a multifactorial disease, characterized by significance increase of blood pressure and the specific cause of this condition is still unknown. Recent research done in Malaysia indicated that the prevalence of hypertension is high in both Malaysian males and females. It poses a serious problem with low awareness, poor treatment and poor control of blood pressure. Besides the environmental factor being the main reason of the disease, genetic factors may best answer the reason why each individual has a different risk towards this disease[1]. The recognition of a number of environmental risk factors has led to important advances in the prevention and treatment of the disease[2]. Our knowledge of heritability is limited to the predictive importance of a positive family history[3,4] and through observation of family aggregation[5,6].

The angiotensin converting enzyme (ACE) gene contains a polymorphism based on the presence of an insertion (insertion [I]) or an absence (deletion [D]) within an intron of a 287 base pair (bp) nonsense DNA domain, resulting in DD and II homozygotes and DI heterozygotes[7]. The angiotensinogen M235T polymorphisms have a missense mutation located at exon 2. This will result in changes of metheonine production to threonine at position 235 for matured angiotensinogen[8].

Several biological actions of ACE could be involved in the pathogenesis of increased blood pressure. The activation of angiotensin I and the inactivation of bradykinin potentially result in vasoconstriction, decreased tissue perfusion, angiotensin induced stimulation of plasminogen-activator inhibitor type 1 may promote the formation of occlusive coronary thrombi and remodeling[9]. The observed codominant association between the D and I polymorphisms and plasma ACE activity could be consistent with the reported increase in any blood pressure and cardiovascular risk associated with the DD genotype[10]. Previous research, has shown a correlation between the disequilibrium of T235

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of the angiotensinogen gene and the molecular variant at the angiotensinogen gene proximal promoter. This affects the basal transcription level of the gene and explains how homozygous T235 has 10% to 20% higher level of plasma angiotensinogen then homozygous M235[11].

Beyond the potential importance of the polymorphism as a predictor of risk, the recognition of a genetic variant of the renin angiotensin candidate genes as a pathogenetic factor in primary hypertension is of particular interest of the ready availability of target specific pharmacologic agents in the form of ACE inhibitors [12]. Clearly, the clinical implications of this finding would be even more far reaching if the different genotypes not only are associated with the occurrence of primary hypertension but also represented a modifiable risk factor for end organ damage by primary hypertension[13].

METHODS

Sampling

The study was approved by the ethical committees from National University of Malaysia and International Medical University, Malaysia.

Subjects were recruited from the medical clinic at two different medical centers. Between January 2006 to January 2007 all patients from three main races in Malaysia (Malay, Chinese, Indian) presented to the clinic were screened for the study. All subjects gave written informed consent for participation. All selected hypertensive patients had a history of hypertension for more than two years. None of those subjects (hypertensive patients / normal control) had chest trauma, pericardial effusion, angina, and renal dysfunction prior to the study. The exclusion criteria are listed in Table 1.

Table 1. Exclusion criteria for the subjects

1. Secondary hypertensive patients.
2. Patients previously diagnosed with any heart diseases.
3. Patients with clinical symptoms / complaints of heart failure.
4. Patients previously diagnosed with myocardial infarction.
5. Patients with other metabolic diseases (eg: obesity, diabetes mellitus.)
6. Patients diagnosed with malignancy.
7. Patients with depressive disorder.

Five milliliters of blood were drawn into EDTA vacutainer test tubes from each subject. DNA extraction was carried out using the QIAamp® DNA minikit by QIAGEN®. The DNA samples were then stored at -20°C. Blood pressure was measured according to standard protocols, and information on age, sex, body mass index, alcohol consumption, and cigarette smoking was collected by trained interviewers using standardized case report forms.

Determination of ACE genotypes

The D and I alleles were identified on the basis of polymerase chain reaction (PCR) amplification of the respective fragments from intron 16 of the ACE gene and subsequent size fractionation and visualization by electrophoresis. Before starting the PCR procedure, 15 µl of PCR master mix was added to 5 µl DNA sample in the 0.5 ml eppendorf tube. The master mix contained 2µl of each primers, 200 µM deoxynucleotide triphosphates, 1.3 mM magnesium chloride, 50mMpotassium chloride, 10mM TRIS hydrochloricacid (ph 8.4 at 25 degrees C), 0.1 percent triton X-100 and 0.35 unit of thermos aquaticus DNA polymerase was added. In this study, an optimized primer pair was used to amplify the D and I alleles, resulting in 319 bp and 597 bp amplicons, respectively (hace 3s; 5' GCC CTG CAG GTG TCT GCA GCA TGT 3', hace 3as; 5' GGA TGG CTC TCC CCG CCT TGT CTC 3'). The thermocycling procedure (PTC 100 apparatus) consisted denaturation at 94^0C for 30 seconds, annealing at 56^0C for 45 seconds and extension at 72^0C for two minutes, repeated for 35 cycles, followed by a final extension at 72^0C for 7 minutes.

The samples were added with 2µl bromophenol blue dye. Then, the mixture was loaded onto a 1.5 percent submarine agarose slab (FMC, Rockland) containing 1M TBE buffer and 0.8µl of ethidium bromide and fractionated according to size at 150V. The amplification products of the D and I alleles were identified by 300nm ultraviolet transillumination as distinct bands.

Determination of the angiotensinogen M235T genotype

Restriction fragment length polymorphism was used to determine angiotensinogen M235T gene and visualization by

the electrophoresis. The master mix contained 2µl of each primers, 200 µM deoxynucleotide triphophates, 1.3 mM magnesium chloride, 50mMpotassium chloride, 10mM TRIS hydrochloricacid (ph 8.4 at 25 degrees C), 0.1 percent triton X-100 and 0.35 unit of thermos aquaticus DNA polymerase was added. The primers used were; F: 5' GAT GCG CAC AAG GTC CTG TC-3' and R: 5' GGT GCT GTC CAC ACT GGA CCC-3'. The restriction enzyme used was PsyI (MBI Fermentas). The thermocycling procedure (PTC 100 apparatus) consisted the predenaturation at 94^0C for 5 minutes, denaturation at 94^0C for 1 minutes, annealing at 71^0C for 1 minute and extension at 72^0C for one minute, repeated for 35 cycles, followed by a final extension at 72^0C for 7 minutes.

RESULTS

Demographic data

There were 126 subjects recruited for this study. All subjects in the study (normotensives and hypertensives) have a family history of hypertension. None of the subjects were smokers. Table 2 summarizes the demographic data collected in this study and range of blood pressure of the subjects.

Table 2. Demographic data of normotensives and hypertensives subjects

Subjects (N: 126)	Normotensives (N: 40)	Hypertensives (N: 86)	p values (<0.05)
Age	30.78 ± 9.61	41.69 ± 10.92	>0.05
Body mass index	23.71 ± 2.41	25.82 ± 2.67	>0.05
Ethnic			
Malay	20 (50%)	46 (53.4%)	
Chinese	10 (25%)	20 (23.3%)	>0.05
Indians	10 (25%)	20 (23.3%)	
Gender			
Male	10 (25%)	53 (61.6%)	>0.05
Female	30 (75%)	33 (38.4%)	
Blood pressure			
SBP- mean mmHg	122 (116-128)	145 (140-150)	
DBP	78 (75-81)	92 (87- 95)	<0.05

Figure 1 shows the ACE gene polymorphism; DD shows a single band at 319bp, homozygous II at 597bp and heterozygous ID at 597bp and 319bp. Figure 2 shows angiotensinogen gene M235T polymorphism; MM showed a single band at 303bp, homozygous TT at 279bp and heterozygous MT at 303bp and 279bp.

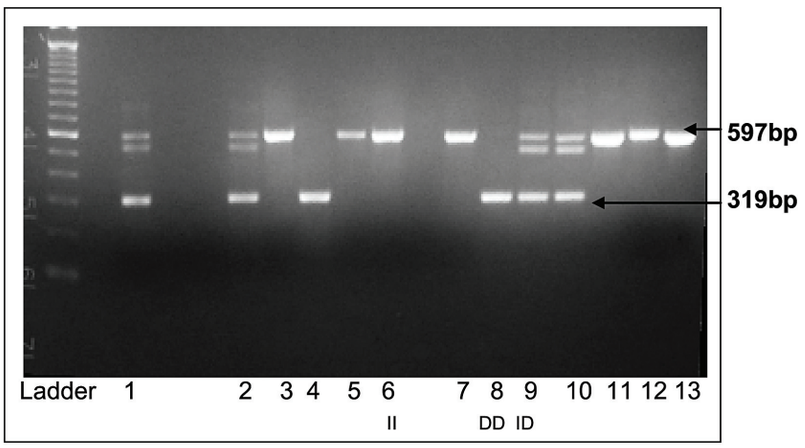

Figure 1. ACE gene polymorphism. II polymorphism is shown at lane 3, 5, 6, 7, 11, 12, 13 (597bp); DD polymorphism is shown at lane 4, 8 (319bp); ID polymorphism is shown at lane 1, 2, 9, 10 (597bp & 319bp)

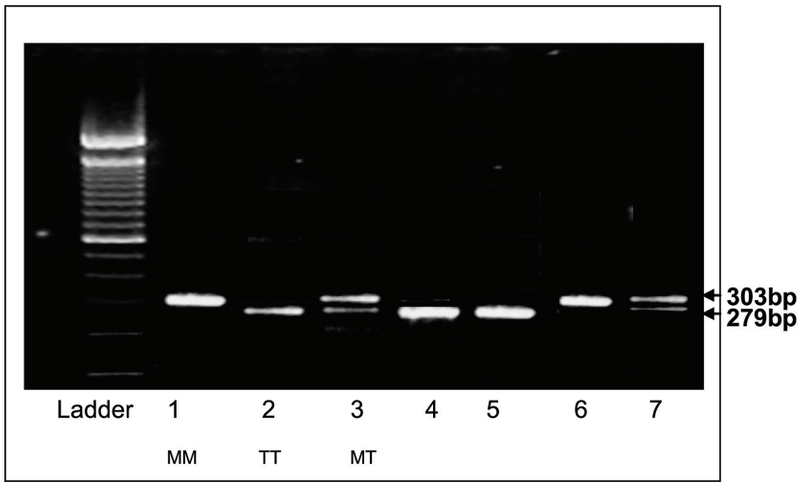

Figure 2. Angiotensinogen M235T gene polymorphism. MM polymorphism is shown at lane 1,6 (303bp); TT polymorphism is shown at lane 2, 4, 5 (279bp); MT polymorphism is shown at lane 3,7 (303bp & 279bp)

The association of the different genotypes and the subjects' condition was analyzed using the chi squared and odds ratio analyses. In the pooled analysis of the two groups, both genes (ACE gene and angiotensinogen gene M235T) showed that the polymorphism in these genes were significantly associated with the incidence of primary hypertension, $p<0.05$. Results showed that the D allele of the ACE gene may be associated with increased risk of primary hypertension ($p<0.05$, O.R: 3.0 [C.I: 1.25 – 5.35]). The angiotensinogen M235T polymorphism also showed a significant result, the T allele may be associated with the increased risk of primary hypertension ($p<0.05$, O.R: 2.56[C. I: 1.55 – 5.28]) (Table 3).

Table 3. Genotypes and alleles frequencies of ACE gene polymorphisms and M235T variant of AGT gene for hypertensives and normotensives group

	Normotensives	Hypertensives	Total	
Genotypes				
II	30	28	58	χ^2= 25.154
ID	10	28	38	df = 2
DD	0	30	30	p<0.001
Total	40	86	126	
Alleles				
I	70	84	154	χ^2= 34.344
D	10	88	98	df = 1
				p<0.001
Total	80	172	252	
Genotypes				
MM	6	26	32	χ^2= 49.74
MT	40	10	50	df = 2
TT	40	4	44	p<0.05
Total	86	40	126	
Alleles				χ^2= 49.25
M	52	62	114	df= 1
T	120	18	138	p<0.05
Total	172	80	252	

DISCUSSION & CONCLUSION

Blood pressure has a bell shaped distribution. There is no clear border dividing the normotensive and hypertensive person. However, determination of blood pressure is a very important factor in deciding treatment. Hence medical specialists have made many suggestions of blood pressure cut off points in order to define hypertension. Nowadays, it is accepted that we should define hypertension by the level of blood pressure at which the benefits of treatment outweigh the risks[14]. Despite many diverse definitions, the specific mechanism contributing towards hypertension remains unsolved[15].

The strong correlation between genetics and primary hypertension as well as heart disease is the main field that is being studied nowadays to solve the puzzle about specific etiology and pathophysiology of primary hypertension[16]. Renin angiotensin candidate genes were chosen for this study because these genes have specific functions in synthesizing Angiotensin II, a potent vasoconstrictor. The action of this hormone as a potent vasoconstrictor is believed to correlate with occurrence of primary hypertension and heart disease[17]. Besides, it is believed the polymorphisms of the genes influence the activity of angiotensinogen and angiotensin converting enzyme. This observation enhanced the focus of interest on these genes[18]. A study in China in contrast to a study in Taiwan showed no significance correlation between these two genes polymorphisms and the occurrence of any vascular or heart disease[19]. This prompted us to choose the two genes to ascertain if the polymorphisms have any impact on a small Malaysian population that constitutes of the three major races; Malay, Chinese and Indian. Previous studies that were carried out among the Malaysian population were focused on one targeted gene in the rennin angiotensin pathway. However, this study attempted to show that there are more than one polymorphism in this pathway that may contribute towards increased risk of primary hypertension[20, 21].

The study holds promise of a potential beneficial therapeutic modality. Based on the results, we may conclude that polymorphisms of rennin angiotensin candidate genes in tandem may increase the individual risk of primary hypertension.

ACKNOWLEDGEMENT

This study was supported by Universiti Industri Selangor (UNISEL) and International Medical University (IMU). We thank the Cancer Research Laboratory group, National University of Malaysia Medical Center for their technical and moral support.

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