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No Association of *BglI* Dimorphism of Human Renin Gene in Hypertensive Subjects in Malaysia

[1]R. Vasudevan, [1]Patimah Ismail and [1]Johnson Stanslas and [2]Norashikin Shamsudin
[1]Molecular Biology Lab, Department of Biomedical Science,
[2]Department of Medicine, Faculty of Medicine and Health Sciences,
Universiti Putra, Malaysia

Abstract: The presence of a dimorphic *BglI* site in the first intron of the Renin (REN) gene is an increased risk in essential hypertension. Several studies have found the association between *BglI* dimorphism and essential hypertension with conflicting results in various populations, which might be due to ethnic and geographical variations. The objective of this study is to determine the relationship between the *BglI* dimorphism of REN gene and Essential Hypertension (EHT) with or without Type 2 Diabetes Mellitus (T2DM) in Malaysian subjects. The study includes 70 EHT, 60 EHT with T2DM and 70 unrelated healthy subjects from the three ethnic groups of Malaysian Subjects. The genotype of *BglI* dimorphism was done by PCR-RFLP method using *BglI* restriction enzyme. The frequency of the *BglI* (+) allele was 37.86% in EHT, 40.83% in EHT with T2DM subjects and 35.71% in control subjects. The results of this study indicate that the *BglI* (+) allele of REN gene is not associated with essential hypertension with or without T2DM in Malaysian Subjects.

Key words: Essential Hypertension, Type 2 Diabetes Mellitus, Renin, *BglI* dimorphism, PCR-RFLP

INTRODUCTION

Essential Hypertension (EHT) is a multifactorial complex disorder caused by genetic, environmental and demographic factors. Nearly 30-50% of genetic variants contribute to the blood pressure variability in human essential hypertension (Garcia *et al*., 2003). Several candidate gene studies have been analyzed in susceptibility to blood pressure in various populations with conflicting results (Jiang *et al*., 2007; Mondorf *et al*., 1998). The Renin Angiotensin-Aldosteone System (RAAS) polymorphisms have been extensively studied in relation to hypertension, since it plays an important role in regulating the blood pressure (Zhu *et al*., 2003). Among RAAS, Renin is an important susceptibility gene for involvement in the development of essential hypertension. Renin is a protease enzyme circulating in the blood stream which hydrolyzes angiotensiongen into the peptide angiotensin I which is further cleaved into angiotensin II, a potent vasoconstrictor and a stimulator for aldosetrone release, by endothelial bound angiotensin converting enzyme (Davis and Robers, 1997).

The renin gene has been mapped to chromosome 1q32 and comprises 10 exons and 9 introns in a 12.5 kb length and *BglI* dimorphic site is in the first intron of Renin (REN) gene (Miyazaki *et al*., 1984). Studies using transgenic rats have demonstrated a strong relationship of renin gene in mouse with blood pressure (Mullins *et al*., 1990). After the first positive association study between REN gene and EHT using MboI restriction enzyme (Okura *et al*., 1993), more studies were carried out in various populations in different polymorphic sites of REN gene. In two independent population study, the variations of the REN (or nearby) gene that may be in linkage disequilibrium with the REN *BglI* (+) marker could play a possible role in contributing to an increased individual's genetic susceptibility to EHT in UAE population and amongst US hypercholesteroleic Caucasians (Frossard *et al*., 1999). Several studies have shown both positive association with EHT, family history of EHT and intermediate phenotypes (Ahmad *et al*., 2005; Chiang *et al*., 1997) and negative results as for association or linkage studies (Morris and Griffiths, 1988; Jeunemaitre *et al*., 1992) have been reported. Frossard *et al*., showed no association of *BglI* polymorphism in REN gene with stroke. The information in related to hypertensive subjects with T2DM and *BglI* dimorphism is still unclear, particularly in Malaysian Subjects. The conflicting results of *BglI* polymorphism in REN gene initiated us to determine the association of

Corresponding Author: Assoc. Prof. Dr. Patimah bt Ismail, Department of Biomedical Sciences, Faculty of Medicine and Health Sciences,Universiti Putra, Malaysia

dimorphic *Bgl1* site of REN gene in Essential Hypertension with or without Type 2 Diabetes Mellitus in Malaysian Subjects.

MATERIALS AND METHODS

Subjects: The study protocol has been approved by Ethical Committee of Faculty of Medical and Health Science; Universiti Putra Malaysia (UPM). Upon the approval, a total of 130 subjects were recruited from the UPM Physician Clinic and the medical wards at Hospital Kuala Lumpur, who was consecutively attending from April 2006 to April 2007. In this study, 70 EHT patients without T2DM, 60 EHT with T2DM and 70 unrelated healthy individual subjects were recruited. Essential Hypertension was defined as an elevated Systolic Blood Pressure (SBP) > 140 mm Hg and sustained Diastolic Blood Pressure (DBP) > 90 mm Hg or who were currently receiving anti-hypertensive therapy. Type 2 Diabetes Mellitus was defined as fasting plasma glucose > 7.0 mmol/l and the levels were obtained from the medical records of all the patients before starting the medication were used. Healthy individuals were randomly collected from the UPM staff and others, categorized with resting SBP<140 and DBP<90 mmHg on at least 2 separate occasions, whereas the plasma glucose level was below 7.0 mmol/l and had a negative family history of hypertension and diabetes. A questionnaire in both Malay and English language was obtained to asses the socio-demographic factors. Informed consent form was obtained from all the subjects who participated in this study.

Sample collection and biochemical analysis: Individual weight and height were obtained to calculate Body Mass Index (BMI) using the formula, weight (kg)/ [height (m)]^2. Peripheral blood samples were collected by a qualified phlebotomist. Plasma was separated from the blood by centrifugation method and stored at -20°C for further analysis. Since, the lipids and cholesterol play an important role in the development of EHT and T2DM. Plasma samples and the fasting plasma glucose levels for controls was measured by the glucose oxidase method which was performed on Hitachi-912 Autoanalyser (Hitachi, Germany) using kits supplied by Roche Diagnostics (Mannheim, Germany) to determine the level of Triglycerides (TG), High Density Lipoprotein Cholesterol (HDL-C), Total Cholesterol (TC). Low Density Lipoprotein Cholesterol (LDL-C) was calculated by Friedewald formula (Friedewald *et al*., 1972).

Genotyping methods: According to the manufacturer's protocol, genomic DNA was extracted from peripheral blood using a DNA isolation kit (BioBasic.Inc, Canada). The extracted DNA was quantified and the purification was checked on Biophotometer (Eppendorf, Germany). The4.8 kb region of *Bgl1* dimorphic site of REN gene was amplified by PCR with the use of the following primers (Frossard *et al*., 1999): sense, 5'-GTGTCATTCAGTCCTTACGAT- 3'; antisense, 5'-AGTACAACCACCTTTAACGTT- 3' (synthesized by Research Biolabs, Malaysia). PCR amplification was performed with a 25-μL reaction mixture that contained 20 pmol of each primer, 0.4 mmol/l each dNTP, 2 mmol/l $MgCl_2$, 1x Taq buffer and 1 unit of NEB Taq DNA polymerase (New England Bio labs, Beverly, MA, USA) and the template DNA. The PCR cycling conditions were carried out on a iCycler machine (BioRad Laboratories, Hercules, California, USA) with an initial denaturation step of 5 min at 94°C followed by 35 cycles of denaturation at 94°C (30 sec), annealing at 65°C (1 min) and extension at 68°C (5 min) and a final step of 5 min at 72°C was added before the storage of the samples at 4°C. PCR products were separated on agarose gel electrophoresis (Promega, Madison, USA). An aliquot of 6 μL PCR product mixture was then incubated at 37°C for 3 h with 2 units of *Bgl1* restriction enzyme, 1× NEB buffer 3, in a total volume of 20 μL reaction mixture. Heat inactivation was done for 20 min at 65°C. The PCR digested products were separated by 1.5% agarose gel and performed in Origins electrophoresis tank (Elchrom Scientific AG, Switzerland). DNA fragments were stained in ethidium bromide and visualized by Alpha Imager (Alpha Innotech, San Leandro, CA) under UV light. The digested fragments of *Bgl1* dimorphism shows 3.9 k base (Kb) pairs fragment in the absence of *Bgl1* site (-), 2.8 and 1.1 kb pairs fragments were shown in the

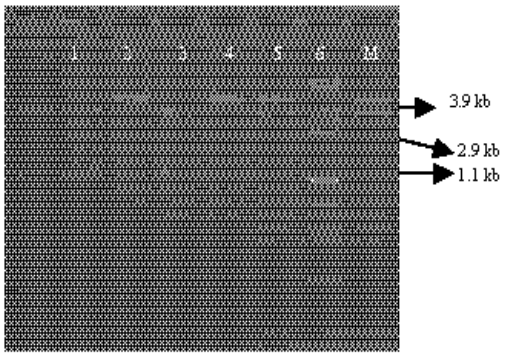

Fig. 1: Restriction Fragments resolved in 1% agarose gel electrophoresis. Lane 1, 2, 4 shows *Bgl1* (+), lane 3, 5 shows *Bgl1* (-), lane 6 and 7 shows *Bgl1* (+/-) genotypes of *Bgl1* dimorphic sites of REN gene. M represents a 1 kb DNA ladder (Promega)

presence of *BglI* sites (+) (Fig. 1). Identical results were obtained when genotyping was performed in two separate occasions.

Statistical analysis: All the statistical analysis was carried out by using SPSS (Chicago, IL) software version 13.0 for Microsoft windows. Clinical characteristics of all the subjects were expressed as mean±SD. Continuous variables were compared between the groups by using two-tailed student's t-test. Allele frequencies were estimated by gene counting. The distribution of genotypes was assessed for deviation from the Hardy-Weinberg equilibrium by using chi-squared tests. A level of $p<0.05$ was considered statistically significant.

RESULTS

The clinical and biochemical characteristics of all the study subjects enrolled in this study are shown in Table 1. Out of 130 patients, 75 of them were males and 55 were females, whereas in controls 33 and 37 were males and females, respectively.

The mean age of the patients was 57.43±10.69 years and the age ranged from 31-84, while controls were ranged from 27-84 with a mean age of 45.88±10.95 years. Among the subjects, Malays (79, 39.50%) comprised more than the other two races Chinese (62, 31%) and Indians (59, 29.50%). Nearly 78 of the subjects had positive history of hypertension while controls were included on the absence of family history of hypertension. The mean BMI of hypertensive subjects were 26.48±3.69 compared to mean BMI 24.23±4.14 of controls. There was significant difference observed in the SBP, DBP, BGL and HDL-C ($p<0.001$) however there was no significant difference found in other risk factors of essential hypertension such as Total cholesterol, LDL cholesterol and Triglycerides ($p>0.05$).

Genotyping results: The distribution of genotypes of *BglI* dimorphic site of REN gene in Malaysian subjects showed no deviation from Hardy-Weinberg equilibrium ($p>0.05$). The genotypic and allelic distributions among the subject are shown in the Table 2.

The BglI (+/+) genotype frequency of REN gene in Malaysian EHT (10%) and EHT+T2DM (10.77) subjects were less compared to control subjects (14.28%). There was no significant difference found in BglI (+) allele frequency of REN gene either in EHT or EHT+T2DM subjects when compared to control subjects ($p>0.05$). Thus, the BglI (+/+) genotypes and BglI (+) allele frequency of BglI dimorphism in REN gene was not associated with essential hypertensive patients with or without Type 2 Diabetes Mellitus in Malaysian subjects.

The effect of gender on hypertension was also considered in this study for *BglI* dimorphism. Table 3, shows the genotype and allelic distributions of *BglI* dimorphism among males and females within the groups. There was no significant difference in either genotypic or

Table 1: Characteristics of all the subjects

Parameters	Controls(N-70)	EHT(N-70)	p-value	EHT + T2DM(N-60)	p-value
Gender, M/F	33/37	55/15		20/40	
Age (years)	45.88±10.95	57.63±11.60	0.00*	57.23±9.78	0.00*
BMI (kg/m^2)	24.23±4.14	25.96±3.89	0.12	27.00±3.49	0.00*
SBP (mm Hg)	125.88±9.00	162.76±20.85	0.00*	159.55±19.51	0.00*
DBP (mm Hg)	77.71±7.14	98.16±10.87	0.00*	93.48±4.41	0.00*
BGL (mmol/l)	4.71±0.92	5.29±0.47	0.00*	11.71±5.02	0.00*
TC (mmol/l)	5.20±1.38	4.86±1.27	0.13	5.16±1.68	0.88
LDL-C (mmol/l)	3.43±1.22	3.48±1.25	0.79	3.85±1.60	0.91
HDL-C (mmol/l)	1.09±0.39	0.86±0.41	0.00*	0.79±0.32	0.00*
TG (mmol/l)	1.71±1.11	2.10±1.17	0.47	2.02±1.06	0.11

Values were shown are the mean ± SD. BMI-Body Mass Index; SBP-Systolic Blood Pressure; DBP-Diastolic Blood Pressure; TC- Total Cholesterol; LDL-C -Low Density Lipoprotein Cholesterol; HDL-C- High Density Lipoprotein Cholesterol; TG-Triglycerides. BGL- Blood Glucose Level. * Significant $p<0.001$, compared between EHT vs. Controls and EHT+T2DM vs. Controls

Table 2: Genotypic and Allelic distribution of *BglI* dimorphism of REN gene in hypertensive subjects

Genotypes	EHT (N-70) n (%)	Controls (N-70) n (%)	EHT + T2DM (N-60)n (%)
BglI (+/+)	7 (10)	10 (14.28)	7 (10.77)
BglI (+/-)	39 (55.72)	30 (42.86)	35 (56.92)
BglI (-/-)	24 (34.28)	30 (42.86)	18(32.31)
Significance	p = 0.30	* p =0.20*	
Alleles			
BglI (+)	53 (37.86)	50 (35.71)	49 (40.83)
BglI (-)	87 (62.14)	90 (64.29)	71 (59.17)
Significance	p = 0.71*	p = 0.39*	
Odds ratio (95% CI)	1.097 (0.675-1.783)	1.242 (0.752-2.05)	

*No significant ($p>0.05$), compared between EHT vs. Controls and EHT+T2DM vs. Controls

Table 3: Genotypic and Allelic distributions of *BglI* dimorphic site of REN gene in Male and Female subjects

	Controls (N-70)		EHT (N-70)		EHT + T2DM (N-60)		Total	
Genotypes	M (N- 33) n/(%)	F (N-37) n/(%)	M (N-55) n/(%)	F (N-15) n/(%)	M (N-40) n/(%)	F (N-20) n/(%)	M (N-128) n/(%)	F (N-72) n/(%)
BglI (+/+)	5 (15.160)	5 (13.510)	5 (9.10)	2 (40)	7 (17.50)	0 (0)	17 (13.28)	7 (9.72)
BglI (+/-)	12 (36.36)	18 (48.65)	32 (58.18)	7 (46.67)	20 (50)	15 (750)	64 (500)	40 (55.56)
BglI (-/-)	16 (48.48)	14 (37.84)	18 (32.72)	6 (40)	13 (32.5)	5 (25)	47 (36.72)	25 (34.72)
	p = 0.57*		p = 0.71*		p = 0.75*		p = 0.67*	
Alleles								
BglI (+)	22 (33.33)	28 (37.84)	42 (38.18)	11 (36.67)	34 (42.50)	15 (37.50)	98 (38.28)	54 (37.5)
BglI (-)	44 (66.67)	46 (62.16)	19 (63.33)	46 (57.50)	25 (62.50)	68 (61.82)	158 (61.72)	90 (62.50)
Significance	p = 0.57*		p = 0.88*		p=0.59*		p=0.87*	
Odds ratio (95% CI)	1.067 (0.410-1.645)		1.232 (0.462-2.462)		0.821 (0.565-2.684)		1.034 (0.678-1.575)	

*No Significant p>0.05, compared between Male and Female within EHT, EHT+T2DM and Controls

allelic distribution in both male and female subjects (p>0.05). When comparison made among the ethnic groups, there was no significant difference (p>0.05) has been seen in the genotype frequencies between Malays, Chinese and Indians in either case or controls (data not shown).

DISCUSSION

In this study, we determined the association of *BglI* dimorphism in essential hypertension with or without T2DM in Malaysian Subjects. The results shows that *BglI* (+) allele of BglI dimorphism is not associated in EHT with or without T2DM in Malaysian subjects. There was no significant gender-mediated preferential distribution of this *BglI* dimorphism in Malaysian Subjects. To our knowledge, there have been no previous reports on *BglI* dimorphism of REN gene in Malaysia was observed and it is the first report in determining the genotypes of *BglI* dimorphism in EHT with or without diabetes in Malaysian subjects.

Renin gene locus has been studied as a candidate gene for essential hypertension by using hypertensive rat models (Mullins *et al*., 1990; Rapp *et al*., 1989). Several studies have confirmed the association of *BglI* dimorphism in renin gene in various populations (Frossard *et al*., 1999). The variants in REN gene has been strongly influence to blood pressure and are directly involved in the pathogenesis of hypertension (Rapp *et al*., 1989). Several case-control studies have been revealed the positive (Frossard *et al*., 1999) and negative (Morris *et al*., 1988) associations of *BglI* dimorphism in renin gene. However, in this study there is no association of *BglI* dimorphism of REN gene with hypertension in Malaysian subjects which is an agreement with other similar studies (Naftilan *et al*., 1989; West *et al*., 1992). Frossard *et al*, (1999) previously reported a significant association between the *BglI* dimorphism of REN gene and essential hypertension in UAE population and US hypercholesterolemic Caucasians in which *BglI* (+) allele was associated with hypertension. But in a large UTAH pedigree there was no linkage found between hypertension and the REN gene (Naftilan *et al*., 1989) as well as in sib-pair method linkage analysis (Soubrier *et al*., 1990). There are several limitations in this study. First, compared with other epidemiological and association studies, the sample size in this study is relatively small; thus, a more extended study with a larger population base and in homogenous population is needed to confirm the association of *BglI* dimorphism in renin gene with EHT in the future. Second, a randomized control design (no age, gender-matched controls) was used in this present study. However, the discrepancies may occur to racial differences or heterogeneity of the population sampling bias or possibly due to the environmental factors may contribute to the negative associations (Persu, 2006).

CONCLUSION

BglI dimorphism was not associated with essential hypertension with or without Type 2 Diabetes Mellitus in Malaysian Subjects. Therefore, *BglI* (+) allele marker is not an independent risk and unlikely to have a major effect on susceptibility to essential hypertension among Malaysian subjects. Further work is needed with a large sample size to confirm the association of *BglI* dimorphism and other polymorphism such as MboI site in REN gene has to be done to determine the genetic background of essential hypertension among Malaysian subjects.

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