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Haplotype Distribution of the β_2-Adrenergic Receptor Gene in Korean Essential Hypertensives

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ABSTRACT : *In view of the effect of β_2-adrenergic receptors (β_2-AR) as a risk factor for essential hypertension, we investigated the Fnu4HI and MnlI RFLPs of β_2-AR gene in the Korean patients with essential hypertension and normal controls. There were no significant differences in the allele and genotype of these polymorphisms between normotensive and essential hypertensive subjects. In ethnic comparison, the allele frequencies of these three sites contained Nde I RFLP, reported the association with essential hypertension in Korean population previously, were very different from those of other ethnic populations studied. The significant linkage disequilibrium was detected only in hypertensive group between Nde I and Fnu4HI sites. The Fnu4HI RFLP was also significantly associated with plasma triglyceride (TG) level. Therefore, our results suggest that the significant association between Fnu4HI variation in the human β_2-AR gene and plasma TG level may reflect the potential role of human β_2-AR gene as one of the genetic components for cardiovascular risk.*

Key Words : *β_2-adrenergic receptor, Essential hypertension, Haplotype, Polymorphism*

I. INTRODUCTION

As well known as, essential hypertension is defined as high blood pressure in which secondary causes such as renovascular disease, renal failure, pheochromocytoma, aldosteronism, or other causes of secondary hypertension or Mendelian forms (monogenic) are not present. The main task in essential hypertension research is to explain genetic causes of raised blood pressure. High blood pressure exacerbates atherogenesis (Alexander *et al.*, 1995; Chobanian *et al.*, 1996) and sustained hypertension increases long-term risk for myocardial infarction to levels comparable to those associated with smoking and elevated serum cholesterol (Wilson, 1998).

The human β_2-adrenergic receptors (β_2-AR) is a member of the G protein-linked seven-transmembrane domain receptor family. The β_2-AR regulates vascular smooth muscle relaxation, and together with the β_1-AR modulates cardiac inotropy and chronotropy in response to endogenous catecholamines. β_2-AR abnormalities have been implicates in human essential hypertension. Higher numbers of β_2-ARs have been reported in lymphocytes from hypertensive patients than in those from normotensive controls (Jia *et al.*, 2000). The human β_2-AR contribute to cardiovascular regulation by influencing several functions and a several studies suggest that a decreased function of the β_2-AR may be involved in essential hypertension.

Recently, several missense mutations (polymorphisms) within the coding block of the (β_2-AR) gene have been identified in the human population. In studies utilizing site-directed mutagenesis and recombinant expression, three loci at amino acid positions 16, 27 and 164 have been found to significantly alter receptor function. The Glu27 genotype results in attenuated downregulation compared with the wild-type Gln27 receptor, whereas Gly16 exhibits enhanced downregulation compared to Arg16. And the

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Ile164 form displays altered coupling to adenylyl cyclase compare to Thr164 (Liggett, 1997; Candy *et al.*, 2000). The very recent study has demonstrated that homozygous Gly16 subjects have increased basal mean blood pressure and decreased salbutamol-induced *in vivo* vasodilatation compared heterozygous subjects (Gratze *et al.*, 1999). Homozygotes for the Gln27 allele showed reduced vascular reactivity to infused isoprenaline, displaying decreased forearm blood flow (Cockcroft *et al.*, 1997). The Gln27 allele was associated with enhanced bronchial reactivity (Hall *et al.*, 1995).

Inspite of these results, the polymorphisms of these sites were no significant differences in the allele and genotype between normotensive and hypertensive subjects in Black African and Caucasians (Candy *et al.*, 2000; Jia *et al.*, 2000; Herrmann *et al.*, 2000). In opposite to these results, we reported that the Arg16Gly polymorphism was significantly associated with the essential hypertension in Korean population previously (Bae *et al.*, 2001). Therefore, we investigated whether the Glu27Gln and Ile164Thr polymorphisms, as the other strong candidate sites, in the β_2-AR gene is associated with essential hypertension in a Korean population. In addition, we looked on a haplotype distribution of both 16 and 27 polymorphisms.

II. MATERIALS AND METHODS

1. Study subjects

A total of 147 unrelated individuals were randomly selected from the Seoul Hygiene Hospital, Seoul, Korea. The essential hypertensives consisted of 71 subjects with higher blood pressure value than 140/90 mmHg, whereas the normotensives consisted of 76 individuals with lower blood pressure value than 140/90 mmHg. In association analysis using genetic markers, subject selection is a critical and difficult problem. Given that blood pressure is influenced by age and sex, we selected closely age- and sex-matched subjects for the normotensive and essential hypertensive groups. Subjects with secondary forms of hypertension and taking antihypertensive drugs were excluded from the study.

2. Biochemical assay

Total cholesterol (TC), triglyceride (TG), apolipoprotein AI (ApoA1), lipoprotein(a) (LP(a)), and high-density lipoprotein (HDL)-cholesterol level were determined by enzymatic method and LDL-cholesterol level was calculated by Friedwald's equation (Friedwald *et al.*, 1972).

3. Genotype analysis of β_2-AR codon 27 (*Fnu*4HI RFLP)

Genomic DNA was prepared from buffy coats from blood (5 m*l*) after lysis of the red blood cells (Sambrook *et al.*, 1989). The Glu27Gly polymorphism was detected using a PCR- *Fnu*4HI digestion. The sequence of the forward primer was 5'-CAC CCA ATA GAA GCC ATG C-3' and the sequence of the reverse primer was 5'-ACA GCA CAT CAA TGG AAG TCC-3' (Jia *et al.*, 2000). PCR was performed in a final volume of 50 μ*l* (100 ng genomic DNA, 20 pmol each primer, 200 μM each of the four dNTPs, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH 8.4, and 2.5 units *Taq* DNA polymerase). The reactions were denatured at 94°C for 1 min, annealed at 60°C for 30 sec, and extended at 72°C for 1 min for a total of 35 cycles. The 309 bp amplified product was subsequently digested using 5 U *Fnu*4HI (New England Biolabs) at 37°C overnight, which recognizes the Gln27 to yield a cleaved 196 bp fragment but does not recognize the Glu27 sequence, leaving a 235 bp fragment produced by the natural *Fnu*4HI analysis was also confirmed by direct sequencing of representative PCR products.

4. Genotype analysis of β_2-AR codon 164 (*Mnl*I RFLP)

The Ile164Thr polymorphism was detected using a PCR-*Mnl*I digestion. The sequence of the forward primer was 5'-GCT ACT TTG CCA TTA CTT CAC CTT-3' and the sequence of the reverse primer was 5'-GTA GAA GGA CAC GAT GGA AGA GG-3' (Kang *et al.*, 2002). PCR was performed using the same method with analysis of codon 27 polymorphism. The 229 bp amplified product was subsequently

Table 1. Polymorphisms of the human β_2-AR and their phenotype

No. of nucleic acid	Nucleic acid	No. of amino acid	Amino acid	Recpetor phenotype
46	A	16	Arg	Wild-type
	G		Gly	Enhanced, downregulation
79	C	27	Gln	Wild-type
	G		Glu	Absent downregulation, immature form
491	C	164	Thr	Wild-type
	T		Ile	Altered binding, coupling, sequestration

The wild-type β_2-AR, as delineated by the original cloning of the receptor (Kobika *et al.*, 1987a), is Arg16, Gln27, Thr164 and is denoted as reference.

digested using 5 U *MnlI* (New England Biolabs) at 37°C overnight, which recognizes the Thr164 to yield a cleaved 125 bp and 104 bp fragment but does not recognize the Thr164 sequence, leaving a 229 bp fragment produced by the natural *MnlI* analysis was also confirmed by direct sequencing of representative PCR products.

5. Statistical analysis

Allele frequencies were estimated by the gene counting method. Deviation in genotype distribution from that expected for Hardy-Weinberg equilibrium was estimated by the χ^2-fitness test. The heterozygosity and polymorphism information content (PIC) was measured by the method of Bostein *et al.* (1980). The significance of differences in genotype or allele frequencies between populations was also estimated by χ^2-independence test. The odds ratio (OR) with 95% confidence interval (CI) of essential hypertension associated with allelic variation was calculated by univariate logistic regression analysis. Maximun likelihood estimate (MLE) of haplotypes were obtained by iterative two-steps algorithm called expectation-maximatization (EM). A Monte-Carlo simulation using the clump (version 1.6) program was performed to test the statistical significance of the association between the haplotype distribution and essential hypertension (Sham and Curtis, 1995). The degree of linkage disequilibrium was determined by calculation of delta (Δ) (Hill and Robertson, 1968) and D' (Lowontin, 1964) values between the polymorphic sites in the β_2-AR gene. To test the significant of linkage disequilibrium, $n\Delta^2$ value was used as the χ^2 distribution with 1 df (degree of freedom). The association between genotypes and quantitative variables was assessed by one-way analysis of variance (ANOVA) with multiple comparison tests by Tukey. All statistical analyses were performed using the SPSSWIN (version 8.0) computer program.

III. RESULTS

1. Genotype distribution

Polymorphic sites of the human β_2-AR gene and their phenotypes were shown in Table 1. The *Fnu*4HI polymorphic site of the β_2-AR gene was located in codon 27, and the genotype pattern of this site was displayed in Fig. 1. The genotype and allele frequencies of the *Fnu*4HI RFLP were shown in Table 2. The genotype frequencies of Glu/Glu, Glu/Gln and Gln/Gln were 1.3, 4.0 and 94.7% in normotensives, and 2.8, 5.7 and 91.5% in essential hypertensives, respectively. The heterozygosity and PIC values of *Fnu*4HI RFLP represented the values of 0.064 and 0.061 for normotensives, and 0.106 and 0.101 for essential hypertensives, respectively. According to the heterozygosity and PIC values, the *Fnu*4HI RFLP showed the relatively low degree of polymorphism in the both groups. The observed genotype distributions were not

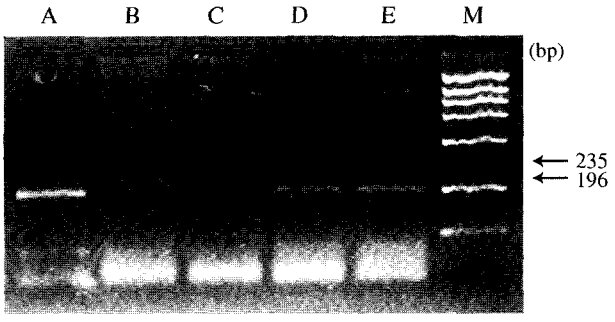

Fig. 1. *Fnu*4HI RFLP of the β_2-AR gene. Lanes A, D, and E: Gln27 allele genotype; lane C: heterozygote genotype; lane B: Glu27 allele genotype.

Table 2. Genotype and allele frequencies of *Fnu*4HI RFLP of the β_2-AR gene in normotensives and essential hypertensives

	No of genotype (%)				No of allele (%)			
	Glu/Glu	Glu/Gln	Gln/Gln	Total	Glu	Gln	H[1]	PIC[2]
Normotensives	1 (1.3)	3 (4.0)	72 (94.7)	76	5 (0.03)	147 (0.97)	0.064	0.061
Hypertensives	2 (2.8)	4 (5.7)	65 (91.5)	71	8 (0.06)	134 (0.94)	0.106	0.101
X^2		0.254			0.955			
P		0.881			0.329			
Odds ratio(CI)[3]				1.76 (0.56-5.50)				

[1]Heterozygosity, [2]Polymorphism Information Content, [3]95% Confidence Interval.
Frequency is given as a percentage in parenthesis.
There were statistically significant differences in genotype and allele frequencies between normotensives and essential hypertensive subjects.

significantly deviated from those expected for Hardy-Weinberg equilibrium. There were no significant differences in allele genotype frequencies between normotensives and essential hypertensives by case-control comparison.

Table 4 showed the genotype frequencies for the Thr and Ile alleles of *MnlI* RFLP of the β_2-AR gene in both groups. The *MnlI* site only showed Thr genotype in all subjects.

Table 3. Comparison of the anthropometrical data and intermediate phenotypes according to *Fnu*4HI genotypes of β_2-AR gene

Variable	Genotype	
	Glu/Glu + Glu/Gln	Gln/Gln
Age (year)	58.8±9.1[8]	59.6±10.1
BMI (kg/m^2)[1]	24.1±1.9	23.9±2.7
ApoA1 (mg/dl)[2]	105.5±24.8	87.9±28.3
LP(a) (mg/dl)[3]	13.2±9.6	16.6±11.4
***TG (mg/dl)[4]**	**232.7±164.3**	**127.7±68.7**
TC (mg/dl)[5]	137.2±21.9	155.0±33.3
LDL-chol (mg/dl)[6]	73.3±40.0	101.7±33.5
HDL-chol (mg/dl)[7]	22.2±6.2	27.3±9.6

[1]Body mass index, [2]apolipoprotein AI, [3]lipoprotein(a), [4]triglyceride, [5]total cholesterol, [6]LDL-cholesterol, [7]HDL-cholesterol, [8]standard deviation.
*There was the significant difference in plasma TG level across the genotype frequencies (by Students t-test. $P < 0.05$).

Table 4. Genotype frequencies for the Thr and Ile alleles of *MnlI* RFLP of the β_2-AR gene in normotensives and essential hypertensives

Genotypes	Hypertensives		Normotensives	
	Number	Frequency (%)	Number	Frequency (%)
Thr/Thr	64	100.0	76	100.0
Thr/Ile	0	0.0	0	0.0
Ile/Ile	0	0.0	0	0.0

There were no statistically significant differences in genotype and allele frequencies between normotensives and essential hypertensive subjects.

2. Association with biochemical parameters

The comparison of anthropometrical data and intermediate phenotypes across *Fnu*4HI RFLP was represented in Table 3. There was the significant difference in plasma TG level across the genotype frequencies (by Students t-test. $P < 0.05$). Others represented no significant association with any biochemical parameters.

3. Haplotype analysis

The haplotype distribution and linkage disequilibrium statistic values reflecting the extent or statistical significance of nonrandom associations between the two polymorphic sites were shown in Table 5. There were no statistically significant difference in haplotype frequency between two groups (Monte-Carlo simulation, $T_2 = 0.9012$, df = 1, P = 0.8471, simulation number = 10,000). However, there was the sig-

Table 5. Haplotype frequencies and linkage disequilibrium statistic (D', Δ) between pairs of two DNA polymorphisms in the β_2-AR gene

Haplotype		Normotensives	Hypertensives
Arg16Gly	Glu27Gln		
Arg	Glu	0.013070	0.000003
Arg	Gln	0.589872	0.576268
Gly	Glu	0.023695	0.025421
Gly	Gln	0.373364	0.398308
Total chromosomes		136	118
Δ		0.0986	**0.1885**
D'		0.4093	**1.0014**
X^2		1.3219	**4.1949**
P		0.2503	**0.0405**

There was the significant linkage disequilibrium between two polymorphic sites in only hypertensive group (Monte-Carlo simulation, $T_2 = 0.9012$, df = 1, P = 0.8471, simulation number = 10,000).

nificant linkage disequilibrium between two polymorphic sites in only hypertensive group.

IV. DISCUSSION

Essential hypertension is known to be caused by polygenes, and its phenotypic expression is modulated by various environmental factors (Bae *et al.*, 2001). Recent advances in molecular biology have allowed investigation of the role of candidate genes for essential hypertension. Specific candidate genes have been tested for linkage and association with a blood pressure or the diagnosis of hypertension. Nevertheless, the genetic alterations responsible for inherited essential hypertension remain largely unknown, and the success to date in identifying susceptibility genes has been very limited. Depending on the genetic factors of human essential hypertension, it appears that DNA polymorphisms at the candidate genes may play a significant role as useful genetic markers in the association study.

The human β_2-AR gene is encoded by an intronless gene on chromosome 5q31-q32 (Kobika *et al.*, 1987a, b). Receptor transcripts have a 5' leader region harbouring an open reading frame that encodes a 19-amino acid peptide; this peptide has been shown to modify translation of β_2-AR mRNA (Parola *et al.*, 1994). The deduced amino acid sequence consists of 413 amino acids. Two common and one rare polymorphisms have been identified in the coding region, first of which, A46 $\rightarrow$ G, lead to the substitution of glycine for arginine at amino acid position 16; second, C79 $\rightarrow$ G, results in the substitution of glutamate, rather than glutamine, at amino acid position 27; third, C $\rightarrow$ T, lead to the substitution of threonine for isoleucine, at amino acid position 164 (Reihsaus *et al.*, 1993; Turki *et al.*, 1995).

Several groups have begun to investigate whether β_2-AR polymorphisms are involved in essential hypertension. Svetkey *et al.* reported a RFLP in the β_2-AR gene, which was associated with salt sensitivity in hypertensive African-American. Kailasam *et al.* genotyped 146 sibpairs from 57 families for autonomic and sympathetic function, and genotyped subjects by microsatellite markers and found that a locus in 5q31-q34, which contains the β_2-AR gene, contributes to phenotypic variations in pressor responsiveness, baroreceptor sensitivity, and blood pressure. Two studies have evaluated the relationship between β_2-AR polymorphisms and essential hypertension and reached opposite conclusion: Kotanko *et al.* examined 136 unrealted African-Caribbeans and observed an increase of Gly16 homozygotes in hypertensive subjects (74%) versus normotensives (52%), whereas Timmermann *et al.* found more frequent expression of the Arg16 allele in 61 offspring from hypertensive versus normotensive Norwegian parents (58% v 28%). Candy *et al.* reported no significant association between either the Arg-Gly16 polymorphism or Gln-Glu27 polymorphism and hypertension status. But, a significant association was seen between Arg16 homozygotes and lower body mass index in hypertensives. Jia *et al.* also found no differences in the genotype or allele frequencies of the β_2-AR polymorphisms between hypertensive and normotensive participants. This discrepant results reported for the same gene may be due to different criteria used in selection of study subjects, difference in study methods or racial differences in study sample. The Gly allele was more frequent in essential hypertensives than in normotensives. A recessive model on Gly allele of β_2-AR gene provides the best fit for this data, so the Gly/Gly genotype could be considered a genetic marker on the risk for hypertension in Korean population (Bae *et al.*, 2001).

In the case of *Fnu*4HI RFLP, Glu allele is significantly higher in plasma triglyceride levels than Gln allele. This result may be a evidence that there is a genotype-specific difference in the association between plasma triglyceride level and β_2-AR activity. This association is suggested that at least some of the effects of genotype on β_2-AR activity may be mediated through the differential, allele-specific effects of plasma lipids on β_2-AR activation. Unfortunately, we could not measure the β_2-AR activity in our subjects, Thus, further studies will be needed in order to investigate whether the interaction between β_2-AR activity and plasma triglyceride concentrations is modified by the Glu allele of β_2-AR gene in Korean population.

By pair-wise haplotype analysis, the significant linkage disequilibrium between two polymorphic sites was detected in hypertensives. This finding suggests that the haplotype occurred by two polymorphisms decreases the information content for linkage analy-

Table 6. Comparison of the allele frequencies of the β_2-AR polymorphisms in other ethnic groups and Korean population

Allele	Korean[1,2]	African-american[3]	Whites[4]	Sweden[5]	Danish[6]	Black african[7]	East anglian caucasians[8]	Caucasians[9]	Eastern germany[10]
Arg16	0.64	0.50	0.45			0.48	0.39	0.48	0.35
Gly16	0.36	0.50	0.55			0.52	0.61	0.52	0.65
Gln27	0.97			0.59	0.60	0.83	0.56	0.48	0.57
Glu27	0.03			0.41	0.40	0.17	0.44	0.52	0.43
Ile164	0.00							0.05	0.00
Thr164	1.00							0.95	1.00

[1]This study; [2]Bae *et al.*, 2001; [3,4]Herrmann *et al.*, 2000; [5]Hellstrom *et al.*, 1999; [6]Echwald *et al.*, 1998; [7]Candy *et al.*, 2000; [8]Jia *et al.*, 2000; [9]Turki *et al.*, 1995; [10]Ulbrecht *et al.*, 2000.

sis, while it did no require the large sample size to perform the association study. Therefore, association study may be better than linkage analysis to discover the disease susceptibility gene in the case of two polymorphisms in the β_2-AR gene. Unlikely to hypertensives, this significant linkage disequilibrium was not detected in normotensives. This may be explained by the significant association between *Nde*I RFLP of the β_2-AR and essential hypertension in previous study. In other word, the discrepancy in the association with essential hypertension between two polymorphic sites might bring about the modification of linkage disequilibrium between the study groups.

Allele frequencies of the RFLPs in Korean subjects differed from those of populations studied previously (Table 6). In general, a Arg16 allele of between Caucasians and Africans was lower than Korean population, and the Gln27 allele of Korean population was especially higher than other racials. A possible explanation for differences of allele frequencies among different populations may be due to different genetic background of populations or various sample sizes. It seems to be important for carefully designed studies to minimize the ethnic heterogeneity of the case control populations.

In conlusion, there was the significant association between *Fnu*4HI variation in the human β_2-AR gene and plasma TG level, and this RFLP may be an useful genetic marker for cardiovascular risk in Korean population, although further studies using large sample size will be required to clarify the precise role of the β_2-AR gene for essential hypertension. Also, association studies in other ethnic populations will be great interest because the distribution of allele frequency between the *Nde*I RFLP and *Fnu*4HI RFLP showed the opposite patterns.

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