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Association Study between the Genetic Variations of the Apo AI-CIII-AIV Gene Cluster and Hypertension among Koreans

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ABSTRACT : *Hypertension is a multifactorial disorder in which the genetic and environmental factors are involved. In a view of the effects of apolipoproteins as a risk factor for hypertension, we investigated the genotype and allele frequencies in the four RFLPs of the apo AI-CIII-AIV gene cluster (G to A mutation at position –75 in the apo AI promoter, SstI RFLP in the apo CIII gene and HincII and HinfI RFLPs in the apo AIV gene) in the Korean patients with hypertension and normal controls. The AA genotype frequency of the G to A promoter polymorphism in hypertensives was significantly higher than that of normotensives ($P < 0.05$). None of the other polymorphisms showed a difference in genotype frequency between two groups. Therefore, our result suggest that the G to A promoter polymorphism of the apo AI gene may be useful as genetic marker in the ethiology of hypertension.*

Key Words : *Apo AI-CIII-AIV gene cluster, Genotype and Hypertension*

I. INTRODUCTION

Hypertension is a major health problem in many industrialized contries, and multiple ethiologic factors is involved in the pathogenesis of this disorder. As well known as, hypertension may be associated with abnormalities of plasma lipid metabolism (Williams *et al.*, 1989). The major lipids of human plasma are phospholipid, cholesterol, cholesteryl ester and triglyceride. Apolipoprotein abnormalities may be of importance as they are essential for normal transport and metabolism of plasma lipid.

Apolipoprotein AI (apo AI), CIII (apo CIII) and AIV (apo AIV) together with apolipoprotein AII constitute the major protein associated with high density lipoprotein (HDL). Each of them has been shown to modify the activity of lecithin : cholesterol acyltransferase *in vitro* (Fielding *et al.*, 1972; Steinmetz and Utermann, 1985). Karathanasis (1985) has described the organization of the apo AI-CIII-AIV gene cluster. These three genes are congregated on the long arm of chromosome 11, covering a segment of DNA of approximately 15 kb in length (Karathanasis, 1985). Also, the apo CIII gene is transcribed in the opposite direction to the apo AI and apo AIV genes despite their proximity.

Multiple DNA sequence variations in the apoAI-CIII-AIV gene cluster have been identified by the presence of restriction fragment length polymorphisms (RFLPs) (Jeenah *et al.*, 1990; Marasco *et al.*, 1993; Rees *et al.*, 1983; Tenkanen *et al.*, 1992). The presence of RFLPs in this gene cluster provided valuable genetic markers which could be used to study the association of particular allele with various diseases such as dyslipidemia or cardiovascular disease (Humphries, 1988; Song *et al.*, 1998). However, few data have been available regarding the role of the apo AI-CIII-AIV gene cluster in hypertension (Frossard *et al.*, 1998). Therefore, our study group investigated the relationship between four common polymorphisms (G to A mutation at position –75 in the apo AI gene promoter, *Sst*I

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RFLP in the apo CIII gene and *Hinc*II and *Hinf*I RFLPs in the apo AIV gene) of the apo AI-CIII-AIV gene cluster and hypertension in Koreans.

II. MATERIALS AND METHODS

1. Study subjects

A total of 200 unrelated individuals were randomly chosen from Seoul Hygiene Hospital, Seoul, Korea. We studied 100 subjects with hypertension. Patients were classified as having hypertension if they had systolic blood pressure above 140 mmHg and diastolic blood pressure above 90 mmHg, and had no clinical signs, symptoms and laboratory findings suggestive of secondary hypertension. In addition, a ramdomly selected normal population (100 individuals) was analyzed as control group (blood pressure value, < 140/90 mmHg). The clinical data were considered in references to determine the association between the genotypes of polymorphic sites and plasma lipid levels.

2. Determination of plasma lipid levels

Blood samples were obtained in EDTA tubes from individuals who had been fasting for 12~16 hr. Concentrations of plasma total cholesterol (TC) and triglyceride (TG) were measured by enzymatic colorimetry methods with commercial kit (Boehringer Mannheim, Germany) and chemical analyzer. The HDL-cholesterol concentration in plasma was determined by measuring cholesterol concentration in the supernatant after precipitation of the plasma with $MgCl_2$ and dextran sulfate, with a Gilford Impact 400E automated analyzer with reagents and calibrators from Boehringer Mannheim. The LDL-cholesterol concentration in plasma was calculated by using the formular of Friedwald *et al.* (1972). Also, plasam Lp(a) concentration was measured by the immunoprecipitation method (SPQ Test System, INCSTAR Corporation, Stillwater, Minnesota, USA). Quantitation of plasma apo AI was performed by immunonephelometric assay (Behring Nephelometer, AG, Germany).

3. DNA analysis

Total genomic DNA was isolated from buffy coat by the method of Sambrook *et al.*, (1989) with slight modification. Polymerase chain reaction (PCR) techniques were used for this study. Briefly, total 50 ul of the reaction mixture contained 200~400 ng genomic DNA, 10 pmol of each primers, 10 nmol of each dNTPs, buffers recommended by the manufacturer, and 1.5 U *Taq* DNA polymerase (Promega Corp., USA). The sequences of the primers and conditions for four polymorphisms are shown in Table 1. PCR amplification was carried out in a DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, CT, USA). Ten ul of each PCR product was restriction-digested overnight with 5 unit of proper enzyme at 37°C. All digested products were size-fractionated after 2% agarose gel electrophoresis in 0.5 X TBE buffer for 40

Table 1. List of the sequences of oligonucleotide primers and PCR conditions to detect the RFLPs in the apo AI-CIII-AIV gene cluster

Polymorphic sites	Sequence of oligonucleotide primer	Amplification condition	Size of amplified DNA
G^{-75} to A[a]	5'-AGGCCCGGCCTGGGGCAAGGCCTGAACCTT-3'	95°C 1 min	290 bp
(*Msp*I)	5'-ACCCCACCCGGGAGACCTGCAAGCCTGCAG-3'	55°C 30 sec	
		72°C 2 min	
*Sst*I[b]	5'-GGAGGGTGATTCCTACCTTA-3'	95°C 1 min	710 bp
	5'-TTTGACTTGTGCTGGGGTTC-3'	60°C 1 min	
		72°C 1 min	
*Hinc*II[c]	5'-TGCCCTCTTCCAGGACAAAC-3'	95°C 1 min	1,012 bp
	5'-GCTCTCCAAAGGGGCCAGCATC-3'	60°C 1 min	
		72°C 3 min	
*Hinf*I[c]	5'-TGCCCTCTTCCAGGACAAAC-3'	95°C 1 min	1,012 bp
	5'-GCTCTCCAAAGGGGCCAGCATC-3'	60°C 1 min	
		72°C 3 min	

[a]From Jeenah *et al.* (1990).
[b]From Marasco *et al.* (1993).
[c]From Tenkanen *et al.* (1992).

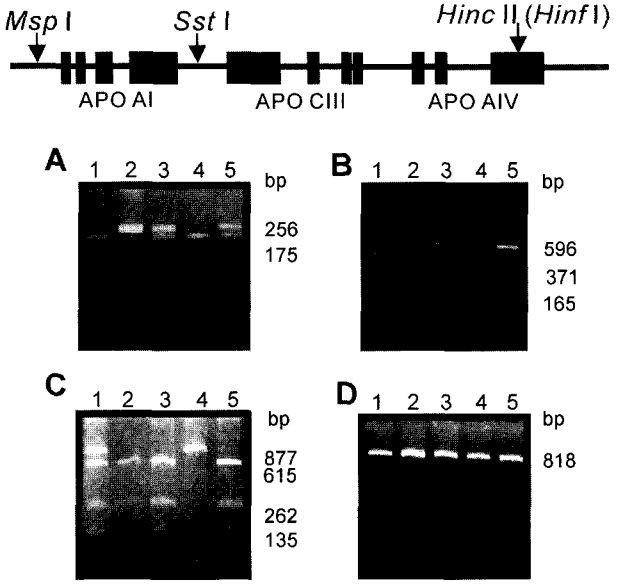

Fig. 1. Polymorphic map of the apo AI-CIII-AIV cluster gene (upper pannel) and electrophoretic patterns (lower pannel, A; *Msp*I, Lane 1, 4, M2M2 genotype; Lane 2, M1M1 genotype; Lane 3, 5, M1M2 genotype, B; *Sst*I, Lane 1, S2S2 genotype; Lane 2, 3, and 4, S1S2 genotype; Lane 5, S1S1 genotype, C; *Hinc*II, Lane 1, SN genotype, Lane 2, 3, and 5, NN genotype; Lane 4, SS genotype, D; *Hinf*I, Lane 1, 2, 3, 4, and 5, TT genotype).

min. Ethidium bromide was incorporated into the gel. The gel was directly photographed on an UV transilluminator and genotyped. Figure 1 shows the map of the apo AI-CIII-AIV gene cluster with the polymorphic restriction site and band pattern.

4. Statistical analysis

Allele frequencies were estimated by the gene counting method. Genotype distribution and allele frequencies were analyzed by χ^2-independence test and logistic regression analysis. Deviation in genotype distribution from the expected value for Hardy-Weinberg equilibrium was estimated by χ^2-fitness test. The heterozygosity and polymorphism information content (PIC) was calculated by the methods of Bostein *et al.* (1980). One-way ANOVA test was performed to compare the mean levels of biochemical parameters among different genotypes. The haplotype frequencies were estimated by using the EH computer program (Terwilliger and Ott, 1994). A Monte-Carlo simulation using the 'Clump (ver. 1.6)' computer program was performed to test the statistical significance of the association between the haplotype distribution and hypertension (Sham and Curtis, 1995). Standardized pairwise linkage disequilibrium, D, was calculated, and the degree of nonrandom association determined by calculation of the delta (Δ) (Hill and Robertson, 1968) and D' (Lewontin, 1964) between the polymorphic sites in the apo AI-CIII-AIV gene cluster. To test whether the observed linkage disequilibrium differed significantly from zero, the statistic $D^2N/p(1-p)q(1-q)$, which is asymptotically distributed as a χ^2 with 1 df under the null hypothesis, was used (p and q are the frequencies of the rare alleles at each locus; N is the number of chromosomes tested). $P < 0.05$ was considered statistically significant. All statistical analyses were performed by the computer program, 'dBSTAT for windows'.

III. RESULTS

1. Genotype distribution

Genotype frequencies of four RFLPs in the normotensives and hypertensives are displayed in Table 2. The G to A polymorphism of the apo AI gene promoter showed the significant differences between normotensives and hypertensives ($P < 0.05$). None of the other polymorphisms indicated a difference in genotype distribution between two groups. At all sites except one, observed genotype distributions did not differ from those expected for Hardy-Weinberg proportions. The *Hinf*I restriction site of apo AIV gene was monomorphic in the both groups. The PIC values of G to A, *Sst*I and *Hinc*II polymorphisms showed relatively high degree of polymorphism in the both groups.

2. Risk assessment of G^{-75} to A polymorphism

For AA genotype, our result had odds ratio, relative risk, sensitivity, specificity, positive predictive value, negative predictive value and total predictive value of 4.53, 1.66, 0.28, 0.92, 0.81, 0.51 and 0.57, respectively, while for A allele, 1.42, 1.17, 0.49, 0.59, 0.60, 0.49 and 0.54, respectively (Table 3).

3. Anthropometrical and biochemical parameters according to the RFLPs

Table 4 represents that the plasma Lp(a) concentra-

Table 2. Comparison of genotype frequencies in 4 RFLPs of the apo AI-CIII-AIV gene cluster in normotensive and hypertensive groups

Site	Geno-types	Normotensives			Hypertensives		
		Freq.	H	PIC	Freq.	H	PIC
G^{-75} to A[a]	GG	0.26	0.4860	0.3660	0.29	0.5000	0.3750
(*Msp*I)	GA	0.66			0.43		
	AA	0.08			0.28		
*Sst*I	S1S1	0.54	0.3660	0.2980	0.54	0.3900	0.3130
	S1S2	0.43			0.40		
	S2S2	0.02			0.07		
*Hinc*II	SS	0.11	0.4433	0.3450	0.11	0.4410	0.3438
	SN	0.44			0.43		
	NN	0.45			0.46		
*Hinf*I	TT	1.00	0.0000	0.0000	1.00	0.0000	0.0000
	TS	0.00			0.00		
	SS	0.00			0.00		

[a]Statistically significant difference (χ^2 = 13.0760, df = 2, P = 0.0010).

Table 3. Risk assessment of selected marker associated with hypertension

Markers	Odds ratio (95%CI)	Relative risk	Sensitivity	Specificity	Predictive power		
					Positive	Negative	Total
Apo AI Gene							
AA genotype	4.53(1.75-11.69)	1.66	0.28	0.92	0.81	0.51	0.57
A allele	1.42(0.92-2.19)	1.17	0.49	0.59	0.60	0.49	0.54

Table 4. Clinical characteristics according to genotypes of three RFLPs in the apo AI-CIII-AIV gene cluster

Variables	P-value by one-way ANOVA test		
	G^{-75} to A	*Sst*I	*Hinc*II
Age (year)	0.264	0.328	0.994
BMI (kg/m^2)[1]	0.678	0.147	0.385
Tg (mg/dl)[2]	0.464	0.511	0.790
TC (mg/dl)[3]	0.084	0.268	0.376
LDL-chol (mg/dl)[4]	0.211	0.400	0.529
HDL-chol (mg/dl)[5]	0.870	0.430	0.360
Lp(a) (mg/dl)[6]	**0.011**[a]	0.320	0.200
apoAI (mg/dl)[7]	0.094	0.444	0.558

[1]Body Mass Index, [2]Triglyceride, [3]Total cholesterol, [4]LDL-cholesterol, [5]HDL-cholesterol, [6]Lipoprotein (a) and [7]apolipoprotein AI.
[a]Statistically significant difference.

Table 5. Extended haplotype distribution of three RFLPs in the apo AI-CIII-AIV gene cluster

Polymorphic Sites			Subjects	
$G^{-75}$to A	*Sst*I	*Hinc*II	Normotensives	Hypertensives
G	S1	S	0.095114	0.010212
G	S1	N	0.262630	0.318048
G	S2	S	0.230479	0.135337
G	S2	N	0.000013	0.000259
A	S1	S	0.047430	0.063140
A	S1	N	0.330120	0.361613
A	S2	S	0.016683	0.098541
A	S2	N	0.017531	0.012851
Chromosome number			136	166
P[1]			< 0.05	

[1]The significant difference between normotensives and essential hypertensives was detected in haplotype frequencies (Monte-Carlo simulation, T_3 = 11.0757, df = 1, P = 0.0009, simulation number = 10,000).

tions were significantly different among the genotypes of G to A polymorphism in the apo AI gene (P < 0.05). Except for this, any parameters were not significantly associated with genotypes in this gene cluster.

4. The haplotype analysis and linkage disequilibrium of 3 RFLPs in the apo AI-CIII-AIV gene cluster

The distribution of extended haplotype in the apo AI-CIII-AIV gene cluster is represented in Table 5. Because the existence of double heterozygotes disturbed the construction of haplotype, we performed the maximum likelihood estimation of haplotype distribution with EH computer program. By Monte-Carlo simulation, there was the significant difference in extended haplotype distribution between two groups (P < 0.05).

The degree of linkage disequilibrium of 3 RFLPs in the apo AI-CIII-AIV gene cluster was shown in Table 6. Among 3 RFLP sites studied, the significant linkage

Table 6. Pair-wise linkage disequilibrium statistic (Δ, D') among RFLP pairs of apo AI-CIII-AIV gene cluster

Δ \ D'	G^{-75} to A	*Sst*I	*Hinc*II
G^{-75} to A		-0.1525[1]	-0.0662
*Sst*I	-0.2759[1]		-0.7412[2]
*Hinc*II	-0.1055	-0.9072[2]	

[1]The significant linkage disequilibrium were detected (χ^2 = 7.1629, df = 1, P = 0.0074).
[2]The significant linkage disequilibrium were detected (χ^2 = 196.6771, df = 1, P < 0.0001).

disequilibrium was detected between G^{-75} to A polymorphism and *Sst*I RFLP pair or *Sst*I RFLP and *Hinc*II RFLP pair (P < 0.05).

IV. DISCUSSION

Hypertension is one of the most common diseases in civilized countries. It is currently seen as a "complex" genetic trait caused by multiple susceptibility genes, which are modulated by gene-gene and gene-environment interactions. 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" hypertention remain largely unknown, and the success to date in identifying susceptibility genes has been very limited. Many previous studies have shown that with environmental factors, genetic mechanisms may play a role in determining susceptibility to essential hypertension. Essential hypertension may result from defective degradation of triglyderide-rich lipoprotein, impaired clearance from plasma, or a combination of both. There were several reports on the association between the RFLPs in the apo AI-CIII-AIV gene cluster and plasma lipid levels (Choi *et al.*, 2000; Hong *et al.*, 1997; Song and Kim, 1998; Song *et al.*, 1998). Aiming at deciphering the genetic architecture of blood pressure regulation and essential hypertension, we investigated the association between the genetic polymorphisms of the apo AI-CIII-AIV gene cluster and hypertension in Koreans by determinig the genotype frequencies of this gene cluster in normotensives and hypertensives, respectively.

Apo AI is an *in vivo* activator of lecithin : cholesterol acyltrancferase and an important component of reverse cholesterol efflux from cells (Reichi and Miller, 1989). The G^{-75} to A polymorphism of the apo AI gene was detected by the restriction enzyme, *Msp*I, and reported to be associated with elevated plasma apo AI and HDL levels (Pagani *et al.*, 1990). We observed that the AA genotype of this polymorphism is significantly higher in the hypertensive group than that in normotensive group (P < 0.05). Lp(a) is a plasma lipoprotein that is associated with coronary heart disease and stroke in Caucasian and Asian populations (Armstrong *et al.*, 1986; Dahlen *et al.*, 1986; Danesh *et al.*, 2000; Hoefler *et al.*, 1988; Murai et al., 1986; Rhoads *et al.*, 1986; Sandholzer *et al.*, 1992; Stein and Rosenson, 1997). Also, the plasma Lp(a) concentration of this study was significantly different among the genotypes of apo AI gene (P < 0.05). Heng *et al.* (2001) found that this polymorphism of the apo AI gene was significantly associated with the plasma Lp(a) level in Asian Indians from Singapore. Our result was similar to this study, and therefore, the G^{-75} to A polymorphism of apo AI gene may influence the pathogenesis of hypertension in Koreans through the mechanism such as high plasma Lp(a) level. According to the the predictive power (Table 3) and adjusted residuals (Fig. 2), AA genotype of the apo AI gene may be a useful genetic marker for hypertension in Koreans, and recessive model may fit our result, respectively.

The Apo CIII may be an inhibitor of LPL, so there is a known positive relationship between the increase of TG and the elevation of apo CIII (Wang *et al.*, 1985). The important causative factor of hypertriglyceridemia is the decreased lipoprotein lipase (LPL) activity (Attman *et al.*, 1993) and the apo CIII is an apolipoprotein that play a role in the elimination of remnants of TG-rich lipoproteins. However, the issue

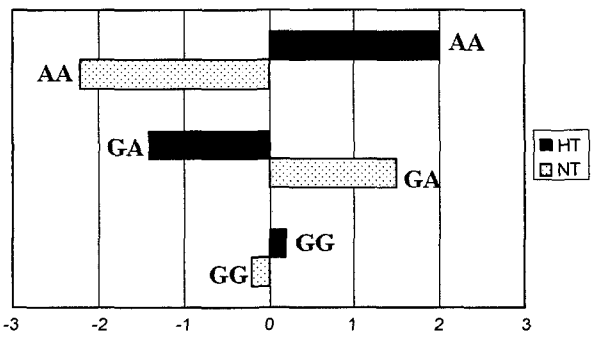

Fig. 2. Adjusted residual of genotypes in the G to A polymorphism of the apo AI gene. Abbreviations; HT, hypertensives, NT, normotensives.

of whether the genetic variation of the apo CIII gene is associated with plasma TG level is still controversial (Aalto-Setala *et al.*, 1992; Surguchov *et al.*, 1996). In the present study, there were no significant difference between the *Sst*I RFLP of the apo CIII gene and hypertension or other cardiovascular risk factors in Koreans. Therefore, it is unlikely that this RFLP may influence the pathogenesis of hypertension or other cardiovascular diseases in Koreans.

Apo AIV is one of the major apolipoproteins of lymph chylomicrons. Its physiological function may be similar to Apo AI and they both share common functions (Steinmetz and Utermann, 1985). The *Hinc*II RFLP of the apo AIV gene did not showed a difference in genotype frequency between normotensive and hypertensive groups in our subjects. Also, there was the no significant difference between this RFLP and other cardiovascular risk factors in our subjects. Like- wise the *Sst*I RFLP of the apo CIII gene, the *Hinc*II RFLP of the apo AIV gene may not be useful as the genetic marker for hypertension or cardiovascular diseases. In the case of The *Hinf*I RFLP of the apo AIV gene, this genetic marker was monomorphic in Koreans.

The distribution of the extended haplotype in our subjects revealed the predominance of A-S1-N haplotype in the both groups. Also, G-S1-S and G-S2-S haplotypes were significantly higher in normotensives than that in hypertensives, while A-S2-S haplotype is overrepresented in hypertensives than that in normotensives ($P < 0.05$). This distribution of the extended haplotypes in our subjects may be due to the linkage disequilibrium among the RFLP pairs. There were the significant linkage disequilibriums between G^{-75} to A polymorphism and *Sst*I RFLP pair or *Sst*I RFLP and *Hinc*II RFLP pair in our subjects ($P < 0.05$), corresponding to other report with Koreans (Song *et al.*, 1998).

In conclusion, our data suggest that the AA genotype of Apo AI gene may be useful as a genetic marker for hypertension in Koreans, and the overrepresentation of A-S2-S haplotype in hypertensives may be due to the linkage disequilibriums among genetic markers studied.

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