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The Haplotype Analyses Using Multiple Markers of the Apolipoprotein B Gene in Patients with Coronary Artery Disease

The high level of low density lipoprotein (LDL) is a risk factor for cardiovascular disease. Apolipoprotein (apo) B is a major protein component of LDL and plays an important role in the maintenance of cholesterol homeostasis. In this study, six polymorphic sites of the *apoB* gene were anlaysed in 235 patients with coronary artery disease (CAD) and 216 normal control subjects. There were no significant differences in the allele frequencies of apoB polymorphisms between the control and patient groups. However, haplotype frequencies were significantly different between the CAD patients and control ($p<0.05$). In addition, the allelic distributions of both *Eco*RI and *Msp*I polymorphisms in Koreans were similar to those in Chinese but significantly different from those in Caucasians. *ApoB* polymorphisms showed no association with plasma lipid levels. In conclusion, haplotype analysis of the apoB gene using multiple diallelic markers might be a useful marker for Korean CAD patients.

Key Words : *Apolipoproteins B; Variation (Genetic); Coronary Disease*

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INTRODUCTION

The major lipids of human body are phospholipids, cholesterol, triglycerides and cholesteryl esters. These insoluble lipids are transported through blood as lipoprotein complexes of lipids and one or more specific proteins, called apolipoproteins. By actively exchanging certain lipids and apolipoproteins with each other, the lipoproteins are synthesized and degraded at a constant rate. Among lipoproteins, low density lipoprotein (LDL) is 75% lipid (cholesterol and cholesteryl esters) and 25% protein. LDL is the metabolic product of very low density lipoprotein (VLDL). The high level of LDL is a risk factor for cardiovascular disease.

ApoB is the major protein component of LDL and plays an important role in the maintenance of cholesterol homeostasis (1). It serves as the ligand for the recognition and catabolism of plasma LDL by the LDL-receptor (2). Elevated levels of serum apoB are associated with an increased risk of premature atherosclerosis (3). Because of its large size and insolubility in water, the gene structure and amino acid sequence of *apoB* have been difficult to determine. The primary structure of the carboxyl-terminal end had been deduced from the nucleotide sequence of the cDNA by Knott et al. (4). The *apoB* gene is clearly distinct from other genes of the soluble apolipoproteins (apoAI, AII, AIV, CI, CII, CIII, and E). The distribution of the introns in the *apoB* gene is asymmetric. No sequence homology has been identified between apoB and other apolipoproteins.

ApoB circulates in two distinct forms (apoB100 and apoB48) encoded by a single gene localized in chromosome 2 pter-24 (5). ApoB100, the larger form, is synthesized in the liver as a translational product of the entire *apoB* mRNA. The smaller form, apoB48, is produced from the small intestine by a novel posttranscriptional RNA editing of a CAA (glutamine) to a UAA (stop) codon in *apoB* mRNA (6, 7). Thus, apoB48 terminates at amino acid residue 2153 and consists of the N-terminal 48% of apoB100. ApoB48 lacks the C-terminal domain of apoB100. As a result, it does not bind to the LDL-receptor. *ApoB* mRNA editing occurs exclusively in the small intestine of most mammalian species tested, although some species produce apoB48 even in the liver (8).

Until now, over ten common polymorphisms within or flanking apoB gene have been detected. Several polymorphic loci have been recognized in association with total cholesterol, LDL cholesterol, and apoB levels. In this study, six polymorphisms (*Hinc*II, *Pvu*II, *Alu*I, *Msp*I, *Eco*RI, and 3′ hypervariable region) of the *apoB* gene were analyzed in Korean patients with coronary artery disease (CAD), and their association with plasma lipid traits was investigated. Secondly, we compared our data to those in other racial or ethnic populations studied previously.

MATERIALS AND METHODS

Study subjects

The study subjects were recruited from the Seoul city in Korea. We selected 235 CAD patients (164 males and 71 females) from Seoul National University Hospital, Korea, documented by coronary angiography because of recent myocardial infarction or angina. None of the selected CAD patients was on a lipid lowering therapy at the time of blood sampling. In myocardial infarction patients, blood samples were obtained two months after the occurrence of the myocardial infarction. Patients with hypertension, diabetes, and endocrine or metabolic disorders were excluded from this group. The control group consisted of 216 individuals (162 males and 54 females), within the same age range as the patients, who were randomly selected by health screening at the same hospital to screen out those who had a history of chest pain, diabetes, hypertension, and general illness. Mean ages of CAD patients and controls were 53.2±9.3 and 51.1 ±9.1, respectively: the age difference was not statistically significant. Clinical details for these groups are summarized in Table 1. Blood samples were collected from all subjects after a fast of 12-16 hr to exclude the fluctuations of lipid levels. Plasma samples were stored at -70°C until the time of lipid assay.

DNA analysis

Total genomic DNA was prepared from leukocytes of 10 mL blood after lysis of red blood cells (9). Polymorphic regions of the *apoB* gene were amplified by polymerase chain reaction (PCR) from genomic DNA (Fig. 1). Primer sequences and procedures for PCR amplification have previously been described (10). Five polymorphic sites are caused by single base substitutions in various exons and introns in the *apoB* gene. The amplified PCR products were digested at specific restriction sites of endonucleases; namely, *Hinc*II, *Pvu*II, *Alu*I, *Msp*I, and *Eco*RI. Genotype analyses were done by electrophoresis on an agarose gel, except for use of 8% polyacrylamide gel in case of 3′ hypervariable region (HVR) polymorphism of the *apoB* gene. Alleles of each polymorphic site were classified as (+) or (-) according to the presence or absence at the cutting site of each restriction enzyme, respectively. The 3′ HVR polymorphism is produced by differences of 15-bp repeat at 491 bp 3′ of the translational termination site. Alleles of the 3′ HVR polymorphism were determined according to the nomenclature of Ludwig et al. (11) based on their 15 base repeats. Haplotype frequencies of the *apoB* gene in Koreans were also determined according to method of Thompson et al. (12).

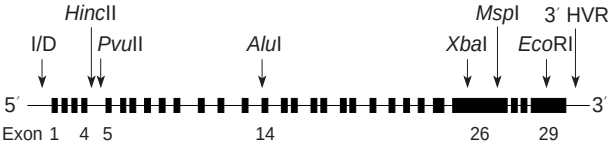

Fig. 1. Schematic diagram showing the polymorphic sites of the *apoB* gene.

Table 1. Mean age (yr±SD) and lipid levels (mmol/L±SD) of the study subjects

Variables	Subjects		*p**
	Control (n=216)	CAD (n=235)	
Age	51.1±9.1	53.2±9.3	NS†
Cholesterol	5.11±0.96	5.72±1.12	0.0026
Triglyceride	1.23±0.61	1.53±0.78	0.0010
HDL-cholesterol	1.51±0.36	0.97±0.27	0.0001
LDL-cholesterol	3.12±0.85	3.21±1.04	NS

**p* value by Chi-square test in lipid levels between the control and CAD groups, †not significant.

Determination of lipid levels

Levels of plasma cholesterol and triglyceride were measured by enzymatic colorimetry methods using commercial kits (Boeringer Mannheim, FRG) on a Hitachi 747 automatic chemistry analyzer. HDL-cholesterol was determined by measuring cholesterol in the supernatant liquid after precipitation of the plasma with $MgCl_2$ and dextran-sulfate, using a Gilford Impact 400E automatic analyzer with reagents and calibrators from Boeringer Mannheim, Germany. LDL-cholesterol levels were calculated by using the formula of Friedwald et al. (13).

Data analysis

Heterozygosity (H) and polymorphism information content (PIC) values of the *apoB* gene were calculated according to the method of Botstein et al. (14). Five diallelic markers within the *apoB* gene were used for haplotyping. The degree of nonrandom association was determined by calculation of the delta value (△) between the two polymorphic sites at the *apoB* gene (15). The χ^2 test was used to apply for Hardy-Weinberg equilibrium and to compare allele frequencies between the CAD and control groups, while the one-way analysis of variance (ANOVA) test was performed to compare the mean levels of lipid parameters among different genotypes. All statistical analyses were performed using the Statistical Analysis System software (SAS Institute, Inc). Statistical significance was accepted at the $p=0.05$ level.

RESULTS

Allele frequencies of the six polymorphic sites of the *apoB* gene in CAD patients and controls are shown in Table 2. Any

Table 2. Comparison of allele frequencies of the *apoB* polymorphisms in the CAD patients and controls

Polymorphic Site	Genotype frequency			Allele frequency			H Control/CAD	PIC Control/CAD
		Control	CAD		Control	CAD		
I/D*	- -	32	16	-	0.38	0.26	0.47/0.38	0.36/0.31
	+ -	99	90	+	0.62	0.74		
	+ +	85	129					
*Hinc*II	- -	160	169	-	0.87	0.86	0.23/0.24	0.20/0.21
	+ -	56	66	+	0.13	0.14		
	+ +	0	0					
*Pvu*II	- -	208	215	-	0.98	0.96	0.04/0.08	0.04/0.08
	+ -	8	20	+	0.02	0.04		
	+ +	0	0					
*Alu*I	- -	148	155	-	0.84	0.83	0.27/0.28	0.24/0.24
	+ -	68	80	+	0.16	0.17		
	+ +	0	0					
*Xba*I*	- -	195	209	-	0.95	0.94	0.10/0.11	0.10/0.11
	+ -	21	25	+	0.05	0.06		
	+ +	0	1					
*Msp*I	- -	0	0	-	0.00	0.00	0.00/0.00	0.00/0.00
	+ -	0	0	+	1.00	1.00		
	+ +	216	235					
*Eco*RI	- -	0	0	-	0.02	0.05	0.04/0.10	0.04/0.10
	+ -	8	22	+	0.98	0.95		
	+ +	208	213					
3′ HVR				<36	0.19	0.14	0.75/0.76	0.68/0.69
				36	0.62	0.66		
				>36	0.19	0.20		

*Insersion/deletion (I/D) and *Xba*I polymorphisms were reported by authors (16).
Abbreviations: CAD, coronary artery disease; HVR, hypervariable region; H, heterozygosity; PIC, polymorphism information content.

Table 3. Haplotype frequencies of the *apoB* gene in the control and CAD group

Haplotype							Number (%)	
I/D	*Hinc*II	*Pvu*II	*Alu*I	*Xba*I	*Msp*I	*Eco*RI	Control	CAD
+	-	-	-	-	+	+		3 (2.9)
+	-	-	-	+	+	-	1 (0.9)	1 (1.0)
+	-	-	-	+	+	+	68 (58.5)	69 (65.6)
+	-	-	+	-	+	+	2 (1.7)	
+	-	-	+	+	+	+	2 (1.7)	5 (4.8)
+	-	+	-	+	+	+	1 (0.9)	1 (1.0)
+	-	-	+	+	+	+	8 (6.9)	10 (9.5)
-	+	-	-	+	+	+	30 (25.9)*	13 (12.3)*
-	-	-	+	+	+	+	1 (0.9)	3 (2.9)
-	-	+	+	+	+	+	1 (0.9)	
-	+	-	-	+	+	+	2 (1.7)	
Total number							116 (100.0)	105 (100.0)

*There is significantly different in the haplotype frequency between controls and CAD patients.

Table 4. Standardized nonrandom association statistics (D', △) between pairs of DNA polymorphisms of the *apoB* gene

D/△	I/D	*Hinc*II	*Pvu*II	*Alu*I	*Xba*I	*Eco*RI
Control						
I/D		0.0470	- 0.0017	0.0687	- 0.0586	- 0.0806
*Hinc*II	0.2282		- 0.0517	- 0.1251	- 0.0523	- 0.0431
*Pvu*II	0.0176	1.0000		- 0.1118	- 0.0247	- 0.0213
*Alu*I	0.1529	1.0000	0.4178		- 0.0326	- 0.0471
*Xba*I	1.0000	1.0000	1.0000	1.0000		- 0.0213
*Eco*RI	1.0000	1.0000	1.0000	0.1000	1.0000	
CAD						
I/D		- 0.1748	- 0.0455	0.1112	- 0.1116	- 0.0633
*Hinc*II	1.0000		- 0.0605	- 0.0985	- 0.0867	- 0.0756
*Pvu*II	1.0000	1.0000		0.0163	- 0.0612	- 0.0502
*Alu*I	0.1609	0.6394	0.0407		- 0.1321	0.0001
*Xba*I	1.0000	1.0000	1.0000	0.3014		- 0.0510
*Eco*RI	1.0000	1.0000	1.0000	0.0072	1.0000	

There was no significant linkage disequilibrium between each pair of polymorphism.

of the polymorphisms in this study did not show significant differences in allele frequencies between patients and controls. The values of heterozygosity (H) and polymorphism information content (PIC) values based on the allele frequencies of each polymorphism were also estimated. The H and PIC values for the 3′ HVR polymorphism were relatively high in the control and CAD groups. Genotype distributions did not differ from those expected for Hardy-Weinberg proportions at all polymorphic sites. Unequivocal assignments of haplotypes can be made for all but the doubly heterozygous individuals. Two polymorphisms (insertion/deletion and *Xba*I) previously reported by authors (16) were added to the haplotype analysis. From 7 diallelic markers within the struc-

tural gene, except the 3′ HVR polymorphism, we could identify 11 different haplotypes out of a possible total of 128 (Table 3). The +/-/-/-/+/+/+ haplotype was the most common in both groups. The second most common haplotype, -/-/-/-/+/+/+, was significantly more frequent in the controls than in patients. However, each polymorphic pair did not show linkage disequilibrium (Table 4).

We also examined whether five diallelic and 3′ HVR polymorphisms of the *apoB* gene were associated with plasma lipid levels in Koreans. Any of the polymorphic sites were not associated with plasma lipid levels.

DISCUSSION

CAD is a multifactorial disease that may differ in each race or ethnic population. For example, the prevalence of CAD vary widely among different population, and the frequencies of the *apoB* gene polymorphisms have been reported to vary among ethnic groups. Thus, we investigated polymorphisms of the *apoB* gene in Korean CAD patients.

Haplotype determination with multiple markers could possibly help to define more specific genotypes associated with high CAD risk than a single marker. From seven allelic polymorphic sites, we could identify the eleven different haplotypes out of a possible total of 128. The -/-/-/-/+/+/+ haplotype was more frequent among controls than in patients. This haplotype may be in a linkage disequilibrium with other functionally important polymorphisms in the *apoB* gene. Thus, it is suggested that, at least in Koreans, this haplotype is likely to be a protective marker against CAD. The haplotype analysis using multiple markers of the *apoB* gene was mainly conducted in 3,500 mutation study (17).

Until now, the genetic variation of *apoB* has been studied extensively. Allele frequencies of the *apoB* gene in Koreans were compared with previous studies in various populations. Among six polymorphisms of this study, the (-) allele frequency of *Eco*RI polymorphism of Mongoloids (2-5%; 18, 19, 20) was significantly lower than that of Caucasoids (11-47%; 21-27). Indians in Singapore had the (-) allele frequency of 10% (28). Also, of interest, *Msp*I polymorphism of this study did not have the mutant compared to that of Caucasians (0.07-0.22%; 21, 23, 29) previously studied. And hence, they seem to be the valuable marker for population study.

Five diallelic *apoB* polymorphisms of this study were not associated with plasma lipid levels. We could not exclude the possibility that an appearance of many subjects having (+ +) or (- -) homozygote in each polymorphic site may cause the failure to show the association with lipid levels. Until now, the association studies between apoB polymorphism and lipid phenotypes have reported contradictory results among populations. Generally, *apoB* diallelic polymorphisms in Asian populations were not associated with lipid levels (20, 30-32). However, *apoB* polymorphisms in Caucasian populations show more polymorphic than those in Oriental populations as we described at above. Actually associations between *apoB* polymorphisms and lipid levels were mainly reported in Caucasian populations. For example, Glisic et al. (33) have reported association between *Eco*RI and *Msp*I polymorphisms of the *apoB* gene and lipid levels in Yugoslavian population. This associations were confirmed in English (34), Finns (35), Canadian (36, 37), Norwegian (38) and Danish (39) populations. Also, HVR polymorphism of this study showed the lack of association with lipid levels. Japanese population show the same trend (40). However, an association between the larger 3′ HVR alleles and CAD patients was reported in Austrian individuals (41). This population also showed associations with serum cholesterol and apoB levels. Alavantic et al. (42) have reported association between 3′ HVR polymorphism and lipid levels. Thus, polymorphisms of the *apoB* gene may be Caucasian-specific. That is, they may originate from Caucasian populations. Therefore, it raises the possibility that lack of association between *apoB* polymorphisms and lipid levels in non-Caucasian populations may, at least in Koreans, partly explain the rarity of CAD.

As a possible explanation for the differences of allele frequency and lipid association of the *apoB* polymorphisms among populations studied, the differences in the genetic background may be a more important factor than environmental variations, such as diet or life-style. That is, the results suggest that this genetic link may, at least in part, explain the differences in prevalence rate of atherosclerosis among populations. Another possibility is that they may be due to the differences in linkage disequilibria between the two polymorphic sites of the *apoB* gene among populations. Genetic drift by a founder effect or selective mechanism may cause different levels of linkage disequilibria. And they could be caused by the differences of sample numbers and the bias of sample selection for the populations studied. In other words, to compare the exact allele heterogeneity among populations, large sample sizes in order to maintain a statistical power is required, making it possible to identify more exact distributions of the polymorphisms among racial or ethnic populations. Thus, polymorphism studies should be performed ideally on samples from a population of homogenous origin. Population admixture may cause a falsely positive genetic association. In this respect, Koreans have had a very low rate of interracial marriage, maintaining a homogeneous population for a long time, suggesting that they are appropriate for polymorphism study.

In conclusion, the results of this study suggest that, at least in the Korean population, a single polymorphism of the *apoB* gene is unlikely to be a useful marker for CAD patients. However, haplotypes of the apoB gene are likely to be more useful markers for Korean CAD patients.

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