Biol. Bull. NTNU. 1997. 32 (2) : 43-51

Human Apolipoprotein B Gene: Polymorphic Haplotype Analysis in Chinese Subjects

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ABSTRACT

The polymorphic DNA haplotype of the human apolipoprotein (apo) B gene in Chinese subjects was analyzed. Genomic DNA extracted from randomly sampled 118 unrelated individuals was used to amplify fragments containing polymorphic change sites of the apo B gene. The polymorphic loci include 6 restriction endonuclease sites (*Apa*LI, *Alu*I, *Mae*I, *Msp*I, *Eco*RI, and *Eco*57I) and an insertion/deletion (I/D) polymorphism involving the signal peptide region of the apo B gene. The polymerase chain reaction (PCR) amplified products were analyzed directly (for I/D polymorphism), or following specific enzyme digestion (for restriction site polymorphism). Among 236 chromosomes examined, no polymorphic allele was detected for *Msp*I locus. All other diallelic loci examined are in Hardy-Weinberg equilibrium. Linkage disequilibrium analysis of the haplotype data revealed strong nonrandom allelic association among I/D, *Apa*LI, and *Alu*I in exons 1, 14 as well as among *Mae*I, *Eco*RI, and *Eco*57I in exons 26-29. On the other hand, little linkage disequilibrium between loci in exons 1-14 and exons 26-29 was observed. The results suggest the apparent recombination in the central region of the human apo B gene with little or no recombination in the 5' or 3' end.

Key words: Apo B, Polymorphism, Haplotype, Linkage disequilibrium

Introduction

Apolipoprotein (apo) B100 is the major protein in low density lipoproteins (LDL) and is essential for the assembly of triglyceride-rich lipoproteins. As the ligand for the LDL receptor, apo B100 mediates the removal of LDL from the circulation by receptor-mediated endocytosis via the LDL receptor (Brown and Goldstein, 1986). The mature apo B100 protein is a 4536-amino acid protein with a 27-amino acid signal peptide (Law *et al.*, 1986). The 43 kb long apo B gene is located on chromosome 2p and contains 29 exons (Knott *et al.*, 1985; Blackhart *et al.*, 1986).

Various common mutations (polymorphisms) in the apo B gene were known. Some of these within exons are listed in Table 1. Studies using antisera from multiply transfused patients have identified five pairs of Ag epitopes resulting from amino acid substitutions of the apo B100 protein. The identification of nucleotide substitutions for Ag epitopes has allowed the detection of Ag polymorphisms by restriction fragment length polymorphism (RFLP) analysis (Ma *et al.*, 1987; Wang *et al.*, 1988; Xu *et al.*, 1989; Young and Hubl, 1989; Wu *et al.*, 1991; Dunning *et al.*, 1992). In addition, a three-amino acid insertion/deletion in the signal sequence was identified (Boerwinkle and

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Chan, 1989). Apo B gene polymorphisms were used to generate haplotypes in linkage studies in pedigrees as well as in population studies to determine the origin of particular FDB (familial defective apo B100) alleles (Ludwig and McCarthy, 1990; Bersot *et al.*, 1993).

In the present work, the genetic heterogeneity of seven polymorphic sites within the apo B locus was studied in 118 Chinese subjects. In addition to genotypes and allele frequencies computed and chi-square tested for Hardy-Weinberg equilibrium, linkage disequilibrium analysis was conducted to test the random association of the polymorphic markers.

Table 1. Polymorphisms within apo B exons

Name	Exon	cDNA position*	Location on Apo B100*	Amino acid variation	Associated antigen polymorphism
I/D	1	162-170	-14 ~ -16	3 aa Ins/Del	
*Apa*LI	4	421	71	Thr/Ile	Ag(c/g)
*Alu*I	14	1981	591	Ala/Val	Ag(a_1/d)
*Mae*I	26	8344	2712	Pro/Leu	Ag(x/y)
*Msp*I	26	11041	3611	Gln/Arg	Ag(h/I)
*Eco*RI	29	12669	4154	Glu/Lys	Ag(t/z)
*Eco*57I	29	13141	4311	Asn/Ser	Ag(x/y)

*The cDNA and amino acid positions were referred to Ludwig *et al.*, 1987.

Materials and Methods

Subject and DNA preparation

One hundred and eighteen unrelated Chinese individuals residing in Taiwan were studied. Blood samples were collected and lysed in a buffer containing 0.32 M sucrose, 10 mM Tris pH 7.5, 5 mM $MgCl_2$, and 1% Triton X-100. Nuclei were separated by centrifugation and digested in buffer containing 50 μg/ml proteinase K, 10 mM Tris pH 7.8, 5 mM EDTA, and 0.5% SDS. Genomic DNA was extracted by using an automatic nucleic acid extractor (Genepure, 341 Nucleic Acid Purification Systems, Applied Biosystems). The DNA was quantified as described (Sambrook *et al.*, 1989) and diluted to a concentration of 100 ng/μl.

PCR amplification

Genomic DNA (250-500 ng) was used for a 50 μl PCR reaction containing 10 mM Tris pH 8.3, 50 mM KCl, 0.1% Triton X-100, 0.2 mM dNTPs, 0.4 μM of each primer, and 2 U *Taq* DNA polymerase (Promega). Specific PCR conditions are listed in Table 2. PCR analyses were performed in an Idaho thermal cycler (1605 Air Thermal Cycler).

Genotype analysis

Seven markers were analysed in the apo B gene (Figure 1). They are: a 9 bp insertion/deletion (I/D) polymorphism in the signal peptide (Boerwinkle and Chan, 1989); a polymorphic *Apa*LI site in exon 4 (Ma *et al.*, 1987; Young and Hubl, 1989); a polymorphic *Alu*I site in exon 14 (Wang *et al.*, 1988); polymorphic *Mae*I (Wu *et al.*, 1991) and *Msp*I (Xu *et al.*, 1989) sites in exon 26; polymorphic *Eco*RI (Ma *et al.*, 1987) and *Eco*57I (Dunning *et al.*, 1992) sites in exon 29. For polymorphism involving restriction enzyme, the PCR amplified products were digested overnight with restriction enzymes and analysed on a 10% polyacrylamide gel (*Apa*LI and *Alu*I) or 2% aragose gel (*Mae*I, *Msp*I, *Eco*RI, and *Eco*57I). The signal peptide I/D polymorphism was detected directly on a 10% polyacrylamide gel. All restriction endonucleases were obtained from New England Biolabs.

Table 2. Specific PCR conditions

Amplified Fragment (exon)	Primer pairs and position *	Annealing temperature(℃)	$MgCl_2$ concentration (mM)	Products size (bp)
1	I/D-5' (120-141) I/D-3' (ac210-190)	64	1.5 plus 3 % formamide	93/84
4	*Apa*LI-5'(367-389) *Apa*LI-3' (507-485)	58	1.5	141
14	*Alu*I-5' (tgattggaaatccatatttacttg) *Alu*I-3' (2161-2138)	55	2.0	292
26	*Mae*I-5' (8142-8168) *Mae*I-3' (8754-8725)	55	1.5	613
26	*Msp*I-5' (10832-10857) *Msp*I-3' (11217-11193)	57	1.0	386
29	*Eco*RI-5' (12410-12434) *Eco*RI-3' (12785-12761)	55	1.5	376
29	*Eco*57I-5' (12982-13011) *Eco*57I-3' (13262-13232)	55	2.0	281

*The nucleotide positions were referred to Ludwig *et al.*, 1987. Sequences in lowercase letters are located within introns.

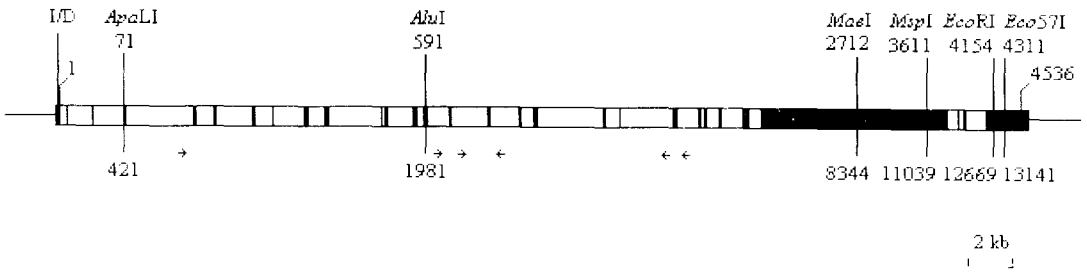

Figure 1. Diagram of the human apo B gene. Exon is represented by a line or a black box and intron by an open box. Location of the haplotype marker is indicated by vertical line above the gene. The marker I/D is a 9 bp insertion/deletion in the signal peptide, and other markers are restriction fragment length polymorphisms caused by single base substitutions. The numbers above and below the gene indicate the position of the polymorphic site on the apo B100 protein and cDNA, respectively. Amino acid 1 and 4536 represent the amino and carboxyl termini, respectively, of the apo B100 protein. The arrow below represents the location and polarity of Alu-type repetitive sequences.

Statistical methods

The allele frequency and heterozygosity for each polymorphism were calculated from the genotype frequency data by standard methods for co-dominant markers. The expected genotypic frequencies under random mating were computed using the algorithm by Levene (1949) and chi-square (χ^2) test was performed for Hardy-Weinberg equilibrium (Popgene version 1.1, Yeh and Boyle, 1996). The expected haplotype frequencies were obtained using gene counting method (Hill, 1974) and chi-square tested for significance.

Results

Genotype analysis

The genotypes at 7 polymorphic sites of the apo B gene for the tested 118 individuals were determined. The results of the examined three sites were shown in Figure 2. The I/D polymorphism in exon 1 is caused by a 9 bp insertion/deletion in coding region for signal peptide of the apo B gene. Upon PCR amplification, 93 bp (Figure 2, lanes 2,3), 84 bp (Figure 2, lane 1), or both (Figure 2, lane 4) fragments were observed.

The exon 14 *Alu*I RFLP is caused by a C-to-T change that alters Ala_{591} to Val in the apo B100 protein. The change creates a new *Alu*I restriction site on the PCR products so that, on digestion, 181 and 49 bp fragments appeared instead of common 230 bp fragments (Figure 2, lanes 6,7). The *Eco*RI RFLP in exon 29 is caused by a G-to-A change that alters Glu_{4154} to Lys in the apo B100 protein. The change abolishes an *Eco*RI restriction site on the PCR products so that, on digestion, 376 bp fragment appeared instead of 260 and 116 bp fragments (Figure 2, lane 8).

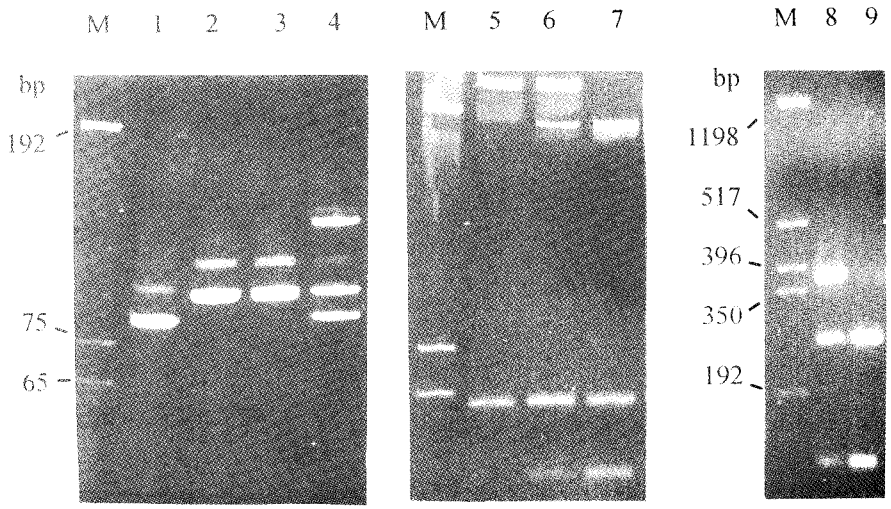

Figure 2. Restriction analysis of I/D, *Alu*I, and *Eco*RI polymorphisms. The PCR amplified products were digested with *Alu*I (lanes 5-7) or *Eco*RI (lanes 8-9) and fractionated on 10% polyacrylamide gel (lanes 1-7) or 2% agarose gel (lanes 8-9). Lane M (*Hin*fI digest of pGEM4 DNA) is used as size markers.

Allele frequency and heterozygosity

The genotypes, allele frequency, and heterozygosity for the above 7 polymorphisms in the apo B gene are shown in Table 3. For all loci studied, alleles without restriction site or 9 bp insertion are termed "−" and those with restriction site or 9 bp insertion termed "+". As shown in Figure 2, genotypes for samples in lanes 1-9 are: +/+ for lanes 2,3,7,9; +/− for lanes 4,6,8; and −/− for lanes 1,5. Among 236 chromosomes examined, no polymorphic allele was seen for *Msp*I locus. For other diallelic loci examined, the less common alleles varying in frequency from 4% to 33%, and heterozygosity from 8% to 43%. The same allele frequency (33%) was observed for *Mae*I− and *Eco*57I−. In fact, for all the 118 individuals tested, the same genotype of *Mae*I and *Eco*57I was observed. When the expected genotypic frequencies under random mating were computed and chi-square tested, all the diallelic loci studied are in Hardy-Weinberg equilibrium (Table 3, $\chi^2 < 3.84$, $P > 0.05$, 1 *df*).

Table 3. Genptype, allele frequency and heterozygosity of 7 polymorphic loci within the apo B gene

		I/D	*Apa*LI	*Alu*I	*Mae*I	*Msp*I	*Eco*RI	*Eco*57I
Genotype *	-/-	9	5	78	13	0	0	13
	+/-	45	35	34	51	0	10	51
	+/+	64	78	6	54	118	108	54
Allele	-	0.27	0.19	0.81	0.33	0.00	0.04	0.33
	+	0.73	0.81	0.19	0.67	1.00	0.96	0.67
Heterozygosity		0.38	0.30	0.29	0.43	0.00	0.08	0.43
χ^2		0.11	0.22	0.89	0.05	–	0.21	0.05
P (1 *df* #)		0.75	0.64	0.35	0.82		0.65	0.82

* + and −, presence and absence of 9 bp insertion (for ID marker) or restriction site (for the rest RFLP).
\# *df*: degree of freedom.

Haplotype analysis

When conducting haplotype analysis for pair of diallelic locus, individuals heterozygous for both loci cannot be defined. The expected haplotype frequencies were obtained by gene counting method (Hill, 1974). Table 4 presents the linkage disequilibrium analysis of haplotype I/D and *Mae*I as well as haplotype *Mae*I and *Eco*RI. When chi-square tested for significance, a random allelic association between I/D and *Mae*I was detected (χ^2 = 0.97, P > 0.90); on the other hand, nonrandom allelic association between *Mae*I and *Eco*RI (χ^2 = 27.05, P < 0.001) was found. Haplotype analysis chi-square value and significance for pairs of diallelic locus are shown in Table 5. A complete allelic association between *Mae*I and *Eco*57I loci was observed (χ^2 = 163.20, P < 0.001). The results suggest that there are two major haplotypes present in Chinese population, one with *Mae*I- and *Eco*57I- and the other *Mae*I+ and *Eco*57I+. A nonrandom allelic association among I/D, *Apa*LI, and *Alu*I loci in exons 1, 4, and 14 was observed (χ^2 = 43.81, 75.46, or 85.09, P < 0.001). For *Mae*I, *Eco*RI, and *Eco*57I loci in exons 26 and 29, a similar nonrandom allelic association was found (χ^2 = 27.05 or 163.20, P < 0.001). On the other hand, little linkage disequilibrium between loci in exons 1-14 and those in exons 26-29 was observed (χ^2 = 0.46, 0.97, 1.49, 1.57, 3.75, or 8.80, P > 0.05).

Discussion

The gene for the human apo B100 protein is split into 29 exons spanning approximately 43 kb (Blackhart *et al.*, 1986).

Table 4. Haplotype analysis using I/D, *Mae*I as well as *Mae*I, *Eco*RI polymorphisms

Haplotype		No. of chromosomes (%)		Haplotype		No. of chromosomes (%)	
I/D	*Mae*I	Observed	Expected*	*Mae*I	*Eco*RI	Observed	Expected*
-	-	13 (5.5)	10.40 (4.4)	-	-	6 (2.5)	1.16 (0.5)
-	+	31 (13.1)	32.28 (13.7)	-	+	65 (27.5)	71.64 (30.4)
+	-	45 (19.1)	46.30 (19.6)	+	-	0 (0.0)	4.63 (2.0)
+	+	109 (46.2)	106.41 (45.1)	+	+	153 (64.8)	150.15 (63.6)
Undefined		38 (16.1)	40.63 (17.2)	Undefined		12 (5.1)	8.43 (3.6)
χ^2 = 0.97, P > 0.90. 4 *df*				χ^2 = 27.05, P < 0.001. 4 *df*			

* The expected frequencies are calculated according to Hill, 1974.

Table 5. Pairwise haplotype analysis by chi-square values (χ^2, above the diagonal) and their significance

	I/D	*Apa*LI	*Alu*I	*Mae*I	*Eco*RI	*Eco*57I
I/D		85.09	43.81	0.97	1.57	0.97
*Apa*LI			75.46	3.75	0.46	3.75
*Alu*I	*	*		8.80	1.49	8.80
*Mae*I					27.05	163.20
*Eco*RI				*		27.05
*Eco*57I				*	*	

$\chi^2 < 9.49$, $P > 0.05$. 4 *df* (the deviations between the observed and expected values are insignificant).
*$\chi^2 > 18.47$, $P < 0.001$. 4 *df*.

The I/D, *Apa*LI, and *Alu*I loci are located in the 5' region of the apo B gene, and the *Mae*I, *Msp*I, *Eco*RI, and *Eco*57I loci in the 3' region. For all diallelic loci examined, the distribution of the genotypes did not differ from those expected under Hardy-Weinberg equilibrium (Table 3). The results indicate random mating and random union of gametes in the population sampled. The genotypes frequencies are therefore not influenced by evolutionary force such as mutation, migration, or natural selection.

When allele frequency was compared between the Chinese and Caucasian populations, the Chinese in Taiwan showed a much lower allele frequency for *Apa*LI- (19% vs 29%), *Alu*I+ (19% vs 47%), *Msp*I- (0% vs 11%) (Ludwig and McCarthy, 1990), *Mae*I- and *Eco*57I- (33% vs 74-81%) (Dunning *et al.*, 1992), and *Eco*RI- (4% vs 14-21%) (Genest *et al.*, 1990; Ludwig and McCarthy, 1990; Paulweber *et al.*, 1990). A similar low allele frequency for *Eco*RI- (6%) in Japanese (Iso *et al.*, 1996), *Mae*I- and *Eco*57I- (29%) in Chinese in Singapore (Dunning *et al.*, 1992), as well as *Msp*I- (1%) and *Eco*RI- (5%) in Chinese in Taiwan (Pan *et al.*, 1995) were also reported. Since haplotype markers are ancient that predate human racial divergence, difference in founding population for Asian and Caucasian could explain the observation.

The *Mae*I and *Eco*57I loci are completely associated in the 134 chromosomes whereas phase could be determined unambiguously (Table 3). The complete allelic association was previously reported in studies of individuals from a number of ethnic groups worldwide (Dunning *et al.*, 1992). The *Mae*I and *Eco*57I polymorphic changes are worldwide and frequency of *Mae*I-/*Eco*57I- allele is greater than 50% in most other ethnic groups (Dunning *et al.*, 1992). The results suggest that mutations occurred on the same chromosome to generate the *Mae*I+/*Eco*57I+ allele. They have not been separated by recombination in the history of modern man, possibly due to the presence of huge exons (7572 and 1906 bp) and only three small introns (403, 107, and 942 bp) between (Blackhart *et al.*, 1986). The results indicate that little recombination occurred in the 3' region of the apo B gene.

When examining the nonrandom allelic association between *Mae*I/*Eco*57I and *Eco*RI loci, there is an apparent complete allelic association between *Mae*I+/*Eco*57I+ and *Eco*RI+ in the 153 unambiguously determined chromosomes (Table 4). The data indicate that mutations creating the *Mae*I+/*Eco*57I+ allele arose on a chromosome carrying the *Eco*RI+ allele.

In addition to the linkage disequilibrium among the *Mae*I, *Eco*RI, and *Eco*57I loci in the 3' region of the apo B gene, the I/D, *Apa*LI, and *Alu*I loci in the 5' region are also nonrandomly associated ($\chi^2 > 18.47$, $P < 0.001$, Table 5). Linkage between I/D and *Apa*LI loci was also reported in other studies (Boerwinkle *et al.*, 1995; Chatterton *et al.*, 1995). The linkage observed could be caused by linkage disequilibrium in the founding population. Since introns separating exons 1-14 are not very long (range from 112 bp to about 3 kb) (Blackhart *et al.*, 1986), little recombination occurred to break the linkage.

No allelic association between polymorphism in exons 1-14 and polymorphism in exons 26-29 was observed. The presence of five Alu-type repetitive sequences in introns 14, 15, 16, 20, and 21 (Figure 1) (Blackhart *et al.*, 1986) could explain the observation. Taken together, the results suggest the apparent recombination in the central region of the human apo B gene, although little or no recombination in the 5' or 3' end.

Acknowledgments

We thank Drs. S. Huang, K. Fang, and F. Kung for their helpful discussions and comments. Gratitude is extended to Drs. F. C. Yeh and T. Boyle for providing the computer software for statistical analysis. This work was supported by grant NSC86-2313-B-003-001 from the National Science Council, Executive Yuan, Republic of China.

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（接受日期：1997.10.17）

人類表面蛋白B基因：中國人族群的多型性單套型分析

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摘　要

本研究在分析中國人族群的表面蛋白 (apo) B 基因的多型性單套型。我們自正常人族群逢機取樣 118 人，萃取其基因組 DNA，以聚合酵素鏈反應 (PCR) 放大包含 apo B 基因多型性點的片段。所檢測的多型性點包含 6 個限制酵素的切割位置 (*Apa*LI、*Alu*I、*Mae*I、*Msp*I、*Eco*RI、*Eco*57I) 與一個訊息胜肽區 9 個核苷酸鹽基對的插入或缺失的多型性點 (I/D)。PCR 放大的含多型性點的 DNA 片段，直接以膠體電泳檢查 (I/D 多型性)，或以限制酵素切割後檢查之 (限制酵素切點多型性)。所檢測的 236 條染色體中，沒有觀察到 *Msp*I 切點的多型性，其餘各多型性的對偶基因頻率及異型合子率皆符合哈溫定律。單套型的連鎖不平衡分析顯示，位於表現子 1-14 上的 I/D、*Apa*LI、*Alu*I 等多型性對偶基因間爲非逢機性的組合，相同的現象亦見於表現子 26-29 上的 *Mae*I、*Eco*RI、*Eco*57I 之對偶基因間。相反的，位於表現子 1-14 和 26-29 上的多型性點間，則無連鎖不平衡現象。本研究結果顯示，雖然 apo B 基因 5' 或 3' 端皆無重組發生，apo B 基因的中間部位則有明顯的基因重組現象。

關鍵詞：表面蛋白 B、多型性標記、單套型、連鎖不平衡