A unique haplotype of the apolipoprotein B-100 allele associated with familial defective apolipoprotein B-100 in a Chinese man discovered during a study of the prevalence of this disorder

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Abstract The prevalence of familial defective apolipoprotein (apo) B-100 (FDB) was determined by sampling 5,160 volunteer subjects from among 14,058 eligible employees of a bank in California. The sample was ethnically diverse (44.6% of the population was non-Caucasian). The prevalence of FDB in the study population was 0.08% with a 90% confidence interval of 0.01–0.14%. Four subjects were found to have the apoB 3500 codon mutation by mutagenic polymerase chain reaction, which creates an *Msp*I site at the 3500 codon of normal alleles but not alleles coding for the Arg→Gln mutation of FDB. Three of these were Caucasian and born in North America. The fourth was a native of China. Haplotype analysis of the affected allele of the Chinese subject using 10 markers described by Ludwig and McCarthy (1990. *Am. J. Hum. Genet.* **47:** 712–720) revealed a unique haplotype that differed from the haplotype of all other subjects with FDB. This unique allele had 30 repeats of a 3' hypervariable element instead of 48 as was found in the allele associated with FDB in other subjects, and in the 3' region there was an *Eco*RI site that was also not present in the allele most commonly found in association with FDB. We conclude that the prevalence of FDB in our ethnically diverse population is lower than that reported in previous studies of predominantly Caucasian populations and that the Chinese subject represents either an independent mutation or possibly recombination at the 3' end of the apoB gene, an event not previously described.—**Bersot, T. P., S. J. Russell, S. R. Thatcher, N. K. Pomernacki, R. W. Mahley, K. H. Weisgraber, T. L. Innerarity, and C. S. Fox.** A unique haplotype of the apolipoprotein B-100 allele associated with familial defective apolipoprotein B-100 in a Chinese man discovered during a study of the prevalence of this disorder. *J. Lipid Res.* 1993. **34:** 1149–1154.

Supplementary key words apolipoproteins • haplotype • alleles • cholesterol • low density lipoproteins • lipids

Familial defective apolipoprotein (apo) B-100 (FDB) is one cause of hypercholesterolemia associated with elevated plasma concentrations of low density lipoproteins (LDL) (1). The disorder was discovered during studies of radioiodinated LDL catabolism in patients with primary moderate hypercholesterolemia (2). The clearance of autologous LDL in the index subject was retarded compared to heterologous LDL from a normal donor, suggesting possible defective binding of the autologous LDL to LDL receptors. This hypothesis was confirmed in LDL-binding studies of skin fibroblasts (3), and the point mutation (CGG→CAG) responsible for the diminished binding was found in the codon for residue 3500 of the apoB-100 gene (4). This mutation results in a glutamine-for-arginine substitution and the associated loss of a positive charge (the arginine guanido group). The substitution occurs in the apoB-100 binding region and is thought to disrupt LDL binding by inducing a conformational change in this critical domain of the apoB-100 molecule (5).

At present, only patients who are heterozygous for this mutation have been identified. Their cholesterol concentrations are usually moderately elevated (250–350 mg/dl), and they are probably hypercholesterolemic from birth; children as young as 1 year of age have been observed to be hypercholesterolemic (T. Bersot, unpublished observation), as have older children (6).

Recognition of FDB as a clinical syndrome has led to speculation about its prevalence both in patients with hypercholesterolemia and in the general population. Previous estimates ranged from 0.17% to 0.2%, based on the incidence of FDB in predominantly Caucasian patients attending lipid clinics because of hypercholesterolemia (1, 7,

Abbreviations: FDB, familial defective apolipoprotein B-100; apo, apolipoprotein; LDL, low density lipoprotein; HVE, hypervariable element.

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8). There are no reports of the prevalence of FDB in the general population or in non-Caucasian persons.

Study of non-Caucasian groups is of interest because all affected persons with FDB described to date have been Caucasian, and apoB-100 gene haplotype analysis using eight restriction fragment-length polymorphism markers and two length polymorphisms (hypervariable regions) suggested that the alleles of 91 subjects from 14 unrelated FDB kindreds were identical (9). Therefore, although this mutation occurred at a potentially hypermutable CpG site, it seems that these individuals shared a common ancestor (10).

A prevalence of 0.2% for FDB suggests that it might be one of the most common inherited disorders of blood cholesterol metabolism. Documentation of this prevalence in the general population would support a need for routine screening of hypercholesterolemic patients for FDB. For this reason, we undertook a screening project to estimate the prevalence of FDB in the general population. The population studied was ethnically diverse, affording an opportunity to identify FDB in non-Caucasian subjects.

METHODS

Recruitment of participants

The study was approved by the Committee on Human Research of the University of California, San Francisco. The population studied was the office work force of Wells Fargo Bank, N.A. Employees were offered free cholesterol determinations at their work sites in four metropolitan California areas (San Francisco, Los Angeles, San Diego, and Sacramento). Employees were notified by mailings and advertising at their work sites. Participation was voluntary. Informed consent was obtained before phlebotomy and the completion of a questionnaire that provided information about personal and family medical history.

Blood samples

A 10-ml sample of blood obtained under nonfasting conditions was placed on ice and delivered to the laboratory for analysis within 4 h after venipuncture. Samples were anticoagulated with potassium ethylenediamine tetraacetic acid, 1.5 mg/ml of blood.

Cholesterol determinations

The plasma was used for determination of cholesterol concentrations using the Abbott Spectrum high-performance diagnostic system with aqueous standards (New England Reagent Laboratory, East Providence, RI).

DNA analysis

Genomic DNA was prepared by the method of Higuchi (11) with the following modification. Instead of beginning with 0.5 ml of fresh whole blood and lysing the total cell mass, we began with frozen white blood cells from approximately 1 ml of whole blood. A segment of the apoB gene (nucleotides 10,799 to 10,823) was amplified by the polymerase chain reaction using the method of Hansen et al. (12). This produced a 140-base pair fragment that contained the codon 3500 mutation site, which was detected by digestion with the endonuclease *Msp*I. Haplotype analysis of the apoB gene was performed using 10 markers, as previously described by Ludwig and McCarthy (9).

Direct automated sequencing of apolipoprotein B PCR fragments (13, 14)

Genomic DNA was obtained from the white blood cells of the Chinese patient by phenol/chloroform extractions and ethanol precipitation. Oligonucleotide primers that flank the codon from amino acid 3500 were synthesized with biotin attached to the 5' end of the 3' amplification primer (biotin—GGCCACTTCCTGGCCAAGGTCAGG) and with the M 13 forward sequence on the 5' end of the 5' primer (TGTAAAACGACGGCCAGTGAGTCATC-TACCAAAGGAGATGTC). Symmetric PCR was performed using 20–60 pm of each primer and 1 μg of genomic DNA. The PCR fragment was attached to magnetic Dynabeads M-280 Streptavidin. The unbiotinylated strand was removed by alkaline denaturation, precipitated, and cycle-sequenced using fluorescently labeled dideooxynucleotides and the 3' amplification primer. The biotinylated strand attached directly to the magnetic streptavidin beads was directly sequenced using T7 polymerase and fluorescently labeled M 13 forward primer. The reactions were loaded onto an Applied Biosystems 373A automated sequencer and the sequence was determined.

Calculation of confidence intervals for prevalence estimate

The 90% confidence interval for the estimate of FDB prevalence was calculated by standard statistical methods as shown below (15):

$$CI_{90\%} = p \pm 1.645 \sqrt{p(1-p)/n}$$

CI, confidence interval; p, proportion of study sample affected by FDB; n, number of participants.

Lipoprotein isolation and fibroblast apoB,E (LDL) receptor binding studies

The control and normal LDL ($1.02 < d < 1.05$ g/ml) were isolated and the cell culture studies of LDL binding to fibroblast LDL receptors were performed by previously described methods (3).

TABLE 1. Ethnic composition and cholesterol concentrations of the study population

Ethnic Group	N	%	Mean Cholesterol Concentration (±SD)	Range	Percentile Values 90th	75th	50th	25th
Total	5160	100.0	193 ± 37	85-430	242	216	190	166
Caucasian	2859	55.4	192 ± 37					
Hispanic	614	11.9	189 ± 38					
Filipino	459	8.9	193 ± 36					
African-American	423	8.2	189 ± 37					
Chinese	315	6.1	192 ± 37					
Vietnamese	41	0.8	168 ± 37[a]					
Other Asian	294	5.7	192 ± 34					
Others	155	3.0	192 ± 36					

N, number of subjects.
[a] Significantly different from mean for all subjects ($P < 0.01$) as judged by Student's t test.

RESULTS

Of 14,058 eligible workers, 5,160, or 36.7%, volunteered to participate. The composition of the study population by ethnic group and gender and the distribution of cholesterol concentrations are shown in **Table 1.** There were no significant differences in the distribution of cholesterol concentrations among the ethnic groups listed in Table 1, except in the case of the Vietnamese participants (41 subjects), whose mean cholesterol concentration of 168 ± 32 mg/dl was significantly lower than the mean cholesterol concentration, 193 ± 37 mg/dl, of the entire group.

Four subjects were identified as having FDB (**Table 2**). One, subject A, was a native of Szechuan, China, and had immigrated to the United States 11 years before our study. Both of his parents were Chinese and he was not aware of any non-Chinese ancestors. The other three FDB subjects were Caucasians born in North America and were unaware of the precise European origins of their ancestors. Subjects B and C thought that some of their forbears were Scots-Irish and subject D believed that some of her ancestors were Germanic. Subject C had had a myocardial infarction at age 36, but coronary arteriography revealed findings typical of Kawasaki's arteritis as the cause of her coronary heart disease.

Based on our sample size of 5,160 subjects and the identification of four subjects with FDB, the prevalence of FDB can be estimated at 1 in 1,290 or 0.08%. Owing to the relatively small sample size, the 90% confidence interval for the prevalence of FDB ranged from 0.01% to 0.14%. The prevalence estimate of 1 in 1,290 was less than that suggested by previous studies (1). However, the ethnic composition of our sample varied from other studies, which involved predominantly Caucasian subjects. Only 55.4% of our subjects were Caucasians. When non-Caucasian subjects are excluded, then the estimate of FDB prevalence for this group becomes 3 in 2,859 (0.1%), which is substantially closer to previous estimates (1, 7, 8).

Haplotype analysis of the four FDB subjects for the 3' hypervariable element (HVE) revealed that three (subjects B, C, and D) of the four subjects possessed at least one 48-repeat allele. This result is consistent with these subjects having the 194 haplotype that previously has been established to be associated with this mutation (**Table 3**) (9). The fourth subject (subject A) displayed 30 and 34 3' HVE repeats, suggesting a new haplotype for the 3500 mutation. From analyses of the proband's son,

TABLE 2. Characteristics of patients with familial defective apolipoprotein B-100

Subject	Cholesterol Level	Gender	Age	Coronary Heart Disease	Family History Coronary Heart Disease[a]	Ethnicity	Birthplace
	mg/dl		*yr*				
A	282	M	40	no	father	Chinese	China
B	202	M	34	no	father	Caucasian	Louisiana
C	190[b]	F	38	yes[c]	none	Caucasian	Nova Scotia
D	251	F	47	no	father	Caucasian	California

[a] Below 60 years of age.
[b] Taking lovastatin and cholestyramine.
[c] Has Kawasaki's arteritis.

TABLE 3. Comparison of apoB haplotypes in subjects with familial defective apolipoprotein B-100

	Haplotype Marker[a]									
Haplotype[b]	5'$(TG)_n$	SP	*Apa*LI	*Hinc*II	*Pvu*II	*Alu*I	*Xba*I	*Msp*I	*Eco*RI	3'HVE[c]
Location	5' Flanking Region	SP E1	aa71 E4	– I4	– I4	aa591 E14	aa2248 E26	aa3611 E26	aa4154 E29	3' Flanking Region
194	14	+	+	–	–	–	–	+	–	48
195[d]	14	+	+	–	–	–	–	+	+	30

[a] 5' $(TG)_n$, number of times the dinucleotide TG is repeated; + and –, presence and absence of restriction site except SP + represents the presence of a 9-base pair insertion in the signal peptide region; 3' HVE, number of times the 15-base pair 3' HVE is repeated. Amino acid (aa), exon (E), intron (I).

[b] Haplotype nomenclature according to Ludwig and McCarthy (6) using the binary system for the eight diallelic markers.

[c] HVE, hypervariable element.

[d] Haplotype 195 is that of Subject A.

who does not carry the mutation, it was possible to deduce the haplotype of the proband's mutant allele (Table 3). With the exception of the 3' HVE, the proband was homozygous for the markers in Table 3. Because his son was homozygous for the HVE 34, the 3500 mutation in the proband can be assigned to the HVE 30 allele. Comparison of this haplotype with the 194 haplotype, previously associated with the 3500 mutation, revealed that the haplotype of the proband, in addition to the 3' HVE, differed by the presence of the *Eco*RI site, which is also near the 3' end of the apoB gene (Table 3). All other markers of Subject A were identical to those of the 194 haplotype. Sequence analysis of the proband's affected allele revealed a CGG→CAG mutation in the codon of amino acid 3500 as has been observed in the 194 haplotype of all other FDB patients. Although it might be informative to study the patient's extended family to aid in determining whether the new haplotype occurred due to a crossover during meiosis, it is not possible to do so because most of his family remains in China. His mother has been studied and does not carry the mutation.

It was of interest to determine that the Chinese patient's LDL exhibited reduced binding to the LDL receptor. Substantially reduced binding of the patient's LDL was observed (**Fig. 1**) even though his unfractionated LDL (the preparation contained both normal and defective LDL since the patient is heterozygous for the FDB mutation) were used.

DISCUSSION

The sample size of 5,160, or 36.7% of eligible employees, represented a reasonable participation rate in our study. Participation of only one-third of those eligible introduces the possibility that self-selection may have occurred, leading to an increased proportion of persons with hyperlipidemia. However, as the mean cholesterol concentration of 193 mg/dl and the 75th percentile value of 216 mg/dl are well below national norms, it is unlikely that our population contained an unusual proportion of hypercholesterolemic persons seeking to have their cholesterol levels remeasured (16). The proportion (69%) of women participants in the subject group was somewhat greater than the proportion (59%) of women of the entire work force.

Previous estimates of FDB prevalence have been based upon studies of hypercholesterolemic patients attending lipid clinics. All of these patients have been Caucasian. In this study we screened over 5,000 individuals who were ethnically diverse and were chosen to be representative of the general population. The prevalence estimate of 0.08%

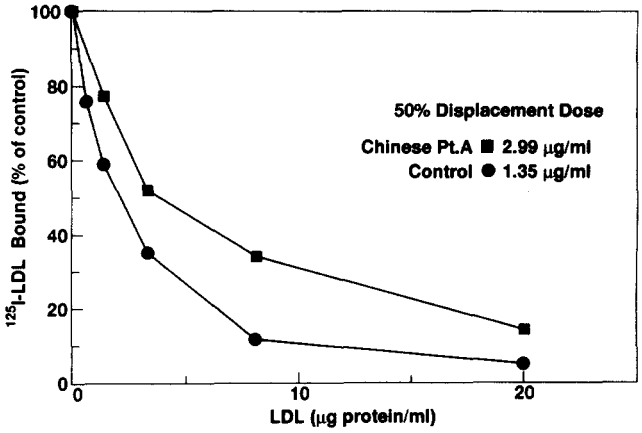

Fig. 1. Ability of unlabeled LDL from a normal control subject and from the Chinese patient A to compete with ^{125}I-labeled LDL for binding to apoB,E(LDL) receptors on normal human fibroblasts. One millimeter of Dulbecco's modified Eagle's medium containing 10% lipoprotein-deficient serum, 2 μg of normal ^{125}I-labeled LDL protein, and the indicated concentrations of unlabeled Chinese Pt.A LDL or normal LDL were added to 35-mm culture dishes containing normal human fibroblasts. After a 2 h incubation at 4°C, the amount of ^{125}I-labeled LDL bound was measured. The 100% control value was 151 ng/mg of cellular protein.

in our ethnically diverse population is less than that suggested previously (1, 7, 8). However, when the 44.6% of our study subjects who were not Caucasian are excluded, the prevalence is 0.1%, a value compatible with earlier estimates.

The cholesterol concentrations of subjects A (282 mg/dl) and D (251 mg/dl) were above the 90th percentile value of 242 mg/dl for all persons in our study. Their concentrations are compatible with the moderately elevated values previously described for FDB patients (1, 6, 7, 8). Subject B, who had a total cholesterol of 202 mg/dl, was interviewed by one (T.P.B.) of us and was found to be eating a vegetarian diet with the exception of fish twice weekly. He adopted this diet after he learned that he had an elevated cholesterol level (approximately 280 mg/dl), at the time of onset of his father's coronary heart disease. The cholesterol level, 190 mg/dl, of subject C was reduced because of her use of hypolipodemic drugs (Table 2).

The screening technique using the artificially created *Msp*I site could not discriminate between the two possible codon 3500 mutations (CGG→CAG or CGG→CAA) that would result in the glutamine-for-arginine substitution characteristic of FDB. For this reason the Chinese patient's mutant apoB-100 allele was sequenced in the region of codon 3500 and the sequence was determined to be CAG. Since the mutation (Arg→Gln) was the same as in other FDB patients (4), it was expected that the Chinese patient's LDL, a mixture of normal and mutant LDL, would exhibit reduced binding to fibroblast LDL receptors. As shown in Fig. 1, this was the case since the amount of the patient's LDL was more than twofold greater than the amount of normal LDL required to cause a 50% reduction in ^{125}I-labeled LDL binding to fibroblast LDL receptors.

An interesting question arises from the observation that the apoB haplotype in one of the four FDB subjects identified in this study differs from the haplotype previously established for this mutation. It was previously concluded that all FDB mutations, which were drawn from North American and European populations (predominantly Caucasian in origin), were derived from a single common ancestral gene (6, 10, 17). Although the arginine 3500 codon contains a hypermutable CG dinucleotide, the data did not indicate that recurrent mutations in other apoB haplotypes had occurred, despite the relatively common occurrence of FDB. Finding a subject of Chinese ancestry having a unique haplotype, designated 195 according to the nomenclature of Ludwig and McCarthy (9), has several possible explanations. First, the 195 haplotype may have arisen from the 194 haplotype by recombination of the extreme 3' end of the apoB gene. Arguing against this possibility is the fact that similar recombination events have not been observed previously in the apoB gene (10). A second possibility is that the 3500 mutation might have arisen from an independent mutation in the 195 haplotype at the potentially hypermutable CG dinucleotide within the arginine 3500 codon. In support of this hypothesis is the observation that mutations at the same site in different haplotypes have been detected in other disorders. For example, in phenylketonuria there are four examples of identical mutations occurring in more than one phenylalanine hydroxylase gene haplotype, and in familial hypercholesterolemia there is one example of an identical mutation occurring in two distinct LDL receptor gene haplotypes (18, 19). Several of these mutations also involve a CpG dinucleotide. Finally, it is also possible that the 3500 mutation antedates diversification of the apoB haplotypes.

Regardless of the explanation for the different haplotype of subject A, the discovery of this mutation in a subject of Chinese ancestry represents the first exception to occurrence of the mutation in subjects of Western European origin. A larger sample of Chinese subjects should be explored for this mutation and the apoB mutation carrying it.

We thank the employees of Wells Fargo Bank, N.A., for participating in the study and Ronald Gristock, Nancy Stent, and Janice Wright of the Personnel Division for their help in implementing the study at the Bank. We also thank Stanley Rall, Jr., Brian McCarthy, and Beatriz Levy-Wilson for reviewing the manuscript. Support was provided by the L. K. Whittier Foundation, South Pasadena, CA, and by NIH Program Project Grant HL41633.

Manuscript received 31 July 1992 and in revised form 1 March 1993.

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