Known mutations of apoB account for only a small minority of hypobetalipoproteinemia

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Abstract **Low LDL cholesterol and apoB levels in plasma cosegregate with mutations of apoB in some kindreds with familial hypobetalipoproteinemia. Approximately 35 apoB mutations, many specifying apoB truncations, have been described. Based on the centile nomenclature where the full-length nature apoB consisting of 4536 amino acids is designated as apoB-100, only those truncations of apoB >25% of normal length are detectable in plasma. Previously, we reported on five unrelated kindreds with familial hypobetalipoproteinemia in whom although no apoB truncations were detectable in plasma, low apoB levels were nevertheless linked to the apoB gene. In one of those kindreds, we reported a donor splice site mutation in intron 5 (specifying apoB-4). We now describe a nonsense mutation in exon 10 (apoB-9) in two of the other unrelated families. Both the apoB-4 and apoB-9 mutations have been reported by others in unrelated families. Recurrent mutations of apoB-40 and apoB-55 also have been reported, suggesting that recurrent mutations of apoB may account for an appreciable proportion of familial hypobetalipoproteinemia kindreds. To test this hypothesis, we searched for four apoB mutations whose products are not detected in plasma including the apoB-4, apoB-9, and two other previously reported mutations in exons 21 and 25. We studied three groups with plasma cholesterols <130 mg/dl in whom no apoB truncations were detected in plasma: *a*) 28 FHBL probands from St. Louis, *b*) 151 individual St. Louisians, and *c*) 28 individual Sicilians. One subject from the 28 kindreds and two subjects among 151 hypobeta individuals from St. Louis harbored the exon 10 mutation. None of the other mutations were detected. Thus, among hypobeta lipoproteinemic subjects without any detectable apoB truncations in plasma, <5% had an apoB truncation-producing mutation. As only about 0.5% of hypobeta lipoproteinemic subjects have plasma-detectable apoB truncations, our data suggest that the known apoB truncations account for only a small proportion of hypocholesterolemia.**—Wu, J., J. Kim, Q. Li, P-Y. Kwok, T. G. Cole, B. Cefalu, M. Averna, and G. Schonfeld. **Known mutations of apoB account for only a small minority of hypobetalipoproteinemia.** ***J. Lipid Res.*** **1999.** 40: **955–959.**

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Familial hypobetalipoproteinemia (FHBL) is a genetic variant of lipid metabolism defined by <5th percentile levels of apolipoprotein B (apoB) and low density lipoprotein (LDL) cholesterol segregating as an autosomal dominant (1–3). ApoB gene defects that specify null alleles or truncated apoB proteins of various lengths have been reported in about 35 FHBL kindreds (1, 3–6). The truncations are designated according to a centile nomenclature (7), with the full-length apoB consisting of 4536 amino acids defined as apoB-100. Truncations corresponding to apoB-25 or longer are detectable in plasma, truncations <apoB-25 are not (5–16). The prevalence of plasma detectable apoB mutations among hypobetalipoproteinemia individuals is approximately 1/200 (2, 3, 16). We previously reported six FHBL kindreds in whom no detectable apoB truncations were detected in plasma (17). We found linkage between low apoB plasma levels and the apoB locus on chromosome 2, using internal and flanking markers of the apoB gene in five of the six kindreds. Linkage to the apoB gene was ruled out in the sixth kindred (17). Previously, we reported an apoB-4 mutation in one of the five kindreds (18). We now report on an apoB-9 defect in two of the other kindreds. Others have reported these same mutations in unrelated families (19, 20). Recurrent mutations apoB-40 (16, and G. Schonfeld, R. Neuman, and B. Yuan, unpublished observations) and apoB-55 (21, 22) also have been reported, suggesting that an appreciable proportion of hypobetalipoproteinemia may be due to mutations of the apoB gene. To test this hypothesis, we estimated the prevalence of the known apoB mutations among low cholesterol individuals and kindreds. We used 151 individuals from St. Louis and 28 from Palermo with total plasma cholesterols <130 mg/dl). In addition, we studied 28 St. Louis kindreds containing 5–8 individuals in whom at least two affected individuals with apoB levels

Abbreviations: apo, apolipoprotein; FHBL, familial hypobetalipoproteinemia; PCR, polymerase chain reaction; SSCP, single strand conformational polymorphism.

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TABLE 1. Screening strategy for the four apoB mutations predicting nondetectable apoBs in plasma

Mutation	Primers[a]	Experimental Manipulation	Expected Product/Pattern	
			Normal (bp)	Abnormal (bp)
Intron 5	14579/DetIn5-1	Primer DetIn5-1 is mutant specific	No amplification	218
Exon 10	exon10-5/exon10-3	PCR + PvuII digestion mutation creates a novel PvuII site	203 + 111 (2 fragments)	203 + 111 + 76 + 35 (4 fragments)
Exon 21	del21-5/del21-3	PCR mutant allele has a 694 bp deletion	1190	495
Exon 25	exon25-5/exon25-3	PCR + XhoI digestion mutation destroys a XhoI site	432	182 + 250 (2 fragments)

[a] Primers used are as follows: 14579/DetIn5-1: GATAAGGACATTTCCGTGACCAT/TCAGCTTTCTAAATCCT CAA; exon10-5/exon10-3: TATGTGCCCTGAAGGTGGTCTG/GACGAGGATCCTCCTCTTTA; del21-5/del21-3: AGCACATTGATTCCTTGGAC/CCCAGCACTGTGCTTGATAC; exon25-5/exon25-3: GGGATGTTATCTTTGTTG TTC/CGGTGCACCCTTTACCTGAGC.

<5th percentile were present. None of the cases had detectable apoB truncations in plasma.

MATERIALS AND METHODS

Subjects and plasma analysis

Over the past 9 years we have screened 2157 volunteers for potential FHBL probands at our Lipid Research Clinic and at various non-clinic sites, using a total plasma cholesterol of 150 mg/dL as the initial cut point (90–95th percentile, ref. 23). The volunteers were obtained by offering a free cholesterol screen in various communications media. Plasma cholesterol was measured by enzymatic methods (Wako Kit), and apoB levels by immunoturbidometory (24). Also studied was a sample of hypobetalipoproteinemric individuals obtained from a blood bank in Palermo, Sicily (Italy). Truncations of apoB detectable in plasma were identified by immunoblotting (10), using monoclonal anti-apoB antibody C1.4 which is directed against an N-terminal epitope of apoB (25). Only those subjects without a detectable plasma in apoB truncation were included in this study. The Washington University Human Studies Committee approved our protocol.

Southern blot analysis of the apoB gene

Genomic DNAs from two hypobetalipoproteinemic individuals and two normal spouses from each of the five kindreds were extracted from whole blood using the Purogene kit (Gentra Systems, Minneapolis, MN). Ten μg of DNA was digested by EcoRI and separated on agarose gel and transferred by Southern blot techniques. The full-length apoB cDNA clone pB100LII (26) was cleaved by restriction enzyme digestion with NotI, EcoRV, and BamHI, yielding four fragments: *a*) 2.6 kb BamHI/NotI, *b*) 8.6 kb (EcoRV), *c*) 3.2 kb (EcoRV), and *d*) 3.6 kb (EcoRV). Each of the fragments was used individually to probe the EcoRI-digested genomic DNAs. Protocols for restriction endonuclease digestion, agarose gel electrophoresis, blotting to nylon membrane, hybridization with ^{32}P-labeled probe and autoradiography were as described previously (27).

Single strand conformational polymorphism (SSCP) analysis

All apoB exons, except exons 26 and 29, were amplified by PCR, giving products sizes ranging from 180 to 400 base pairs. The PCR was done in 10 μl volumes containing 50 μm KCl; 10 μm Tris, pH 8.0; 1.5 μm $MgCl_2$; 200 μm each of dCTP, dGTP, and dTTP; 25 μm dATP; 0.2 μCi α^{35}S-dATP; and 0.5 units of AmpliTaq (Perkin Elmer). Amplification was performed at 94°C for 30 s, 56–60°C for 30 s, and 72°C for 30 s, for 35 cycles. The samples were then diluted with one volume of SSCP buffer (0.2 m NaOH, 1% SDS) and three volumes of loading buffer (95% formamide, 15 mm EDTA, 0.03% each of xylene cyanol and bromophenol blue), denatured at 95°C for 5 min, and 10 μl was loaded. Electrophoresis was performed in 6% non-denaturing polyacrylamide gels at room temperature for ~20 h at 10 W. Gels were then dried and exposed to X-ray films for 1–5 days.

Nucleotide sequencing of SSCP positive regions

When SSCP revealed a different pattern in both affected individuals as compared to the unaffected spouses, direct nucleotide sequencing was performed. Polymerase chain reaction (PCR)-amplified fragments were separated on a 1.5% TAE agarose gel, DNA bands of interest were cut out, and the fragments were purified using the Wizard PCR Preps kit (Promega, Madison, WI). Direct nucleotide sequencing was done on ~1 μg of purified DNA using the automatic ABI377 Sequencer, using the same primers as those used in the PCR reactions.

Mutation screening

Screening for the four plasma non-detectable truncation mutations was based on PCR methodology and protocols are detailed in **Table 1**. DNA sequence differences between mutant and wild-type alleles, manifesting as alterations either in a restriction enzyme recognition site or in the size of the amplified fragment were used to differentiate between normal and mutant genotypes.

RESULTS

The mean lipid values for the study subjects are similar to the TC and LDL-C levels reported for individuals with familial hypobetalipoproteinemia (**Table 2**).

TABLE 2. Plasma lipoprotein lipids of the study populations

Population	n	TG	TC	LDL-C	HDL-C
St. Louis individuals	151	80 ± 65	113 ± 16	53 ± 15	44 ± 12
Palermo individuals	28	57 ± 33	113 ± 17	62 ± 15	39 ± 11
St. Louis probands	28	89 ± 71	120 ± 19	53 ± 14	48 ± 12

Results (in mg/dl) are means ± 1 SD. TG, triglycerides; TC, total cholesterol; LDL-C and HDL-C, low and high density lipoprotein cholesterol, respectively.

TABLE 3. ApoB genetic sequence variants in five FHBL kindreds with no detectable apoB truncation in plasma

Exon	Kindred	Variants	Predicted a.a. Change	Comment
4[a]	T	ACC-ATC	Thr-Ile	Missense mutation[b]
10[a]	De & C	CTA-TGA	Arg-Term	Nonsense mutation[b]
18	Z	AAC-AAT	nil	Silent mutation
26	De	GGC-GGT	nil	Silent mutation[c]

The De family is of Italian origin and the C family of English origin.
[a] Mutation previously reported (8).
[b] Mutations segregating with low apoB levels.
[c] Mutations not segregating with low apoB levels.

TABLE 4. Results of screening for the four plasma non-detectable apoB truncation mutations

Mutation	Hypobeta Subjects		St. Louis Small Hypobeta Families
	St. Louis Sporadic Cases	Italian Cases	
Intron 5	0/151	0/28	0/28
Exon 10	2/151	0/28	1/28
Exon 21	0/151	0/28	0/28
Exon 25	0/151	0/28	0/28

The two sporadic cases are of German and Slavic (Russian-Ukranian) origins. The family member is of Polish origin.

Exclusions of gross deletions of the apoB gene in the D, De, C, T, and Z kindreds

As the first step in searching for mutations, we carried out Southern blot analysis. No differences were noted in hybridization patterns, detectable either as a reduction in hybridization signal intensity or as an alteration in band sizes (not shown).

Identification of apoB variants

SSCP analysis revealed variant patterns in exons 1, 4, 10, 12, 18 and 26 (**Table 3**). However, the variant in exon 1 was not present in both affected members of the T kindred and was present in one of the unaffected spouses, suggesting no connection to the FHBL trait. On the other hand, variants in exons 4, 10, 18, and 26 were present in both of the tested affected members of the kindreds as indicated, but absent in Table 3 from the two unaffected spouses. The variants therefore represented possible mutations (Table 3). The exon sequences corresponding to these SSCP variants were PCR amplified and subjected to direct nucleotide sequencing. The variants in exons 4 and 10 predicted a missense and nonsense mutation, respectively. Variants in exons 18 and 26 predicted silent mutations. The exon 10, nonsense mutation (Arg_{412}–Term) was present in both the De and C kindreds (**Fig. 1**). The mutation predicts a truncated apoB (apoB-9) too small to be detectable in plasma. The affected members in these two kindreds possess different apoB haplotypes (17), suggesting the kindreds are not related. We have not yet found the genetic defects in the T and Z kindreds.

Prevalence of the splice-donor site mutations in hypobetalipoproteinemic individuals

One of the 28 FHBL families had the exon 10 mutation (**Table 4**). The exon 10 mutation was also present in two of the 151 St. Louis individuals. None of the four mutations was found in the 28 Sicilian individuals.

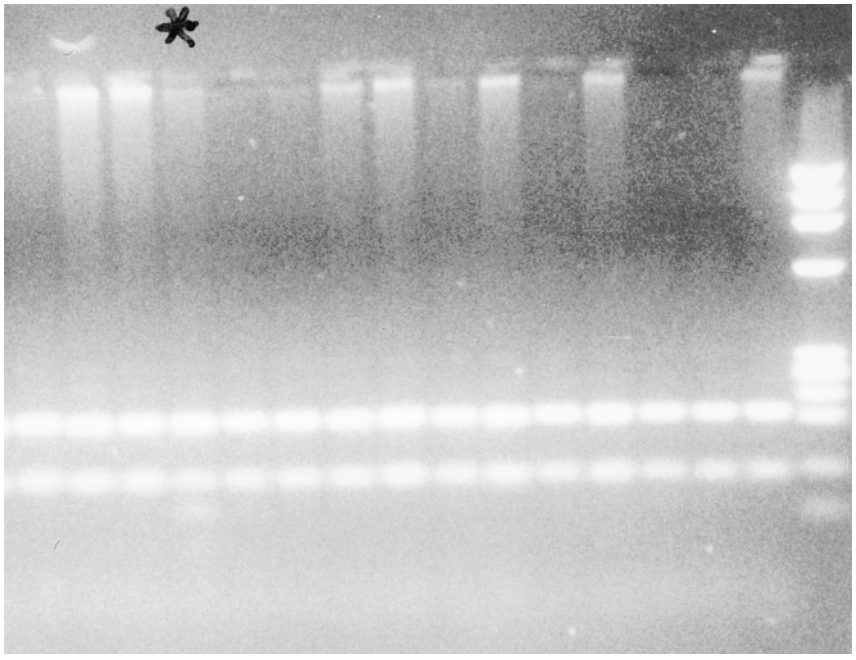

Fig. 1. PCR/restriction enzyme digestion screening for exon 10 mutation. The wild-type PCR product (314 bp) were cut into two fragments (203 and 111 bp) by PvuII digestion, while in mutant the 111 bp fragment was further broken down into two smaller fragments (76 and 35 bp). Therefore, the heterozygote for the exon 10 mutation (the lane with *) has all the four fragments (203, 111, 76, and 35 bp).

DISCUSSION

In well characterized kindreds, FHBL is known to be associated with ~35 different mutations of the apoB gene (1, 2, 6), most of which specify apoB truncations of various lengths. Only those truncations longer than apoB-75 can be detected in plasma (5–16). The genetic bases of most cases of hypobetalipoproteinemia are not known. We previously reported five FHBL kindreds in which linkage was present between low levels of plasma apoB and markers of the apoB locus (17). One of those kindreds has an intron 5 donor splice site mutation (18). We now report identical nonsense mutations in exon 10 in two more of the 5 kindreds, C and De, that differ in apoB haplotype and hence are not related. Both the exon 10 (19, 20) and the intron 5 (8) mutations have been reported by others in unrelated kindreds. Neither of these products of these mutations are detected in plasma. Recurrent apoB truncation mutations detectable in plasma also have been reported in unrelated kindreds, e.g., apoB-55, (21, 22) and apoB-40 (16 and G. Schonfeld, R. Neuman, and B. Yuan, unpublished results). We tested the hypothesis that the recurrent apoB truncation mutations not detectable in plasma could be responsible for an appreciable proportion of FHBL in individuals and families where no apoB truncation was detectable in plasma.

We found mutations in 1 of 28 kindreds (~3%) and in 2 of 179 (~1%), implying that plasma non-detectable truncations do not account for most of the low cholesterol in individuals and kindreds with no detectable apoB truncations. In addition, during the last 9 years, we have screened 2157 individuals with total cholesterols <150 mg/dl for apoB truncation mutations by immunoblotting of plasma, and found nine probands with detectable truncations for a prevalence of 0.5% (3). Our numerical estimates are subject to some error because the base population consists of volunteers, and because we report only on mutations identified to date. Also, we may have missed some possible mutations due to silent mutations that may produce exon skipping (28). Nevertheless, it is likely that only a small minority of familial or "sporadic" cases of hypocholesterolemia is due to any of the >35 known truncation mutations of apoB.

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