

Hypobetalipoproteinemia with an apparently recessive inheritance due to a “de novo” mutation of apolipoprotein B

Sandra Lancellotti^{a,1}, Enza Di Leo^{a,1}, Junia Y. Penacchioni^a, Fiorella Balli^b, Laura Viola^b, Stefano Bertolini^c, Sebastiano Calandra^a, Patrizia Tarugi^{a,*}

^aDepartment of Biomedical Sciences, University of Modena and Reggio Emilia, Via Campi 287, I-41100 Modena, Italy

^bDepartment of Pediatrics, University of Modena and Reggio Emilia, Via Campi 287, I-41100 Modena, Italy

^cDepartment of Internal Medicine, University of Genoa, Viale Benedetto XV, I-16132 Genoa, Italy

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Abstract

Familial hypobetalipoproteinemia (FHBL) is a co-dominant disorder either linked or not linked to apolipoprotein (apo) B gene. Abetalipoproteinemia (ABL) is a recessive disorder due to mutations of microsomal triglyceride transfer protein (MTP) gene. We investigated a patient with apparently recessive hypobetalipoproteinemia consistent with symptomatic heterozygous FHBL or a “mild” form of ABL. The proband had fatty liver associated with LDL-cholesterol (LDL-C) and apo B levels <5th percentile but no truncated apo B forms detectable in plasma. MTP gene sequence revealed that he was a carrier of the I128T polymorphism and an unreported amino acid substitution (V168I) unlikely to be the cause of hypobetalipoproteinemia. Apo B gene sequence showed that he was heterozygous for two single base substitutions in exon 9 and 22 resulting in a nonsense (Q294X) and a missense (R1101H) mutation, respectively. Neither of his parents carried the Q294X; his father and paternal grandmother carried the R1101H mutation. Analysis of polymorphic genetic markers excluded non-paternity. In conclusion, the proband has a “de novo” mutation of apo B gene resulting in a short truncated apo B form (apo B-6.46). Sporadic cases of FHBL with an apparently recessive transmission may be caused by “de novo” mutations of apo B gene.
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1. Introduction

Familial hypobetalipoproteinemia (FHBL) is a “relatively” frequent genetic disorder characterized by plasma levels of total cholesterol (TC), LDL-cholesterol (LDL-C) and apolipoprotein B (apo B) below the 5th percentile of the distribution in the general population. FHBL segregates as an autosomal co-dominant trait [1]. The majority of FHBL subjects are simple heterozygotes who are asymptomatic or mildly symptomatic; a large proportion of them have non-alcoholic fatty liver and a few may have diarrhea and intestinal fat malabsorption [1–5]. FHBL appears to be genetically heterogeneous; it may be linked or not linked to the apo B gene [5]. The best characterized FHBL cases are

those due to nonsense/frameshift mutations of the apo B gene which prevent the complete translation of apo B mRNA leading to the production of truncated forms of apo B. The size of truncated apo Bs, those identified so far, varies from apo B-2 to apo B-89; truncated apo Bs shorter than apo B-27/B-29 are not detectable in plasma as they are not secreted as constituents of lipoproteins [1,5]. Recently a missense mutation in apo B (R463W) was found to be the cause of FHBL in a large kindred [6]. The apo B harbouring R463W substitution is not secreted as it is retained in the endoplasmic reticulum [6]. In other FHBL families, where the apo B gene has been excluded, linkage to a susceptibility locus on chromosome 3p21 has been shown [7–9]. In some FHBL families, linkage to both apo B and chromosome 3 locus (3p21) is absent [5]. In all FHBL cases, regardless of the gene/locus involved, FHBL lipoprotein phenotype co-segregates as a co-dominant trait [5].

Abetalipoproteinemia (ABL) is an “exceedingly rare” disorder characterised by extremely low levels of apo B-

* Corresponding author. Tel.: +39-059-2055-416; fax: +39-059-2055-426.

E-mail address: tarugi@unimore.it (P. Tarugi).

¹ Both authors contributed equally to the results of this work.

containing lipoproteins. From the clinical standpoint, ABL is more severe than symptomatic heterozygous FHBL; ABL patients usually have steatorrhea, failure to thrive and variable neurological manifestations [1]. ABL is due to a deficiency of the microsomal triglyceride transfer protein (MTP), which is required for the assembly of apo B-containing lipoproteins in the liver and intestine [10]. It is conceivable that the severity of ABL phenotype is related to the residual activity of MTP and the capacity to form chylomicrons and VLDL. ABL has an autosomal recessive transmission; obligate heterozygotes appear to have a normal lipid profile [1].

In this study we report a patient with primary hypobeta-lipoproteinemia which appeared to be inherited as a recessive trait suggesting the diagnosis of a “mild” ABL phenotype. In fact the patient was found to have heterozygous FHBL due to a “de novo” mutation in apo B gene, predicted to produce a short truncated apo B (apo B-6.46) not detectable in plasma.

2. Subjects and methods

2.1. Clinical data

2.1.1. S.M. kindred

The proband S.M. (subject III-1 in Fig. 1) was a 12-year-old boy born from non-consanguineous parents. He had a normal growth. He was referred to the hospital at 11, for the presence of hepatomegaly, a mild elevation of serum transaminases, and a mild steatorrhea (fecal fat = 10–15 g/24 h) associated with low TC (102 mg/dl), triglycerides (27 mg/dl) and apo B (<30 mg/dl). Liver ultrasound examination revealed the presence of fatty liver. These clinical features associated with low plasma lipids at that time suggested the diagnosis of a “mild” form of ABL. Intestinal biopsy revealed normal intestinal mucosa with no lipid infiltration of the enterocytes. At the time of the observation (1998) the proband was slightly overweight (BMI = 24.3 kg/m²) and had mild hepatomegaly. Neurological examination, including retinal fundus, was negative. Routine blood chemistry

(with the exception of serum lipids and transaminases) including HBsAg, HBcAb, HCV were within the normal range or negative. Liver biopsy showed the presence of steatosis in 40% of the hepatocytes (macro/micro fat droplets), but no signs of inflammation, necrosis and iron deposits.

Proband's parents were healthy with a negative family history for gastrointestinal and liver disorders or plasma lipid abnormalities. Proband's grandparents were alive and in good health. The proband was treated with a low fat, high carbohydrate diet supplemented with fat-soluble vitamins.

Proband's pedigree spans three generations and includes seven living members (Fig. 1). All members of the kindred available for the study were subjected to the evaluation of the lipid profile and to the analysis of MTP and apo B genes for the search of the sequence variants found in the proband. Normal subjects were selected among laboratory staff and medical students. All gave their informed consent for the study. The study protocol was approved by the institutional human investigation committee of each participating institution.

2.1.2. Analysis of plasma lipids and lipoproteins

Blood was collected after overnight fasting, unless otherwise specified. Plasma TC, TG, direct HDL-C, direct LDL-C, apo A-I and apo B were measured enzymatically using Roche Diagnostic GmbH reagents on a Hitachi 912 analyzer. Plasma lipoproteins were separated by continuous density gradient ultracentrifugation [4].

2.1.3. Apolipoprotein analysis

Aliquots (15–50 µg of protein) of lipoprotein fractions were precipitated in 10% trichloroacetic acid and extracted with ethanol–diethyl ether 3:2 (v/v). For the analysis of apo B, the samples were separated by a linear (3.5/5–10%) gradient sodium dodecyl sulfate (SDS)-PAGE [4]. For the analysis of the other apolipoproteins, the samples were separated by a linear 5–20% gradient SDS-PAGE [4].

2.1.4. Apo B immunoblot analysis

Aliquots of total plasma and plasma lipoproteins (50–60 µg of protein) were delipidated and separated by linear 3.5/5–10% or 5–20% gradient SDS-PAGE [4]. For immunoblotting, apolipoproteins were electro-transferred from the gel to a Zeta Probe membrane (BioRad Laboratories, Richmond, CA). The membranes were incubated with anti-human apo B-100 sheep polyclonal antiserum, as previously described [4].

2.1.5. Analysis of MTP gene

Genomic DNA was isolated from whole blood (NucleoSpin Blood L, Macherey-Nagel, Duren, Germany). The entire MTP coding region, including the 5'-flanking region, all exons and at least 50 bp of intronic sequence at each

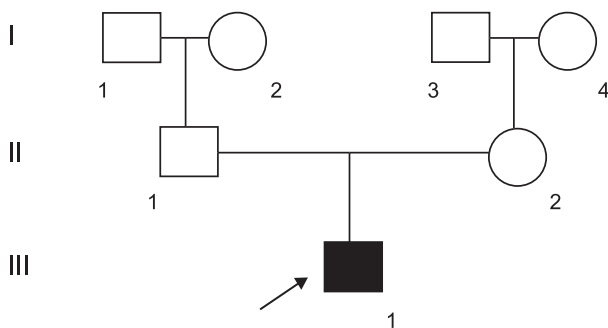


Fig. 1. Pedigree of the S.M. family. The proband is indicated by an arrow.

Table 1
Plasma lipids and lipoproteins in S.M. family

Subjects	Age (years)	TC	TG	LDL-C	HDL-C	Apo B	Apo A-I	Apo E genotype
Proband (III-1)	12	88	18	24	61	18	153	$\epsilon 3\epsilon 3$
Father (II-1)	47	170	94	97	59	62	143	$\epsilon 3\epsilon 3$
Mother (II-2)	40	227	78	148	67	84	194	$\epsilon 3\epsilon 3$
Paternal grandfather (I-1)	79	207	60	149	46	105	131	nd
Paternal grandmother (I-2)	78	274	73	150	110	91	263	nd
Maternal grandfather (I-3)	73	224	121	142	58	105	156	nd
Maternal grandmother (I-4)	69	222	67	139	69	96	165	nd

nd, not determined. Lipid and apolipoprotein concentrations are expressed as mg/dl.

intron–exon boundary, were amplified using polymerase chain reaction (PCR). The sequence of the primer pairs for PCR amplification was kindly provided by Dr. J. Wetterau (Bristol-Myers Squibb, Pharmaceutical Research Institute, Princeton, USA). For all PCR amplifications, 0.1 μg of genomic DNA was added to 25- μl mixture containing 0.2 mmol/l of each deoxynucleoside triphosphate, 25 pmol of each primer and 1.5-U Taq DNA polymerase (Expand High Fidelity, Roche Diagnostics GmbH, Germany) in PCR buffer (50 mmol/l KCl, 10 mmol/l Tris-HCl, pH 9, and 1.5 mmol/l MgCl_2). The amplification conditions were: 95 °C for 5 min followed by 30 cycles at 95 °C for 1 min/51 °C for 1 min/68 °C for 1.5 min with a final elongation at 72 °C for 5 min. The amplification products were purified with High Pure PCR purification kit (Roche Diagnostics), analysed by 2% agarose gel electrophoresis and directly sequenced in two directions using the amplification primers, with BigDye Terminator Cycle sequencing Kit (PE, Applied Biosystems, Warrington, UK). Sequences were detected on an Applied Biosystems 3100 DNA sequencer and results were analysed with ABI PRISM SeqScape software (Applied Biosystems). Sequence variants found in automated sequencing were always checked by using a second independent amplifica-

tion of the affected DNA region and resequencing in both directions or, when possible, by restriction enzyme digestion (see below).

2.1.6. Analysis of apo B gene

Since no truncated apo B forms were detected in plasma lipoproteins, the whole apo B gene was analysed as described previously [4]. Sequence analysis was performed as indicated above.

2.1.7. Screening of the MTP and apo B gene mutations

The screening of family members for the MTP and apo B gene variants found in the proband (see results) was performed by automated sequencing of PCR-amplified exons harbouring these variants (see above). The screening of the sequence variant (V168I) of MTP gene was performed by PCR amplification of exon 5 followed by digestion with the restriction enzyme BsaBI.

2.1.8. Complementary genetic analysis

Several polymorphisms of genes involved in lipid metabolism [apo E ($\epsilon 2/\epsilon 3/\epsilon 4$); CETP (B1/B2); FABP-2 (54 A/T); apo A-V (-1131T/C); HL (-250G/A); ABCA1 (R219K)] were examined [11–16]. In addition, multiplex reactions

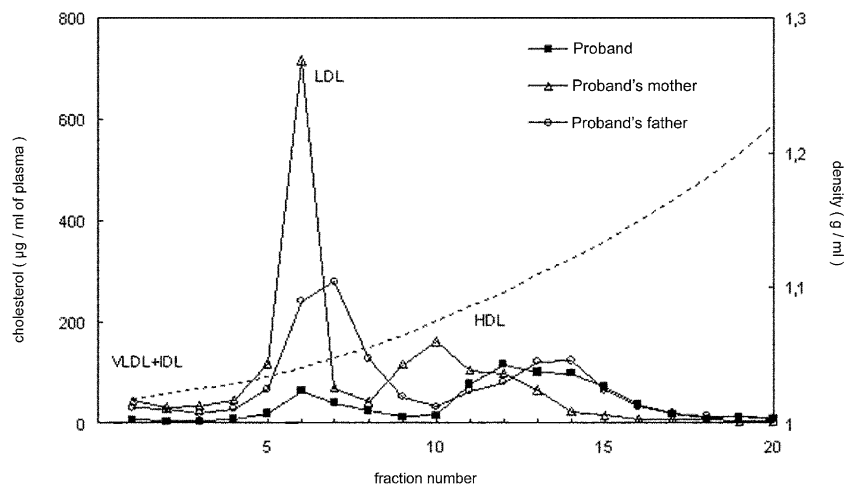


Fig. 2. Density profile of plasma lipoproteins. Plasma lipoproteins were separated by density gradient ultracentrifugation from the proband and his parents. VLDL + IDL (fractions 1–3); LDL (fractions 4–9); HDL (fractions 10–18).

Table 2
Sequence variants of MTP gene in S.M. family

	Sequence variant	Proband	Mother	Father
Promoter	– 493 G/T	G/T	G/T	G/T
Promoter	– 400 A/T	A/T	A/T	A/T
Promoter	– 164 T/C	T/C	T/C	T/C
Exon 3	c.383 T/C (I128T)	T/C	T/C	T/C
Exon 5	c.502 G/A (V168I)	G/A	G/G	G/A
Exon 7	c.891 C/G (H297Q)	C/C	C/C	C/G

were performed for paternity testing as specified in our previous reports [17,18].

3. Results

3.1. S.M. kindred

Plasma levels of TC and LDL-C of proband S.M. (Table 1) were below the 5th percentile for age and sex (TC = 131.3 mg/dl; LDL-C = 63.8 mg/dl). The plasma levels of LDL-C and apo B found in the proband were similar to those we observed in FHBL heterozygotes for a short truncated form of apo B (apo B-8.15) [4]. Plasma TC and LDL-C levels of proband's father (Table 1) were below the mean values found in age- and sex-matched controls (TC = 214.4 ± 39.2 mg/dl; LDL-C = 140.9 ± 33.5 mg/dl) but above the 5th percentile of the general population. The plasma levels of TC, LDL-C and apo B found in proband's mother (Table 1) were within the control range (TC = 205.5 ± 33.2 mg/dl; LDL-C = 130.3 ± 32.1 mg/dl; apo B = 120.0 ± 23.8 mg/dl).

The density profile of plasma lipoproteins isolated from the proband (Fig. 2) was characterised by a marked reduction of the LDL peak (fractions 4–9, $d = 1.035–1.060$ g/ml), similar to that found in FHBL heterozygotes of our series [2–4]. The LDL peak of proband's father was intermediate between that of the proband and that observed in proband's mother (Fig. 2).

The apo B present in each lipoprotein density fraction was analysed by SDS-PAGE (5–10% and 5–20% polyacrylamide gradient gels). In the proband, the only detectable forms of apo B were apo B-48 and apo B-100 (data not shown). Immunoblot of 5–10% and 5–20% polyacrylamide gradient gels of lipoprotein fractions and lipoprotein infranate failed to show truncated forms of apo B (data not shown).

In view of these findings, we suspected that the proband might have a “mild” form of ABL. For this reason we sequenced the MTP gene first.

3.2. Analysis of MTP gene

The sequence of MTP gene (Table 2) showed that the proband was heterozygous for two nucleotide substitutions in the coding region that result in amino acid changes: (1) c.383 T>C transition in exon 3 (I128T), a previously re-

ported rare allele of a common polymorphism [19] and (2) c.502 G>A transition in exon 5 (V168I) not reported previously. He was also homozygous for the most frequent polymorphic allele (c.891 C/C in exon 7) (H297) [19]. Proband's father was heterozygous for I128T, V168I and for the rare polymorphic allele in exon 7 (c.891 C/G, H297Q) [19,20]. Proband's mother was heterozygous only for the I128T (Table 2). The sequence of the promoter revealed that the proband was heterozygous for the following polymorphic sites: – 493 G/T, – 400 A/T, – 164 T/C [19] (Table 2). The variant V168I is a conservative amino acid substitution likely to have no effect on MTP function. We screened a sample of 100 randomly selected healthy subjects for this mutation and found four carriers. This suggests that V168I is a rare polymorphism in our population.

In view of these findings, we thought that the diagnosis of a “mild” form of ABL was unlikely. We considered the option that the proband had a heterozygous FHBL (either linked or not linked to the apo B gene) possibly inherited from his father, whose plasma TC and LDL-C levels were in the lower range of the normal values. We therefore decided to sequence the whole apo B gene.

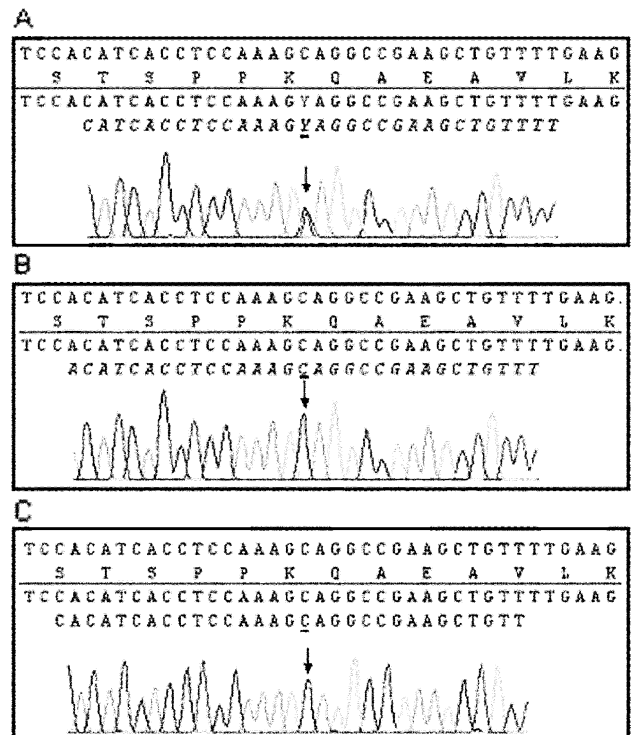


Fig. 3. Partial nucleotide sequence of exon 9 of apo B gene in proband S.M. (panel A), his mother (panel B), and his father (panel C). The proband was found to be heterozygous for a C → T transition (indicated by Y) that converts the glutamine codon (CAG) at position 294 into a termination codon (TAG) (Q294X). This mutation was not detected in proband's parents. Amino acids are indicated as single-letter code below the reference nucleotide sequence. The arrow indicates the nucleotide involved in the substitution.

3.3. Analysis of apo B gene

The sequence of apo B gene revealed that the proband was heterozygous for a C>T transition (c.1089 C>T) in exon 9, which causes the conversion of the glutamine codon (CAG) into a termination codon (TAG) at position 294 of the mature protein (Q294X) (Fig. 3, panel A). This is a novel nonsense mutation predicted to generate a truncated peptide of 293 amino acids (apo B-6.46). The proband was also heterozygous for a G>A transition (c.3511 G>A) in exon 22, which converts the arginine codon (CGT) into a histidine codon (CAT) at position 1101 (R1101H) (Fig. 4, panel A). This missense mutation has not been reported in previous studies [21,22].

The sequence of apo B gene in proband's parents revealed that neither of them was heterozygous for the nonsense mutation Q294X (Fig. 3). Proband's father, however, was heterozygous for the R1101H substitution (Fig. 4). This mutation was also found in proband's paternal grandmother (data not shown). No sequence variants causing amino acid substitutions were found in apo B gene of proband's mother.

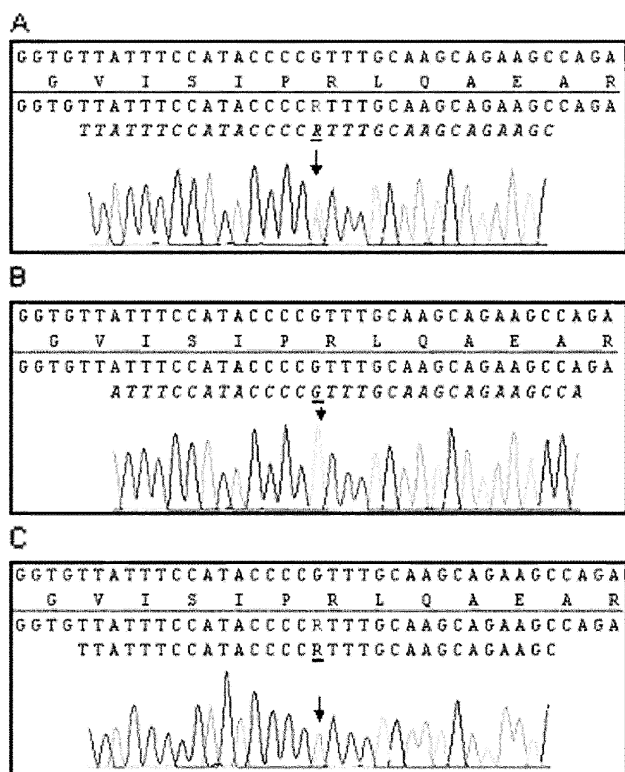


Fig. 4. Partial nucleotide sequence of exon 22 of apo B gene in proband S.M. (panel A), his mother (panel B), and his father (panel C). The proband and his father were found to be heterozygous for a G→A transition (indicated by **R**) which converts the arginine codon (CGT) at position 1101 into a histidine codon (CAT) (R1101H). Amino acids are indicated as single-letter code below the reference nucleotide sequence. The arrow indicates the nucleotide involved in the substitution.

Table 3

Polymorphisms of genes involved in lipoprotein metabolism

Polymorphism	Proband	Mother	Father
CETP B1/B2	B2/B2	B2/B2	B1/B2
FABP-2 54A/T	A/A	A/T	A/T
Apo A-V-1131 T/C	T/T	T/T	T/T
HL-250G/A	G/G	G/A	G/G
ABCA1 R219K	R/K	R/K	R/K

CETP, cholesteryl ester transfer protein; FABP-2, fatty acid binding protein-2; HL, hepatic lipase; ABCA1, ATP-binding cassette transporter 1.

3.4. Complementary genetic analysis

The proband and his parents were genotyped for several polymorphisms of genes involved in lipoprotein metabolism (Table 3). These results combined with those of MTP gene (Table 2) were all consistent with paternity. Formal paternity testing carried out with additional genetic markers [17,18] indicated a likelihood of paternity of 99.99%.

4. Discussion

In this study we report a patient with fatty liver disease and mild steatorrhea associated with severe hypobetalipoproteinemia, as we observed in symptomatic FHBL heterozygotes carrying short apo B truncations [4]. Since there was no clear indication of a vertical transmission of hypobetalipoproteinemia in the kindred, we thought that the proband might have: (i) recessive hypobetalipoproteinemia suggestive of a “mild” form of ABL; (ii) heterozygous FHBL (possibly transmitted by his father) with a different phenotypic expression in the proband with respect to his father; (iii) heterozygous FHBL due to a “de novo” mutation in apo B gene (or, for that matter, in other candidate genes/loci).

The analysis of proband's MTP gene revealed that he was a carrier of a rare allele of a previously reported polymorphism (I128T) [19] and a new rare polymorphism in exon 5 (c. 502 G/A) predicted to result in a conservative amino acid substitution (V168I). We considered also the hypothesis that the c.502 G/A in exon 5 might result in a splicing defect of MTP pre-mRNA; this mutation involves the first nucleotide of an exon, which in most of the human genes in a G [23]. A splicing defect might lead to a reduced MTP content in liver and intestine and, as a result, an impaired secretion of apo B-containing lipoproteins. Although this is an attractive hypothesis, the effect of a splicing defect would be undetectable (in terms of reduced plasma LDL-C and apo B) in heterozygotes. Proband's father carried the same MTP variants as his son, despite that he did not have the same lipoprotein phenotype, thus indicating that these MTP variants were not the main cause of the hypobetalipoproteinemia phenotype.

As an alternative, we thought that the proband might have heterozygous FHBL due to a mutation in apo B gene, causing a short truncated apo B. We assumed that the mutation was transmitted by the father, whose plasma lipids were close to the lower limit of the normal range. The sequence of apo B gene revealed that the proband was heterozygous for a novel nonsense mutation (Q294X), predicted to generate a short truncated apo B (apo B-6.46). Surprisingly neither of the proband's parents carried the same mutation. As non-paternity was excluded, we concluded that the proband carried a “de novo” mutation in apo B gene causing symptomatic heterozygous FHBL. To the best of our knowledge, this is the first report of a “de novo” mutation in the apo B gene causing FHBL. This novel mutation involves a GC dinucleotide (GCAG>GTAG), which is the third in the rank order of relative mutability resulting in base-pair substitutions [24]. Whether this mutation has occurred in the maternal or paternal germ line cannot be established at present.

In view of the large size of apo B gene and the heterogeneity of mutant alleles causing FHBL [1,5], one would expect a large number of “de novo” mutations. It is thus surprising that, up to now, only one “de novo” mutation has been discovered. Interestingly a similar situation seems to occur in familial hypercholesterolemia (FH), where few “de novo” mutations of LDL-receptor gene have been reported [17,18,25,26]. This unexpected situation is probably related to the fact that the proportion of dominant disorders due to “de novo” mutations is inversely related to the biological fitness (i.e. the ability of an individual to generate children who survive to adult life and reproduce) [27]. Since FHBL and FH in the heterozygous state do not impair biological fitness, one might expect the percentage of “de novo” mutations in these disorders to be very low.

Our proband was also a carrier of a previously unreported missense mutation of apo B (R1101H) which had been inherited from his father. This mutation, as frequently observed in many genetic disorders, involved a CG dinucleotide (a hot spot for nucleotide substitutions) [24]. We do not know whether R1101H is a rare neutral polymorphism or a mutation which has measurable effects on apo B metabolism. The fact that arginine and histidine are both polar and positively charged amino acids and that the R1101H substitution is outside the binding sites of apo B for MTP and the LDL-receptor [28] would all support the idea of a neutral rare polymorphism. One can argue, however, that R1101H substitution might have some effect on apo B metabolism, as the proband's father (a R1101H carrier) has borderline hypocholesterolemia. This hypothesis seems unlikely since the paternal grandmother, a carrier of the same mutation, has hypercholesterolemia and an LDL-C level in the upper limits of the control values.

The nonsense mutation Q294X is the fourth apo B mutation reported so far as the cause of very short truncated apo Bs (with a size <10% of apo B-100) [4,29–31], which are not secreted into the plasma as lipoprotein constituents

or in lipid-free form. Apo B-6.46 is completely devoid of the lipid associating domain present in the NH₂-terminal region of apo B-100 and of the multiple region involved in MTP binding [28]. It is most likely therefore that apo B-6.46 does not associate with lipids and, for this reason, is rapidly degraded intracellularly in both liver and intestine. Thus, in our proband, the only apo B form synthesized in both tissues is the one encoded by the normal allele. However, the plasma level of apo B in our proband, as in other heterozygous FHBL subjects with apo B truncations, is much lower than the expected 50% value (i.e. 50% of the level found in controls). This probably reflects a reduced production rate of wild-type apo B (dominant negative effect) [5].

In conclusion, we report the first “de novo” mutation of apo B gene causing a short truncated apo B (apo B-6.46) in a subject with symptomatic heterozygous FHBL. Although a “de novo” mutation is probably a rare event, it is possible that some sporadic cases of hypobetalipoproteinemia with an apparent recessive transmission are in fact caused by “de novo” mutations in apo B gene.

Acknowledgements

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