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Case report

Homozygous familial hypobetalipoproteinemia: A Turkish case carrying a missense mutation in apolipoprotein B

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ABSTRACT

The autosomal co-dominant disorder familial hypobetalipoproteinemia (FHBL) may be due to mutations in the *APOB* gene encoding apolipoprotein B (apoB), the main constituent peptide of chylomicrons, very low and low density lipoproteins. We describe an 11 month-old child with failure to thrive, intestinal lipid malabsorption, hepatic steatosis and severe hypobetalipoproteinemia, suggesting the diagnosis of homozygous FHBL, abetalipoproteinemia (ABL) or chylomicron retention disease (CMRD). The analysis of candidate genes showed that patient was homozygous for a variant (c.1594 C>T) in the *APOB* gene causing arginine to tryptophan conversion at position 505 of mature apoB (Arg505Trp). No mutations were found in a panel of other potential candidate genes for hypobetalipoproteinemia. In vitro studies showed a reduced secretion of mutant apoB-48 with respect to the wild-type apoB-48 in transfected McA-RH7777 cells. The Arg505Trp substitution is located in the $\beta\alpha_1$ domain of apoB involved in the lipidation of apoB mediated by microsomal triglyceride transfer protein (MTP), the first step in VLDL and chylomicron formation. The patient's condition improved in response to a low fat diet supplemented with fat-soluble vitamins. Homozygosity for a rare missense mutation in the $\beta\alpha_1$ domain of apoB may be the cause of both severe hypobetalipoproteinemia and intestinal lipid malabsorption.

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1. Introduction

Familial hypobetalipoproteinemia (FHBL, OMIM 615558) is an autosomal co-dominant disorder characterized by plasma levels of low density lipoprotein cholesterol (LDL-C) and apolipoprotein B (apoB) below the 5th percentile of the general population [1,2]. FHBL is genetically heterogeneous: it may be due to mutations in *APOB* gene (*APOB*-linked FHBL) or, less frequently, to loss of function mutations in *PCSK9* gene (*PCSK9*-linked FHBL) [3]. However, in many subjects the genetic basis of FHBL remains unexplained (Orphan FHBL) [3]. Most *APOB* gene mutations lead to the formation of C-terminally truncated forms of apoB [1–3] which lose, to a variable extent, the capacity to form plasma lipoproteins in liver and/or intestine and to export lipids from these organs. Only a few amino acid substitutions of apoB have been reported as the cause of FHBL [4–6]. In FHBL heterozygotes, carrying either truncating or missense mutations of apoB, the plasma levels of total cholesterol, LDL-C and apoB are close to 30% of the corresponding values found in non-carriers [1–3]. FHBL heterozygotes may be asymptomatic or have non-alcoholic fatty liver disease (NAFLD) as the main clinical manifestation [7,8] due to a reduced secretion of VLDL by the liver.

Homozygous FHBL due to homozygosity/compound heterozygosity for *APOB* gene mutations is a rare disorder characterized by extremely low or undetectable levels LDL-C and apoB in plasma [3]. The clinical manifestations of homozygous patients with *APOB*-linked FHBL carrying truncated forms of apoB show great variability [9–13]. Patients carrying apoB truncations longer than apoB-48 (the wild-type apoB form secreted by the intestine as the main protein constituent of chylomicrons) are usually asymptomatic or have NAFLD of variable severity. Patients carrying apoB truncations shorter than apoB-48 have severe NAFLD, intestinal fat malabsorption and accumulation of lipids in enterocytes (due to impaired formation of chylomicrons), fat soluble vitamins deficiency, growth retardation, acanthocytosis and late onset neurologic and ocular dysfunctions [13], as observed in patients with abetalipoproteinemia

Abbreviations: ABL, abetalipoproteinemia; FHBL, familial hypobetalipoproteinemia; CMRD, chylomicron retention disease; HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol; TC, total cholesterol; TG, triglyceride.

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(ABL, OMIM 200100) (a recessive disorder due to mutations in *MTTP* gene) [10,13]. The rare homozygotes for an apoB missense mutation reported so far had extremely low levels of LDL-C and apoB and hepatic steatosis but did not show signs of intestinal lipid malabsorption and related clinical manifestations [4].

In the present study we describe a child presenting with failure to thrive and intestinal lipid malabsorption who was found to be homozygous for a missense mutation in apoB.

2. Material and methods

2.1. Patient HBL-162

The patient was a Turkish girl (HBL-162) born of consanguineous (first cousins) healthy parents. She was delivered at term by caesarean section after a normal pregnancy. The birth weight was 3500 g. She was breastfed from birth. Feeding problems, consisting of attacks of projectile vomiting after feeding, appeared in the first week of life. She was thought to have gastro-esophageal reflux and anti-reflux medication was started. Despite this treatment her symptoms continued. At 11 months of age she was referred to the hospital because of failure to thrive, worsening of vomiting and chronic diarrhoea (see result section). The study of the patient and her family members was approved by the Institutional Ethics Committee.

2.2. Plasma lipids

Plasma TC, TG, HDL-C and apoB concentrations were measured using standard procedures [14]. LDL-C concentration was calculated by the Friedewald's formula.

2.3. Gene analysis

Blood for genetic analysis was obtained after written informed consent from patient's parents. Isolation of genomic DNA, PCR (polymerase chain reaction) amplification of exons, and Sanger sequencing of *APOB* (FHBL), *MTTP* (ABL) and *SAR1B* (CMRD) genes were performed as previously described [9,14].

In addition a NGS methodology was used for the parallel sequencing of a panel of candidate genes for hypobetalipoproteinemia. To this purpose the Ion AmpliSeq™ technology (https://www.ampliseq.com/browse.action) was used to generate an optimized primers design encompassing the coding DNA sequence of potential candidate genes for primary hypobetalipoproteinemia (*APOB*, *PCSK9*, *ANGPTL3*, *ANGPTL4*, *ANGPTL8*, *MTTP*, *APOC3* and the promoter regions of *APOB* and *MTTP* genes). Primer pairs were divided into two pools in order to optimize coverage and multiplex PCR conditions. For Ion AmpliSeq™ libraries preparation and sequencing, up to 10 ng of genomic DNA per primer pool was used. Libraries were prepared using the Ion AmpliSeq™ Library Kit 2.0 and equalized using the Ion Library Equalizer™ kit according to manufacturer's recommendations. The Ion Sphere Particles template positive (ISPs+) were prepared by emPCR onto the Ion One Touch 2 System following the Ion PGM™ Template OT2 200 kit manual. ISPs+ were sequenced on the Ion Torrent™ Personal Genome Machine System (Life Technologies Ltd., Paisley, UK) using an Ion 314™ Chip v2 following the Ion PGM™ Sequencing 200 Kit v2 manual.

2.4. Bioinformatics

Samples were processed using Ion Torrent Suite™ Software for raw data processing and sequence alignment to human genome reference hg19. The plugin TS Variant Caller was used for variant detection. Variants with low coverage or low allele burden (<50 reads or <30%, respectively) were filtered out. Annovar [15] and Variant Effect Predictor [16] were used to functionally annotate variants, retrieving RefSeq gene annotation, dbSNP rs identifiers, ClinVar accession, and allele frequency observed in the population (1000 Genome Project, NHLBI-ESP 6500 exomes, Exome Aggregation Consortium). Variants were further annotated with conservation scores and functional predictions listed in dbNSFP [17] a database which compiles scores from various prediction algorithms, among which are: SIFT, Polyphen2, LRT, MutationTaster, MutationAssessor and FATHMM. All databases and software were used with the last available versions.

In silico prediction of novel splice site variants was performed using Automated Splice Site and Exon Definition Analysis (ASSEDA) (http://splice.uwo.ca/), Human Splicing Finder (http://www.umd.be/HSF/) and Alternative Splice Site Predictor (ASSP) (http://wangcomputing.com/assp/). The mutation in *APOB* gene was designated according to the Human Genome Variation Society, 2013 version (http://www.hgvs.org/mutnomen/recs-DNA.html).

Nucleotide numbers are derived from APOB cDNA sequence (GenBank accession no. NM_000384.2) considering the A of first ATG translation initiation codon as nucleotide +1.

ApoB protein sequence variants were designated according to http://www.hgvs.org/mutnomen/recs-prot.html. The *APOB* mutation found in the proband was screened by direct Sanger sequencing in all family members available for study and in a group of 200 normolipidemic subjects of the Italian population as well as in 50 normolipidemic subjects of the Turkish population.

2.5. In vitro expression of mutant apoB-48

2.5.1. Construction of mutant apoB-48 cDNA

The expression plasmid (pCMV5) containing the coding sequence of human apoB-48 [kindly provided by prof. Zemin Yao, university of Ottawa, Canada] [6] was used as a template for site-directed mutagenesis which was performed using the QuikChange II XL site-directed mutagenesis kit (Stratagene, Agilent technologies company, Santa Clara, CA) according to manufacturer's instructions. The primers used to generate the genomic *APOB* variant (c.1594 C>T) are shown in Supplemental material. The expression plasmid was purified by Qiagen plasmid purification kit (Qiagen, Hilden, Germany) and the *APOB* cDNA region encoding apoB-48 was authenticated by sequencing

2.5.2. Transient transfection of hepatic cells and detection of human apoB-48

McA-RH7777 cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco ®, Life Technologies, Carlsbad, CA, USA) containing 20% Fetal Bovine Serum (FBS, Lonza, Verviers, Belgium) and 1% Penicillin/Streptomicin (PS). McA-RH7777 cells were transiently transfected with wild-type and mutant apoB-48 containing plasmid by using the calcium phosphate precipitation technique [6]. The untransfected cells as well as the cells transiently expressing wild-type and mutant apoB-48 were incubated in DMEM containing 20% FBS and 0.4 mM oleic acid (Sigma-Aldrich, Saint Louis, MO, USA) for 4 h. At the end of the incubation, culture media were collected, centrifuged at 1200 ×*g* for 10 min and the supernatant was supplemented with 1 μl/ml phenylmethanesulfonylfluoride (PMSF, Sigma-Aldrich, Saint Louis, MO, USA), 40 μl/ml Complete EDTA-free Protease Inhibitor Cocktail (Roche, Mannheim, Germany) dissolved in water. The cells were washed with PBS (2.7 mM KCl, 1.8 mM KH_2PO_4, 137 mM NaCl, 10.1 mM $Na_2HPO_4 \cdot 12\ H_2O$) and lysed using lysis buffer (0.2% of 0.5 mM EDTA pH 8.0, 1% Triton X-100, 1% sodium deoxycholate, 6% of 2.5 M NaCl, 5% of 1 M Tris HCl pH 8.0 supplemented with 0.1 ml/ml of 10% SDS, 1 μl/ml of 15% PMSF, 0.5 μl/ml of 2 M dithiothreitol and a Complete Protease Inhibitor Cocktail). Cell lysates were heated twice at 75 °C for 15 min. Cell lysates and incubation media were subjected to gradient SDS-PAGE (5%–10% polyacrylamide gradient gel) under reducing conditions and blotted onto a polyvinylidene fluoride (PVDF) membrane (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Human apoB-48 in cell and media was detected by Western blotting using the anti-human apoB monoclonal antibody 1D1 [6] as primary antibody.

β-actin was detected using the anti-human β-actin mouse monoclonal antibody (Sigma-Aldrich, Saint Louis, MO, USA) as primary antibody. The secondary antibody used to detect human apoB-48 and β-actin was ECL Anti-Mouse IgG Horseradish Peroxidase-Linked Species-Specific Whole Antibody (GE Healthcare, Little Chalfont, Buckinghamshire, UK) from sheep. The blotting membranes were treated with ECL™ Prime Western blotting Detection Reagent (GE Healthcare, Little Chalfont, Buckinghamshire, UK), exposed on X-ray film (CL-XPosure™ Film, Thermo Scientific, Rockford, USA); the protein bands were quantified by densitometry using Molecular Imager Gel Doc System (Bio Rad, Hercules, CA, USA).

2.5.3. APOB mRNA quantification in transfected cells

Total RNA was isolated from McA-RH7777 cells transiently expressing wild-type or mutant apoB-48 by using the RNeasy®Mini Kit (Qiagen, Hilden, Germany) and treated with RNase-free DNase (Roche, Mannheim, Germany). The first-strand of cDNA was synthesized using SupersScript™ III Reverse Transcriptase Kit (Invitrogen™, Life Technologies, Carlsbad, CA, USA). Primers (Supplemental material) specific for rat *GAPDH* and human *APOB* genes were used for quantitative real-time PCR. Rat GAPDH mRNA was used as internal control. The reaction was carried out in the IQ5 Cycler Real Time PCR instrument (Bio Rad, Hercules, CA, USA) and the data were analysed by IQ5 System Software.

2.6. In vitro expression of APOB minigenes

2.6.1. Construction of APOB minigenes and their expression in transfected cells

To assess whether the *APOB* gene mutation c.1594 C>T caused the formation of a novel donor splice site in exon 12, two minigenes, designated mutant (MT) and wild-type (WT) minigene respectively, were constructed through PCR-amplification of the candidate *APOB* gene region (i.e. the region encompassing the c. 1594 C>T substitution in exon 12) from the genomic DNA of the proband and a control subject respectively. The primers used for genomic DNA amplification and the PCR amplification conditions are reported in Supplemental material. WT and MT minigenes (1937 bp in size) contained two third of exon 12 (137 bp), intron 12 (1100 bp), exon 13 (212 bp), intron 13 (261 bp), and the 5′ half of exon 14 (227 bp). Minigenes were cloned into a pTargeT (Promega, Madison, Wisconsin, USA) expression vector and transfected into COS-1 cells using Lipofectamine™ 2000 (Invitrogen™, Life Technologies, Carlsbad, CA, USA). Forty-eight hours after transfection total RNA was extracted using RNAzol (EuroClone S.p.A., Pero, Milan, Italy). RT–PCR amplification of *APOB* minigene transcripts was performed using the same primers used for genomic DNA amplification (Supplemental material). The RT–PCR conditions were: 95 °C for 5 min followed by 30 cycles at 95 °C for 1 min, 55 °C for 1.5 min, 68 °C for 5 min. The transcripts were separated on 2% agarose gel electrophoresis and sequenced [14].

3. Results

3.1. Case description

At the admission to the hospital, the physical examination of the patient revealed a cachectic appearance; the body weight was 5170 g (<3rd percentile), the length 58 cm (<3rd percentile), and the head circumference 38.5 cm (<3rd percentile). The patient had hepatomegaly (liver margin 3 cm below the right costal margin), confirmed by abdominal ultrasound that also showed the presence of hepatic steatosis. Splenomegaly was not detected. Ophtalmologic and neurologic examinations did not reveal abnormalities. Neoplastic diseases, common mother-to-child transmission of infectious disease (TORCH: T, Toxoplasmosis; O, Other infections; R, Rubella; C, Cytomegalovirus; H, Herpes simplex virus-2 or neonatal herpes simplex) and other infections due to hepatotropic viruses or HIV were ruled out. The liver function tests showed a mild increase of serum transaminases (AST 93 U/l, ALT 81 U/l). Haemoglobin levels ranged from 7.2 g/dl to 8.1 g/dl. Mean corpuscular volume was 85 fl. Total bilirubin was 2.1 mg/dl. Peripheral blood smear showed polychromasia, poikilocytosis and acanthocytosis. The prothrombin time was slightly increased (International Normalized Ratio 1.3) (reference range 0.85–1.10). Alpha-1 antitrypsin and ceruloplasmin were normal; the search for anti-nuclear and anti-mitochondrial antibodies was negative. Sweat chloride test was normal. Stool examination revealed numerous fat globules, no reducing substances and no blood cells. The search for pathogenic bacteria or parasites in the stools was negative. The levels of fat-soluble vitamins were reduced. Liver biopsy demonstrated extensive macro-vesicular steatosis, expansion of portal tracts and mild inflammation. Intestinal endoscopy was not performed because patient's parents did not give their consent to this invasive procedure.

The plasma lipid profile (Table 1) showed a severe hypocholesterolemia due to a reduction of all plasma lipoprotein fractions, specifically LDL. This profile, combined with the clinical manifestations, suggested at the diagnosis of homozygous FHBL, abetalipoproteinemia or chylomicron retention disease [13]. The lipid profile of proband's parents (Table 1) was characterized by a mild reduction of TC, LDL-C and HDL-C suggesting that they might be FHBL heterozygotes. Liver ultrasound examination did not reveal the presence of fatty liver in proband's parents. The reduced level of plasma LDL-C found in the parents was consistent with the hypothesis that the child had homozygous FHBL.

3.2. Treatment and follow up

Since the age of 11 months the child has been treated with high oral doses of fat-soluble vitamins (Vitamin A 100 IU/kg/day, Vitamin D 800 IU/day, Vitamin E 100 mg/kg/day, Vitamin K 10 mg/week). Total fat intake was restricted to 25% of the total caloric intake. A medium-chain triglyceride containing formula diet was given [13]. During the following 60 months, patient's growth steadily increased (reaching 25th–50th percentile for height and 10th–25th percentile for weight). Her motor and cognitive milestones were normal. She had neither ophthalmologic nor neuromuscular manifestations. Serum transaminases were slightly elevated. Liver ultrasound examination revealed a mild steatosis as frequently observed in homozygous FHBL [18].

3.3. Sequencing of candidate genes

The Sanger sequencing of *APOB* gene revealed that the patient was homozygous for a rare nucleotide substitution in exon 12 (c.1594 C>T). This substitution converts arginine to tryptophan at position 505 in the mature apoB protein (Arg505Trp, R505W) or p.Arg532Trp (p.R532W) in the pre-protein (GenBank accession no. NP_000375.2) (Fig. 1). This non-conservative amino acid substitution is reported in dbSNP database (www.ncbi.nlm.nih.gov) as rs13306194 with a global MAF (minor allele frequency) of 0.0274 by the 1000 Genome Project, 0.000231 by the NHLBI-ESP 6500 exomes and of 0.01138 by the Exome Aggregation Consortium (ExAC). (http://exac.broadinstitute.

Table 1
Plasma lipid profile of patient HBL-162 and her family.

Subject	Age	TC	LDL-C	HDL-C	TG	ApoB
	(Years)	(mg/dl)	(mg/dl)	(mg/dl)	(mg/dl)	g/l
Mother	23	142	65	30	101	0.68
Father	25	120	46	38	45	0.59
Patient	11 months	37	11	23	15	<0.2
Reference range		70–175	60–130	35–75	35–110	0.55–1.4

ApoB, apolipoprotein B; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglycerides.

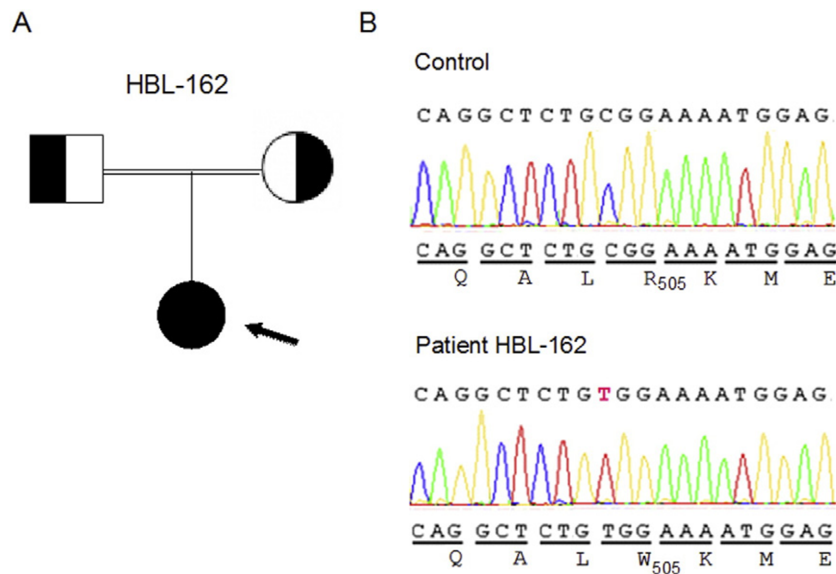

Fig. 1. Pedigree of the HBL-162 kindred and partial nucleotide sequence of exon 12 of *APOB* gene in the index patient and in a control subject. Panel A shows the pedigree of the patient (indicated by an arrow). The filled symbol indicates homozygous and half-filled symbols indicate heterozygous subjects. Panel B shows the electropherograms of a partial sequence of exon 12 of *APOB* gene in the patient and in a control subject. The single nucleotide substitution (in red) in exon 12 (c.1594 C>T) results in the conversion of the arginine to tryptophan at position 505 in the mature protein (Arg505Trp, R505W).

org). No phenotype associated with the R505W variant is reported in the databases. In *silico* evaluation tools indicated that this amino acid substitution was "damaging" (SIFT score 0; Provean score −5.64), "probably damaging" (Polyphen-2 score 1.000) or disease causing (Mutation Tester). The Sanger sequence of *APOB* gene also revealed that the patient was homozygous for several common variants listed in Supplemental Table 1. Patient's parents were heterozygous for the mutation. No carrier of the mutation was found in 200 normolipidemic subjects of the Italian population and in 50 normolipidemic subjects of the Turkish population. No carriers of this variant were found in our series of 191 subjects with the clinical diagnosis of FHBL. Only one healthy blood donor with hypocholesterolemia (plasma TC 102 mg/dl) was found to be heterozygous for this mutation [2]. The Sanger sequencing of *MTTP* and *SAR1B* genes did not reveal the presence of rare variants (Supplemental Table 1).

In order to extend the analysis to other potential candidate genes for hypobetalipoproteinemia and to detect *MTTP* and *APOB* variants that might have been missed by Sanger sequencing, we performed the analysis of an FHBL disease-comprehensive AmpliSeq panel to accomplish the next-generation sequencing (NGS) on an Ion Torrent Personal Genome Machine platform. This analysis confirmed that the patient was homozygous for the R505W missense mutation and other common polymorphisms of apoB (Supplemental Table 1). No pathogenic mutations were found in the other genes analysed in the panel (Supplemental Table 1). The NGS analysis of *MTTP* gene revealed the presence of an homozygous variant in intron 4 (c.502-42 C>T) not detected by Sanger sequencing. The in silico analysis indicated that this variant had no effect on mRNA splicing. No variants were found in *ANGPTL3* and *ANGPTL8* genes (Supplemental Table 1). In view of these results ABL, CMRD and familial combined hypolipidemia were excluded.

3.4. Synthesis and secretion of mutant apoB-48 in McA-RH777 cells

To assess whether the missense mutation R505W affected the secretion of apoB-containing lipoprotein by hepatocytes we looked at the secretion of mutant apoB-48 in transiently transfected McA-RH7777 cells. The Western blot shown in Fig. 2 indicates that the amount of mutant apoB-48 detected in cell extract and in the medium was lower than that of the wild-type apoB-48. The intracellular content of endogenous β-actin was comparable in wild-type and mutant transfected cells. Densitometric analysis of the blot revealed that the secretion efficiency of mutant apoB-48, calculated as the ratio between apoB-48 in media and total apoB-48 (cell plus media), was 60% of that of the wild-type apoB-48 (Fig. 2).

3.5. Possible effect of c.1594 C>T mutation on mRNA splicing

The C>T transition at position c.1594 generates a GT dinucleotide within exon 12 (Fig. 1) raising the possibility that a new donor splice site is created within this exon. To test this hypothesis we transfected COS-1 cells with a mutant minigene containing the *APOB* gene region spanning from exon 12 to exon 14 and containing the c.1594 C>T mutation. The transcripts corresponding to the mutant and wild-type minigenes were amplified by RT–PCR, separated on 2% agarose gel electrophoresis and sequenced. The transcript of the mutant minigene had the same size and the same nucleotide sequence of the wild-type minigene transcript (Supplemental Figs. 1 and 2), thus indicating that the c.1594 C>T mutation does not generate a novel donor splice site in exon 12.

4. Discussion

In this study we describe a child born of consanguineous parents, who at 11 months of age presented with a severe growth retardation, intestinal lipid malabsorption, hepatic steatosis and a severe hypolipidemia. After the exclusion of secondary hypolipidemias, the child was suspected to have homozygous FHBL, abetalipoproteinemia or chylomicron retention disease. The diagnosis of homozygous FHBL appeared to be the most likely in view of the reduced plasma levels of TC and LDL-C found in the parents, which suggested a co-dominant transmission of the hypobetalipoproteinemia trait. For this reason we sequenced the *APOB* gene, the main candidate gene in FHBL. We found that the child was homozygous for a nucleotide substitution in exon 12, leading to the conversion of arginine at position 505 to tryptophan (Arg505Trp) (R505W). ABL and CMRD were excluded as no rare variants were found in *MTTP* and *SAR1B* genes respectively. The NGS analysis of a panel of FHBL candidate genes was negative for the presence of other mutations.

In *silico* analysis indicated that the R505W substitution in apoB was probably damaging. This mutation is located in the $\beta\alpha_1$ domain of apoB

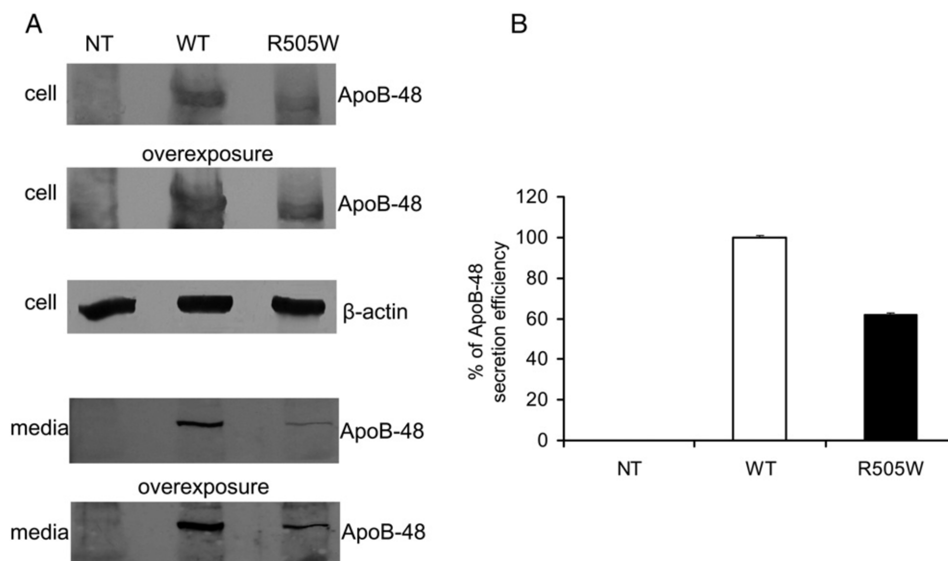

Fig. 2. Expression and secretion of human ApoB-48 in transiently transfected McA-RH7777 cells. A) Western blot analysis of human ApoB-48 expressed in McA-RH7777 cells. The cells were transfected with a plasmid expressing human wild-type ApoB-48 (WT) and the mutant ApoB-48 (R505W). Two days post-transfection cells were incubated in DMEM containing 20% FBS and 0.4 mM oleic acid for 4 h. Cells and media were collected and subjected to SDS-PAGE. Human ApoB-48 was detected by Western blotting using anti-human ApoB-48 monoclonal antibody 1D1. The intracellular content of β-actin was used as control. NT: untransfected cells. Blots shown are representative of two independent experiments performed in triplicate. B) Secretion efficiency of mutant ApoB-48 (R505W) expressed as percentage of the secretion efficiency of wild-type ApoB-48. The secretion efficiency was calculated as the ratio between ApoB-48 in media and total ApoB-48 (cell plus media) [6]. The error bars represent the mean ± standard deviation (SD) of triplicates of two independent experiments.

involved in the interaction with the microsomal triglyceride transfer protein (MTP). In view of this location the mutation might interfere with the initial lipidation of nascent apoB mediated by MTP, a key process required for the assembly of VLDL in the liver and chylomicrons in the intestine [19–20]. In the last decade some apoB missense mutations have been identified as the cause of FHBL [4–6]. So far three of them, Leu343Val (L343V), Arg463Trp (R463W) and Ala31Pro (A31P) all located in the $\beta\alpha_1$ domain, were found to impair the secretion of apoB-containing lipoproteins in vitro [4–6]. Most prominent in this respect is the Arg463Trp (R463W) mutation, originally identified in a large FHBL kindred (which included also two homozygous subjects) [4]. This mutation consists in the same amino acid substitution as the one found in our patient HBL-162 (Arg505Trp, R505W). In *vitro*, the Arg463Trp mutation was found to increase the binding of nascent apoB to MTP and to increase the retention of apoB in the endoplasmic reticulum, a condition which facilitates its proteasomal degradation [4, 5]. In view of these findings it is conceivable that the Arg505Trp substitution found in our patient has the similar effect on nascent apoB, thus preventing the formation of VLDL in the liver and chylomicrons in the intestine. Surprisingly however, the two homozygotes for the Arg463Trp mutation were asymptomatic (presenting only a modest elevation of liver enzymes in addition to the severe hypobetalipoproteinemia) [4], in sharp contrast with our patient who had a severe lipid malabsorption and marked growth retardation similar to that seen in abetalipoproteinemia [10,14]. The reason for this discrepancy is unclear. It is possible that the Arg505Trp substitution causes a more pronounced disruption of the assembly of apoB-containing lipoproteins (specifically chylomicrons) with respect to the Arg463Trp substitution. It should be emphasized that the arginine residue at position 505 is highly conserved among species, and is followed at position 506 by a lysine residue, while other lysine residues are present at position 510 and 512 (Supplemental Fig. 3). The presence of four positively charged amino acids in close proximity may be crucial for the proper folding of the $\beta\alpha1$ region of apoB and for its interaction with MTP. By using transiently transfected McA-RH7777 cells we found that the amount of mutant apoB-48 present in cell and incubation medium was lower than that of wild-type apoB-48. In addition the secretion efficiency of mutant apoB-48 was 60% of that of its wild-type counterpart. This suggests that newly synthesized mutant apoB-48 is rapidly degraded and less available for formation of TG-rich lipoproteins.

We have also considered the possibility that the c.1594 C>T substitution, by generating a GT dinucleotide within exon 12, affected mRNA splicing. This hypothesis was ruled out as the in vitro transcript of a minigene harbouring this mutation had the same sequence as the transcript generated by the wild-type minigene.

The R505Wsubstitution (rs13306194) has been reported in the public databases with a MAF that differs among investigated populations. The MAF reported in the European population is 0.001246 and even lower in African (0.00048), Latino (0.00043) populations. A notable exception is East Asian population (Chinese subjects) in whom MAF was found to be much higher (0.1403) and several homozygotes have been found (without reported clinical manifestations) [ExAC, Exome Aggregation Consortium]. The R505W (rs13306194) has also been reported to have higher frequency in a small cohort of Malaysian subjects with the clinical diagnosis of definite, probable or possible familial hypercholesterolemia (FH) [21]. Surprisingly in these FH patients rs13306194 was found to be significantly associated with a higher plasma HDL-C level [21]. However, the reason why this amino acid change in apoB affects plasma HDL-C was not addressed in that study [21]. In view of these observations our assumption that homozygosity for R505W substitution is the primary cause of the patient's phenotype is open to question. One possible explanation is that in populations (like the European population) in which R505W is rare this amino acid change is in linkage with some other mutation in *APOB* gene that has not been detected by the methods we used for gene analysis.

A limitation of our study is given by the fact that we did not check for major rearrangements (large deletions/insertions) in the *APOB* gene, which might be in linkage with the (c.1594 C>T) (R505W) mutation. However the presence of a partial deletion of *APOB* gene in homozygous state is unlikely as the 29 PCR amplification products we obtained in the patient's *APOB* gene were comparable in size and intensity to those of the wild type *APOB* gene. It is also possible that homozygosity for R505W variant reduces apoB-48 production by liver and intestine thus contributing to hypobetalipoproteinemia but it may be not sufficient to determine the severe lipid malabsorption which would occur only

in the presence of mutations in unknown genes regulating intestinal chylomicron formation.

5. Conclusions

Our observation emphasizes the importance of investigating the plasma lipid profile in an infant with feeding problems, poor weight gain and gastrointestinal symptoms. After the exclusion of secondary hypolipidemias, these clinical features should suggest the diagnosis of homozygous FHBL, ABL or CMRD. The patient reported in this study was found to be homozygous for a rare amino acid substitution in apoB predicted in silico to be probably damaging. In vitro expression studies indicated that this mutation reduced the secretion of human apoB-48 in McA-RH7777 cells, suggesting a defective formation of apoB-48 by the intestine as the basis for a reduced chylomicron formation. However it cannot be excluded that the patient's severe lipid malabsorption may be caused by another mutation in *APOB* gene linked to R505W or to a mutation in other unknown genes affecting intestinal lipid absorption. An early diagnosis of FHBL followed by a prompt and appropriate dietary treatment and vitamin supplementation, overcomes the growth retardation and may prevent the late onset neuromotor and ophtalmologic complications [12].

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