Clinica Chimica Acta 452 (2016) 185–190

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Case report

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

Berna Seker Yilmaz [a,*], Neslihan Onenli Mungan [a], Enza Di Leo [b,c], Lucia Magnolo [b], Lucia Artuso [c], Isabella Bernardis [c], Gokhan Tumgor [d], Deniz Kor [a], Patrizia Tarugi [b,c,**]

[a] Department of Pediatric Metabolism and Nutrition, Cukurova University, Adana, Turkey
[b] Department of Life Sciences, University of Modena and Reggio Emilia, Modena, Italy
[c] Centre for Genome Research, University of Modena and Reggio Emilia, Modena, Italy
[d] Department of Pediatric Gastroenterology, Cukurova University, Adana, Turkey

ARTICLE INFO

Article history:
Received 21 April 2015
Received in revised form 17 November 2015
Accepted 18 November 2015
Available online 22 November 2015

Keywords:
Apolipoprotein B
Familial hypobetalipoproteinemia
Hepatic steatosis
Lipid malabsorption
Missense mutation

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.

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.

* Corresponding author.
** Correspondence to: Department of Life Sciences, University of Modena and Reggio Emilia, Via Campi 287, I-41100 Modena, Italy.
E-mail addresses: berna_seker@yahoo.co.uk (B.S. Yilmaz), munganhno@gmail.com (N.O. Mungan), enzadileo76@libero.it (E. Di Leo), lucillamag@libero.it (L. Magnolo), lucia.artuso@unimore.it (L. Artuso), isabella.bernardis@unimore.it (I. Bernardis), gtumgor74@yahoo.com (G. Tumgor), dozonur@yahoo.com (D. Kor), tarugi@unimore.it (P. Tarugi).

http://dx.doi.org/10.1016/j.cca.2015.11.017
0009-8981/

(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.