Material and Methods

FHBL patients (TC < 5th percentile) are routinely identified and studied in the Lipid Clinic of our Center for Genetic Dyslipidemias at the AOUP “P. Giaccone”, University of Palermo, Italy. Over the years we have collected a large number of patients who underwent a clinical, biochemical and genetic evaluation. Among these, one FHBL family with a high prevalence of associated hepatic diseases was selected for exome sequencing.

Study participants and pedigree

The proband (Figure 1 - subject IV:5) is a 25 year-old female of European ancestry who first came to our attention at the age of 18. Her clinical history was unremarkable except for low plasma TC and triglycerides (TG) detected in several occasions (86 mg/dl and 44 mg/dl respectively). Moreover, an ultrasound evaluation of the abdomen showed liver steatosis.

The analysis of plasma lipids of family members showed low plasma levels of TC and TG in several others. Noticeably in subjects who underwent to ultrasound evaluation, fatty liver was observed (subjects III:1, III:7, III:11, III:13, III:15, III:16 IV:5, IV:6 and IV:7) and in subject III:16 the diagnosis of liver cirrhosis was made after he suffered an acute gastroesophageal bleed.

It was also reported that four more subjects (II:1, II:5, III:3 and III:11) died of either hepatocarcinoma (III:3 and III:11) or carcinoma on cirrhosis (II:1 and II:5). In particular subject III-11 was referred to have a long-lasting unexplained history of hypocholesterolemia who died at the age of 58 from massive gastroesophageal bleeding after he was diagnosed with a hepatocarcinoma with a histologic finding of fibrolamellar hepatocellular carcinoma.

Chronic infection due to hepatitis B virus (HBV) or hepatitis C virus (HCV) and alcohol abuse (mean alcohol intake was < 15g/day) were excluded in all the studied subjects. For family members who had died of hepatocarcinoma, the exclusion of either chronic viral infection or alcohol abuse was made by analyzing the clinical records available and information provided by the relatives.

Moreover, as far as possible, other potential causes of chronic liver diseases, including hepatic disorders of iron and copper metabolism, were excluded by analyzing the clinical records.

Plasma Lipid analysis

Blood samples were collected after an overnight fast. Blood (10 mL) was collected into a plain tube and a tube containing EDTA (1 mg/mL) to obtain serum and plasma, respectively, and buffy coat by centrifugation at 3000 rpm for 15 min. Plasma TC, TG and high-density lipoprotein cholesterol (HDL-C) were measured using standard enzymatic–colorimetric procedures (Roche Diagnostics, Basel Switzerland) on a COBAS MIRA plus auto-analyzer (Roche Diagnostics, Basel Switzerland). LDL-C was calculated by the Friedewald formula. ApoB plasma levels were measured by immuno-nephelometry using a dedicated kit (Radim, Rome, Italy) on a DELTA (Radim, Rome, Italy) instrument.

Fatty liver

Ultrasound (US) examination of the liver was performed to assess for fatty liver.

All US examinations were performed by a single operator with experience in liver disease ultrasonography for more than a decade.

The US of the liver was performed in the morning after a 10 h fasting using a realtime apparatus with a 2–5 MHz multi-frequency or 3.5 MHz convex probe.

The fatty liver diagnosis was made in the presence of fine, packed high amplitude echoes that confer the brightness to the liver (bright liver) and hepatorenal echo contrast

Fatty liver severity was scored as follows: grade 1 = increased echogenicity or bright liver with normal visualization of diaphragm and intrahepatic vessel borders; grade 2 = increased echogenicity with posterior beam attenuation, but with slightly impaired visualization of the intrahepatic vessels and diaphragm; and 3 = marked increase in echogenicity and marked posterior beam attenuation resulting in failure to demonstrate the intrahepatic vessels, diaphragm, and posterior right lobe of the liver [1].

Exome sequencing

Two participants with hypocholesterolemia and liver steatosis (Subjects III:13 and IV:5) underwent exome sequencing. DNA from the two selected individuals was sent to the Broad Institute in accordance with protocols put in place by the institutional review boards of the AOUP "P. Giaccone" at University of Palermo and the Broad Institute. The DNA was subjected to solution hybrid selection using oligonucleotides synthesized on an Agilent array [2] in order to isolate the exonic genomic DNA, which was then sequenced using the Illumina HiSeq platform with 76-nucleotide paired-end reads. A total of 32,950,014 bases were targeted for sequencing. The sequence data was mapped to HG19 using BWA (which implements the Burrows-Wheeler transform) [3], and single nucleotide variants were called using the UnifiedGenotyper module of the Genome Analysis Toolkit (GATK) [4]. The variants were then filtered as follows: (1) Phred-scaled probability that a polymorphism exists at a site > 30; (2) ratio of variant quality score to number of reads > 5; (3) nonreference allele present in >25% of reads; (4) maximum contiguous homopolymer run of the variant allele in either direction on the reference < 5 bases; and (5) strand bias (as described in [30] less than -0.10. The variants were then annotated with a custom pipeline [5].

Polymerase chain reaction (PCR) and DNA sequencing

Genomic DNAs from all subjects were extracted from whole blood using the Wizard DNA Purification System (Promega Italia, Italy). A partial region of exon 26 of APOB gene was sequenced using the primer pairs for PCR amplification and the amplification conditions as previously described [6]. PCR fragments were purified with a commercial kit (Wizard PCR Preps–DNA Purification System; Promega Italia, Italy) then sequenced directly in both directions using BigDye Terminator Cycle sequencing kit 1.1 in a ABI 310 DNA sequencer (Applera Italia, Italy) and the results were analyzed with the Seqed software (Applied Biosystems, Warrington, UK).

The direct sequencing of the region of exon 1 of *RHOB* gene variant was sequenced as described above by using the following primers: EX1F GGGCCAGGAGGACTACGA and EX1.R CAGTTGATGCAGCCGTTCT.

Genotyping for rs738409 (I148M) in *PNPLA3* gene

Genotyping for PNPLA3 rs738409 was conducted in a StepOne Real time Apparatus (Life Technology) by using a commercial genotyping assay (cat. C_7241_10, Life Technology). The genotyping call was done with SDS software v.1.3.0 (ABI Prism 7500, Foster City, CA, USA). Genotyping was conducted in a blinded fashion relative to subject characteristics.

References

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