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Novel missense *MTTP* gene mutations causing abetalipoproteinemia

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ABSTRACT

Objective: The microsomal triglyceride transfer protein (MTTP) plays a critical role in the formation of hepatic very low density lipoprotein. Abetalipoproteinemia (ABL) is a rare, naturally occurring extreme form of MTTP inhibition, which is characterized by the virtual absence of apolipoprotein (apo) B-containing lipoproteins in blood. The goal of this study was to examine the effect that four novel MTTP missense mutations had on protein interactions, expression and lipid-transfer activity, and to determine which mutations were responsible for the ABL phenotype observed in two patients.

Approach and results: In two patients with ABL, we identified in MTTP a novel frameshift mutation (K35Ffs*37), and four novel missense mutations, namely, G264R, Y528H, R540C, and N649S. When transiently expressed in COS-7 cells, all missense MTTP mutations interacted with apoB17, apoB48, and protein disulfide isomerase. Mutations Y528H and R540C, however, displayed negligible levels of MTTP activity and N649S displayed a partial reduction relative to the wild-type MTTP. In contrast, G264R retained full lipid-transfer activity.

Conclusions: These studies indicate that missense mutations Y528H, R540C, and N649S appear to cause ABL by reducing MTTP activity rather than by reducing binding of MTTP with protein disulfide isomerase or apoB. The region of MTTP containing amino acids 528 and 540 constitutes a critical domain for its lipid-transfer activity.

1. Introduction

Abetalipoproteinemia (ABL; OMIM 200100) is a rare, autosomal recessive disorder of lipoprotein metabolism, which is characterized by a virtual absence of plasma apolipoprotein (apo) B-containing lipoproteins, such as chylomicrons, very low density lipoprotein (VLDL) and low density lipoprotein (LDL) [1,2]. ABL is caused by mutations in the microsomal triglyceride transfer protein (*MTTP*) gene [1–3] and affected individuals often present in childhood with failure to thrive, fat malabsorption, acanthocytosis, and vitamin E deficiency [4,5]. Patients with ABL cannot be distinguished clinically from compound heterozygous and homozygous familial hypobetalipoproteinemia (OMIM 107730), which are caused by mutations in the *APOB* gene [5–7].

MTTP is an endoplasmic reticulum protein that catalyses the transfer of neutral lipids between membrane vesicles and is essential for the assembly of apoB-containing lipoproteins by the liver and small intestine [8]. MTTP is a heterodimer composed of a P subunit (~55 kDa), which is a multi-functional, ubiquitously expressed protein, protein disulfide isomerase (PDI), and a larger M subunit (~97 kDa, 894 amino acids), which is primarily expressed in hepatocytes and enterocytes. Based on the sequence homology of MTTP with lipovitellin, the M subunit is proposed to have three major structural domains: N-terminal β-barrel (amino acid residues 22–297), which mediates the interaction with apoB, the central α-helical domain (amino acid residues 298–603), which associates with both PDI and apoB, and the C-terminal lipid-binding domain (amino acid residues 604–894), which is involved in lipid-binding and lipid-transfer activity [8–12].

To date, more than 30 *MTTP* mutations causing ABL have been described. The majority of these are frameshift, nonsense, and splice-site mutations that are located throughout the entire *MTTP* gene and are predicted to encode truncated forms of MTTP resulting in a complete loss of function [1,2,13–17]. Five missense MTTP mutations: L435H, R540H, S590I, G746E, and N780Y have been found to cause ABL and

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in vitro studies of these mutations has provided a valuable insight into the structure and function of MTTP [15,17–22].

R540H, the first MTTP missense mutation described to cause ABL, prohibits the association of the M subunit with the PDI subunit, causing the mutant MTTP to remain as an insoluble aggregate that does not support apoB secretion. R540H is thought to disrupt the R540-H531-E570 internal salt bridge, thereby affecting binding with PDI and subsequently the loss of both lipid-transfer activities [10,15,20].

The four other mutations previously described were all found to lack lipid-transfer activity, but still maintained the ability to bind to the PDI subunit. Mutations N780Y and G764E are thought to affect the tertiary structure of the C-terminal lipid-binding domain, thus disrupting its lipid-binding capabilities [18,20]. L435H is located in the predicted α-helical domain and is thought to affect MTTP folding [22]. S590I is also located in the α-helical domain and it is thought to potentially augment a hydrophobic patch, consisting of residues S590, F585, M587, and A589 by increasing the hydrophobicity of the pocket [20].

In the present study, we report two patients with ABL. The first was found to be a compound heterozygote for two novel mutations in the *MTTP* gene, one a deletion (c.103_127del25) and the other a missense mutation (Y528H). The second was found to carry three novel missense mutations: G264R, R540C, and N649S. To determine whether any of these missense mutations are responsible for the ABL phenotypes, a series of *in vitro* experiments were performed to assess the effect that each of these missense mutations had on protein interactions, expression levels, and lipid-transfer activity.

2. Materials and methods

2.1. Clinical data

2.1.1. Patient 1

A 4-month-old girl was referred for evaluation of failure to thrive. Her plasma biochemistry results revealed a strikingly low total cholesterol at 1.0 mM, with extremely low levels of LDL-cholesterol and apoB (Table 1). The child's parents were in good health and had normal plasma lipid profiles, consistent with a recessive mode of inheritance, i.e. ABL. She has been supplemented with high-dose vitamin E (100 mg/kg/day) up through to her most recent follow-up visit at age 7, and has remained healthy with normal development and function.

2.1.2. Patient 2

A 37-year-old man was referred for investigation after marked hypocholesterolemia was found on routine biochemical testing (Table 1). His plasma total cholesterol was 1.3 mM, with detectable, but extremely low LDL-cholesterol and apoB. Family members were unavailable for study.

2.2. MTTP gene sequencing and mutation analysis

Patient 2 and the family of Patient 1 gave informed consent to genetic testing. In brief, genomic DNA was extracted from peripheral blood using a standard Triton X-100 salting out procedure. All 18 exons and flanking intronic junctions of the *MTTP* gene were amplified by PCR using AmpliTaq Gold (Invitrogen) and sequenced in forward and reverse directions using Big Dye Terminator chemistry (Applied Biosystems). Chromatograms were aligned to reference sequence NM_000253. Mutations were confirmed by sequencing of a second PCR product.

In order to exclude compound heterozygous and homozygous familial hypobetalipoproteinemia in Patient 2, plasma was screened for truncated apoB variants by immunoblotting using the apoB monoclonal antibody 1D1, kindly provided by Prof. Ross Milne (University of Ottawa Heart Institute, Ottawa, Ontario, Canada), using chemiluminescent detection (Amersham Biosciences). In addition, exons 1 to 25 of the *APOB* gene were PCR-amplified and sequenced.

Table 1
Biochemistry of patients with abetalipoproteinemia.

Analyte	Patient 1	Patient 2	Reference limit/interval
Total cholesterol (mM)	0.96	1.30	<5.5
HDL-cholesterol (mM)	0.78	0.81	>1.0
LDL-cholesterol (mM)	Undetectable	0.13	<3.0
ApoB (g/L)	Undetectable	0.14	<1.00
ALT (U/L)	109	52	<35
AST (U/L)	137	49	<35
α-Tocopherol (μM)	Undetectable	10	18–46

ALT, alanine aminotransferase; AST, aspartate aminotransferase; HDL, high-density lipoprotein.

2.3. In silico analysis

Mutations were analyzed using PolyPhen-2 [23] (http://genetics.bwh.harvard.edu/pph2/) and SIFT [24] (http://sift.jcvi.org/).

2.4. Modeling

The MTTP model was built using the human MTTP sequence and the high-resolution structure of lamprey lipovitellin [25] as inputs to the program Modeller 9.12 [26]. The Modeller program's alignment differs from that of Read *et al.* [11], primarily after the two regions of lipovitellin sequence that are missing from the electron density in the crystal structure in the C-sheet region. Modeller does not align any MTTP sequence to these regions whereas the Read *et al.* [11] model does. This offset allows Modeller to fit the entire sequence of MTTP to the lipovitellin structure with reasonable sequence correspondence.

2.5. Construction of mutant MTTP cDNA

The plasmid vector pMTTP, containing the entire coding sequence of the large subunit of MTTP subcloned into plasmid pRC/neo (Invitrogen), was used as a template to prepare mutant pMTTP plasmids containing Y528H, G264R, R540C, and N649S. Site-directed mutagenesis was performed using the Quikchange® II Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. Plasmid DNA was harvested using the QIAGEN Maxiprep Kit and sequenced to confirm the presence of all mutations. Plasmid pMTTP was kindly provided by Prof. Zemin Yao, University of Ottawa Heart Institute, Canada.

2.6. Transient transfection and detection of MTTP

COS-7 cells, an immortalized African green monkey fibroblast-like kidney cell line, were cultured in 6-well plates to an 80% confluency and transfected with 3 μg of wild-type or mutant pMTTP using the fuGENE® 6 transfection reagent (Promega). After 48 h, cells were washed three times with ice-cold phosphate buffered saline (PBS) and harvested in PBS. Cells were lysed using EBC lysis buffer (50 mM TRIS-HCl [pH 8], 120 mM NaCl, 0.5% NP-40, 100 mM NaF, 100 μM leupeptin hemisulphate, 1 mM phenylmethylsulfonyl fluoride) on ice for 15 min and insoluble material was removed by centrifugation (12,000 ×*g*, 10 min). Protein concentration was determined by Bradford Assay (Sigma). Protein samples were subjected to SDS-PAGE (8%) under reducing conditions and blotted onto a supported nitrocellulose membrane (Bio-Rad). MTTP was detected using a primary anti-MTTP Ab, SC-33116 and a secondary donkey anti-goat IgG-HRP Ab, SC-2020 (Santa Cruz Biotechnology). MTTP was visualized using Luminata Classico Western HRP substrate (Millipore) and exposed on x-ray film. Protein sizes were estimated using a broad range protein ladder (Bio-Rad).