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Human Mutation

A severe form of abetalipoproteinemia caused by new splicing mutations of microsomal triglyceride transfer protein

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Key Words:	abetalipoproteinemia, MTTP, triglyceride transfer, endoplasmic reticulum, intestinal HDL

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A severe form of abetalipoproteinemia caused by new splicing mutations of microsomal triglyceride transfer protein

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ABSTRACT

Abetalipoproteinemia is a rare autosomal recessive disease characterized by low lipid levels and by the absence of apoB-containing lipoproteins. It is the consequence of microsomal triglyceride transfer protein (MTTP) deficiency.

We report 2 patients with new *MTTP* mutations. We studied their functional consequences on the triglyceride transfer function using duodenal biopsies. We transfected *MTTP* mutants in HepG2 and HeLa cells to investigate their association with protein disulfide isomerase (*PDI*) and their localization at the endoplasmic reticulum.

These children have a severe abetalipoproteinemia. Both of them had also a mild hypogammaglobulinemia. They are compound heterozygotes with c.619G>T and c.1237-28A>G mutations within *MTTP* gene. mRNA analysis revealed abnormal splicing with deletion of exon 6 and 10, respectively. Deletion of exon 6 (*Δ6-MTTP*) introduced a frame shift in the reading frame and a premature stop codon at position 234. Despite *Δ6-MTTP* and *Δ10-MTTP* mutants were not capable of binding PDI, both MTTP mutant proteins normally localize at the endoplasmic reticulum. However, these two mutations induce a loss of MTTP triglyceride transfer activity.

These two mutations lead to abnormal truncated MTTP proteins, incapable of binding PDI and responsible for the loss of function of MTTP, thereby explaining the severe abetalipoproteinemia phenotype of these children.

Keywords: abetalipoproteinemia, *MTTP*, triglyceride transfer, intestinal HDL, endoplasmic reticulum

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INTRODUCTION

Abetalipoproteinemia (ABL, OMIM 200100) is a rare autosomal recessive disease caused by the deficiency of the microsomal triglyceride transfer protein (MTTP, OMIM 157147). ABL is characterized by the absence of apolipoprotein B (apoB)-containing lipoproteins from plasma. MTTP is a 97 kDa protein, containing 894 amino acids, that forms a heterodimer with the 55 kDa protein disulfide isomerase (PDI). MTTP is mainly found in the endoplasmic reticulum (ER) of hepatocytes and enterocytes (Wetterau, et al., 1992). MTTP is required for the transfer of neutral lipids to nascent apoB-lipoproteins, resulting in the secretion of very low-density lipoproteins (VLDL) in the liver or chylomicrons (CM) in the intestine. When *MTTP* is deficient, nascent apoB is degraded by the proteasome (Benoist and Grand-Perret, 1997). It differs from familial hypobetalipoproteinemia, displaying dysfunctional apoB and leading to the absence of apoB-lipoproteins in the plasma. Familial hypobetalipoproteinemia is a co-dominant disorder and even if heterozygotes can be asymptomatic, they had low levels of total cholesterol (TC), low density lipoprotein (LDL)-cholesterol and apoB (OMIM 107730).

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ABL was characterized as the consequence of MTTP absence or dysfunction in 1992 by Wettereau *et al.* (Wetterau, et al., 1992). The genetic defects in *MTTP* gene, located on chromosome 4q22-24, were confirmed one year later (Sharp, et al., 1993; Shoulders, et al., 1993). ABL is a very uncommon disease, which highlights the crucial role of this protein. Indeed, the mttp-deficient mice died at embryonic day 10.5 with abnormal neurological development (exencephalia) (Raabe, et al., 1998). When a specific inducible intestinal deletion of mttp is generated in mice, they presented a phenotype similar to ABL, with steatorrhea, growth arrest and decreased cholesterol absorption (Xie, et al., 2006).

During evolution, MTTP has progressively acquired TG transfer activity in vertebrates whereas invertebrate MTTP is known to only transfer phospholipids (Rava and Hussain,

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2007). Another way to study MTTP functions is to characterize human mutations. This approach could help us to determine its active domains that transfer phospholipids, cholesterol esters or TG, as well as its binding domains to PDI or to apoB. To our knowledge, so far, 40 mutations have been described in 52 patients (Supp. Table S1) (Najah, et al., 2009; Zamel, et al., 2008). Here, we report 2 children with a severe form of ABL that are compound heterozygotes for *MTTP*. The new mutations induce deletion of exon 6 and 10 and translation of truncated proteins.

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MATERIALS AND METHODS

Patients:

We report 2 new cases of ABL, whose diagnosis was performed when AM was a 13 month-old boy and his sister PM was 6 years-old. They were born from Caucasian non-consanguineous parents. We received the agreement of both parents for genetic investigations.

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Endoscopy and duodenal biopsies:

Upper gastrointestinal endoscopy was realized under general anesthesia with a Pentax EG-1870K. Duodenal biopsies were fixed in Bouin for pathology or immediately frozen in liquid nitrogen for further investigations (Western blot and MTTP activity assay). For routine microscopy, biopsies were embedded in paraformaldehyde and sections were hematoxylin/eosin-stained.

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Intestinal MTTP analyses:

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Proteins were extracted from duodenal biopsies to perform a western blot of MTTP. MTTP activity was measured by radiolabelled TG transfer from donor to acceptor vesicles as previously described (Levy, et al., 2002).

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Plasma sample analyses:

Blood samples were collected for lipids, lipoproteins, vitamin A and E investigations, and ALT. TG and TC were measured by an enzymatic method (Roche). HDL and VLDL-cholesterol were measured by a dextran/PEG method (Roche). LDL-cholesterol was calculated by Friedwald method. ApoA-I and apoB were determined by immuno-turbidimetry (Roche).

Lipid chromatography:

The FPLC system consisted of a Hitachi L7250 auto-sampler, a L7100 pump system and two detectors: L7400 (Hitachi) and UVD170U (Dionex). 20µL of plasma were injected and

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lipoproteins were separated on SuperoseTM6 10/300GL column (GE Healthcare) with 0.15M NaCl solution at a flow rate of 0.4mL/min. The column effluent was split equally into two lines by a microsplitter 50:50, mixing with cholesterol or triglyceride reagents (Biolabo), thus achieving a simultaneous profile from single injection. The two enzymatic reagents were each pumped at a rate of 0.2mL/min. Both enzymatic reactions proceeded at 37°C in a Teflon reactor coil (15mLx0.4mm id). Proteins were measured directly at 280nm.

Genomic DNA sequencing of *MTTP*:

DNA from patients was extracted from blood samples and then exons and introns of *MTTP* gene (NCBI Reference Sequence: NG_011469.1) were sequenced with Applied ABI Prism.

mRNA sequencing of *MTTP*:

Total RNA was extracted using RNeasy Mini Kit (Qiagen) from either EBV-immortalized B lymphocytes for both children or primary lymphocytes for both children and their parents. Primary lymphocytes were isolated from blood samples using a ficoll technique. A reverse transcription was performed and nested PCR was used to amplify exon 6 and exon 10 with 5' and 3' flanking regions (see primers in Supp. Table S2). PCR products were separated on agarose gel, then extracted with a QIAquick Gel Extraction Kit (Qiagen) and directly inserted into pCR2.1 vector (Invitrogen) before sequencing analyses.

Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence (www.hgvs.org/mutnomen). The initiation codon is codon 1.

Constructs:

WT-MTTP cDNA was a generous gift of Dr M.Hussain (New-York). We produced by PCR *WT-MTTP* and *MTTP* mutants with deletions of patients: *Δ6-MTTP* and *Δ10-MTTP*. PCR fragments (*WT-MTTP*, *Δ6-MTTP* and *Δ10-MTTP*) were digested using BamHI and HindIII restriction enzymes, purified with a QIAquick Gel Extraction Kit (Qiagen) and then inserted

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in pEGFP-N1 or in p2xMyc-N1, generated by replacing the EGFP from pEGFP-N1 with a 2xMyc epitope. pDsRed2-ER vector (Clontech) was used to encode an ER marker.

Cell culture and transfection:

HepG2 (human hepatocellular carcinoma), HeLa (human cervix carcinoma) or Caco-2 (human epithelial colorectal adenocarcinoma) cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 0.1 mM non essential amino acids (Invitrogen), and 100 µg/ml penicillin/streptomycin (Invitrogen) in a humidified atmosphere containing 5% CO_2. HepG2 and HeLa cells were transfected using GenJuice (Novagen, Merck) or Fugene (Roche) respectively.

Immunoprecipitation:

The cells were lysed for 30 min in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol) containing protease and phosphatase inhibitors. Soluble material was then incubated overnight at 4°C with 5 µg of monoclonal anti-Myc antibody (clone 9E10) or polyclonal anti-PDI antibody (DL-11). The antigen-antibody complex was incubated with a mix of protein-G and protein-A Sepharose for 1h and then washed in lysis buffer containing 0.1% Nonidet P-40. The bound proteins were eluted by boiling the beads in Laemmli buffer and analyzed by immunoblotting.

Immunoblotting:

The proteins were separated by SDS-polyacrylamide gel electrophoresis under reducing conditions, transferred onto nitrocellulose membrane, and analyzed by immunoblotting according to standard protocols using indicated antibodies. Anti-MTTP antibody was a generous gift of Drs. J. R. Wetterau and H. Jamil. We used a mouse monoclonal antibody against Myc (clone 9E10) from BD Biosciences, a rabbit polyclonal antibody against PDI (DL-11) from Sigma and a mouse monoclonal antibody against GFP from Roche. HRP-labelled secondary antibodies were from Santa-Cruz or Roche Diagnostics.

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Immunofluorescence experiments:

HepG2 and HeLa cells were grown on collagen I coated or glass coverslips respectively. Cells were fixed in 4% paraformaldehyde, permeabilized with 0.05% saponin, saturated with PBS containing 10% fetal bovine serum and incubated with the anti-PDI rabbit polyclonal antibody (Sigma) or the anti-Golgin 97 mouse monoclonal antibody (Molecular Probes) and then with Cy3-labelled secondary antibody (Jackson Immunoresearch Laboratories). Pictures were captured using a Zeiss LSM 510 META confocal microscope equipped with a 63x Plan-Apochromat objective.

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Immunological investigation:

Serum immunoglobulin levels were determined by nephelemetry. B lymphocyte phenotype and function were analyzed as previously described (Peron, et al., 2008).

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RESULTS

Severe form of abetalipoproteinemia:

When AM was 13-months-old, he presented with a failure to thrive. An intestinal malabsorption was suspected on the basis of abdominal distension, abnormal stools (diarrhea or constipation) and hydro-aeric levels on abdominal X-ray. An upper gastrointestinal endoscopy was performed and revealed a white aspect of duodenal villi characteristic of intestinal fat malabsorption and abnormal lipid storage (Figure 1A), as confirmed by histology (large fat inclusions located within enterocytes, Figure 1B). Blood analyses revealed a severe hypocholesterolemia and hypotriglyceridemia without LDL particles and apoB (Table 1). Since parents have normal lipid values as well as apoB and LDL levels, we ruled out a hypo-betalipoproteinemia which is a dominant inheritance disease. Thus, ABL was suspected. They also had an increased level of alanine amino-transferase (ALT) and a hyperechogeneic aspect of the liver on ultra-sound scan. Furthermore, both children presented a severe vitamin E deficiency secondary to intestinal fat malabsorption. His sister (PM), 6-years-old at the time of diagnosis, had no rotula reflex (the very early diagnosis of AM prevented the development of peripheral neuropathy). However, both of them have no sign of retinopathy.

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Abnormal lipoprotein profiles:

We assessed the blood lipoprotein profiles by FPLC analysis. Both children presented a complete absence of LDL and an almost complete absence VLDL in TC and TG moieties (Figure 1C and 1D). Although HDL-cholesterol levels are only 20% of control, HDL particles remain the only lipid class, with a normal ratio of cholesterol ester to TC (Table 1) reflecting a functional lecithin cholesterol acyl-transferase (LCAT) activity. The HDL pattern differs between patient and control. Patient had 2 peaks of HDL particles. The early peak is specific of the patient while the later one appears at the same time than control, as it is suggested by

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the concordance of protein chromatogram. HDL particles of the early peak seem to be cholesterol-enriched and to have less protein than HDL of the second peak.

Immunoglobulin synthesis abnormalities:

Both of these children had also a congenital hypogammaglobulinemia requiring regular immunoglobulin substitution. They were diagnosed as immunodeficient on serum IgG, IgM and IgA levels that were performed because of a failure to thrive in the first months of age (Table 2). There was no protein loosing enteropathy. Mild hypogammaglobulinemia was discovered, affecting IgM and IgG in both children. IgA was also slightly decreased in the brother AM during the first years of age. AM and PM had normal T and B cell populations (data not shown). B cell phenotype was found normal in PM but absence of memory switched B cells was observed in the younger brother AM. In contrast, *in vitro* B cell immunoglobulin production was found normal (data not shown).

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MTTP **mutations:**

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Genomic DNA sequencing of *MTTP* gene showed 2 different mutations: c.619G>T and c.1237-28A>G (Supp. Table S3). The first one c.619G>T was located at the first nucleotide of exon 6 and the second one c.1237-28A>G was located within intron 9. In order to understand the consequences of such mutations, we extracted mRNA from lymphocytes and analyzed *MTTP* transcripts. MTTP is mainly expressed in the liver and in the intestine, but can also be detected from EBV-immortalized B lymphocytes. After reverse transcription and PCR amplification, products were analyzed by gel migration, thereby revealing a decrease in size for both mutations in patient compared to the control (Figure 2A and 2C). As expected, we obtained in control a band at 228 pb and 888 pb for exons 6 and 10 respectively, whereas lower fragments were revealed in patient. Those fragments were detected at the size of ~90pb and ~780pb, suggesting the loss of exons 6 and 10 respectively. Sequencing confirmed that these two mutations are responsible for abnormal splicing of exons 6 and 10

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respectively (Figure 2B and 2D). The first genomic DNA mutation c.619G>T induces a frank deletion of exon 6 and a shift in the open reading frame leading to a premature stop codon at position 234 and resulting in an abnormal truncated protein consisting of 233 amino acids (~25kDa). The second DNA mutation c.1237-28A>G induces a frank deletion of exon 10 (36 amino acids), with no shift in the reading frame, leading to a protein of 858 amino acids (~94kDa) (Figure 2E).

These mutations were also confirmed with sequencing analyses from primary lymphocytes from parents and children, meaning that abnormal splicing of *MTTP* was a consequence of genomic mutations and not of EBV immortalization.

As children are compound heterozygotes, these results confirm the genetic diagnosis of ABL.

Loss of function of MTTP protein:

Duodenal biopsies were performed and samples were analyzed for MTTP expression and activity. In patient, we detected a band with a slight decrease of the apparent MTTP size compared to control or Caco-2 cells (Figure 3A), which probably corresponds to Δ10-MTTP with the loss of 36 amino acids (3kDa) due to exon 10 deletion. We observed no band at a size of 25kDa, corresponding to the expected size of Δ6-MTTP (data not shown), suggesting that Δ6-MTTP might be degraded by the proteasome. MTTP activity was also measured on these samples and showed a complete loss of TG transfer activity (5-8%/mg protein in control *versus* 0.08-0.8%/mg protein in AM, Figure 3B).

We further investigated the ability of both Δ6-MTTP and Δ10-MTTP mutants to interact with PDI and their subcellular localization by first generating constructs encoding GFP- or 2xMyc-tagged WT-MTTP, Δ6-MTTP or Δ10-MTTP. Tagged MTTP mutants as well as WT-MTTP were expressed in HeLa cells and analyzed by immunoblotting. We observed that Δ6-MTTP and Δ10-MTTP were detected, as well as WT form (Figure 4A and 4B). This

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is in contrast to the mutant MTTP analysis in duodenal biopsy since Δ6-MTTP could not be detected. The overexpression level of tagged proteins might overcome the degradation machinery, thereby allowing the detection of a part of Δ6-MTTP and Δ10-MTTP. However, Δ10-MTTP seemed to be lower expressed compared to WT-MTTP, either expressed as GFP- or 2xMyc-Δ10-MTTP (quantification in Figure 4C), probably because a part of abnormal proteins was degraded by proteasome.

Since MTTP is localized in the ER through its binding with PDI, we tested the association of MTTP mutants with PDI. 2xMyc-tagged MTTP mutant and WT proteins were expressed in both HeLa and HepG2 cells and immunoprecipitation was performed using anti-myc antibody (Figure 4D) or anti-PDI antibody (Figure 4E). As expected, we found that WT-MTTP interacted with PDI (Figure 4D and 4E, upper panel). By contrast, neither Δ6-MTTP nor Δ10-MTTP was able to associate with PDI (Figure 4D and 4E, middle and lower panels).

We then investigated the cellular localization of WT-MTTP as well as Δ6-MTTP and Δ10-MTTP. After expression in both HeLa and HepG2 cells, WT-MTTP, Δ6-MTTP and Δ10-MTTP displayed an identical pattern, showing a reticular localization throughout the cytosol, reminiscent of ER staining (Supp. Figure S1). However, in HepG2 cells but not in HeLa cells, Δ6-MTTP also displays a non specific localization both in the nucleus and throughout the cytosol (Supp. Figure S1B, see inset in Δ6-MTTP-GFP), much like GFP alone. This suggests that in HepG2 cells, Δ6-MTTP-GFP protein can be degraded, as we observed by immunoblotting in duodenal biopsies. Surprisingly, WT-MTTP as well as Δ6-MTTP and Δ10-MTTP co-localized with ER markers such as ER-DsRed, a red marker consisting of a fusion of the DsRed and the ER retention sequence, KDEL (Figure 5A) and PDI (Figure 5B). By contrast, no co-localization was observed with cis-Golgi marker such as Golgin 97 (Supp. Figure S2) or trans-Golgi marker such as TGN46 (data not shown).

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Altogether, these data showed that MTTP mutants lost TG transfer activity. They are still localized in the ER despite their inability to interact with PDI.

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DISCUSSION

MTTP is mainly expressed in enterocytes and hepatocytes where it participates in the synthesis of chylomicrons and VLDL respectively. These patients presented fat storage in the small intestine (Figure 1A and 1B) and in the liver (as suggested by increased ALT levels and hyperechogeneic aspect), as a consequence of MTTP dysfunction.

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Lipids are almost exclusively transported in blood into HDL lipoproteins in ABL patients (Figures 1C and 1D and Table 1). These particles are mainly synthesized in peripheral tissues to ensure the reverse cholesterol transport to the liver. But, HDL are also produced at the basolateral membrane of enterocytes, via ATP Binding Cassette-A1 (ABCA1) efflux of cholesterol to apoA-I (Levy, et al., 2007). Cholesterol is transported by enterocytes in two different pathways: the apoB-dependent and the apoB-independent pathways resulting respectively in the production of CM or HDL lipoproteins (Iqbal, et al., 2003). Some differences exist between these two pathways. Cholesterol ester is only secreted within CM while free cholesterol is secreted by both pathways. The apoB-dependent pathway is also regulated by MTTP and contributes to cholesterol absorption during post-prandial states. MTTP is not required for cholesterol secretion by the apoB-independent pathway that may be more important during fasting states. In the human epithelial Caco-2 cell line, Liver X Receptor/Retinoid X Receptor activation increases *ABCA1* gene expression and basolateral efflux of cholesterol in intestinal HDL (Murthy, et al., 2002). In ABL patients, neurological impairment (ataxia and peripheral neuropathy) and retinopathy are the consequences of vitamin E deficiency. Like cholesterol, these two routes also exist for vitamin E absorption by enterocytes (Anwar, et al., 2007). In *MTTP*-deficient enterocytes, the HDL pathway is used to deliver vitamin E. Vitamin E secretion within HDL lipoproteins is not increased by MTTP inhibition (Anwar, et al., 2007). This pathway is not as important as chylomicron synthesis but, in ABL patients, intestinal HDL represent the only route for lipids or vitamin E from

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intestine to the liver (Anwar, et al., 2007; Anwar, et al., 2006). These ABL patients have a specific class of HDL particles that have a lower density and a lower protein content.

MTTP is also expressed in natural killer T (NKT) cells. Inhibition of MTTP in fetal thymocyte organ culture results in a complete loss of NKT cells (Dougan, et al., 2007). In these antigen presenting cells, MTTP loads lipids onto nascent CD1d and regulates presentation of glycolipid antigens (Dougan, et al., 2005). MTTP deficiency could impair the recycling of CD1d from lysosome to the plasma membrane (Sagiv, et al., 2007). Interestingly, both patients present with a hypogammaglobulinemia that was mild but however required immunoglobulin substitution. We only found an absence of memory switched B cells in one patient while both of them had a normal *in vitro* immunoglobulin production (Table 2). Since such an association between mild B lymphocyte immunodeficiency and abetalipoproteinemia has not been previously reported in ABL patients, it is unlikely that MTTP defect is directly involved in hypogammaglobulinemia. However, this point should be further investigated, looking for a subtle defect in immunoglobulin levels in abetalipoproteinemia patients. Recently, Zeissig *et al.* characterized the loss of CD1 function in ABL patients (Zeissig, et al., 2010). They found a defect of all antigen-presenting CD1 family members in dendritic cells from ABL patients. Similarly to apoB, MTTP deficiency in the ER leads to the degradation of group 1 CD1 by the proteasome pathway, which altered activation of NKT cells.

In these new patients with ABL, we characterized mutations of *MTTP* by analyzing genomic DNA and mRNA products. Sequencing revealed abnormal splicing leading to deletion of exon 6 or 10, as a result of two genomic mutations, c.619G>T and c.1237-28A>G respectively. c.619G>T is not only a missense mutation (207 Val>Phe), but induces as well an abnormal splicing (Mitchell, et al., 1986). The deletion of 140 bp of exon 6 causes a shift in the reading frame and a truncated protein because of a premature stop codon in position 234. Recently, Najah *et al.* (Najah, et al., 2009) found similar results with c.619-3T>G mutation

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located in intron 5 of *MTTP*. We showed that this truncated protein is not detected in duodenal biopsies (data not shown), suggesting that Δ6-MTTP is degraded by the proteasome pathway (Pan, et al., 2007). c.1237-28A>G located in intron 9 leads to the frank deletion of 108 bp of exon 10, with no shift in the reading frame, resulting in the loss of 36 amino acids.

Human MTTP structure contains an N-terminal β-barrel (β^N) (residues 22-297), a central α-helical domain (α) (residues 298-603), and two C-terminal β-sheets (β^C and β^A) (residues 604-894). β^N is conserved in apoB, lipovitellin, and apolipophorin and may be one of the two phospholipid binding sites of MTTP. Helices 4-6, β^C and β^A domains of MTTP are conserved in vertebrates, but not in invertebrates, suggesting that they are involved in TG transfer activity (Rava and Hussain, 2007). β^N mediates the interaction with the N-terminus of apoB; the middle α-helical domain mediates the interaction with both PDI (residues 520-598) and apoB (residues 517-603); and the C-terminal mediates the lipid-binding and transfer catalytic activity of MTTP (Mann, et al., 1999; Read, et al., 2000). Expression of *MTTP* constructs in HepG2 and HeLa cells showed that Δ6-MTTP and Δ10-MTTP proteins are located at ER (Figure 5) but are not associated with PDI (Figure 4D and 4E). MTTP mutants might be retained in the ER as defective or misfolded proteins and a part might be retrotranslocated to the cytosol for proteasomal degradation. The truncated Δ6-MTTP protein only conserved one apoB-binding domain. By contrast, Δ10-MTTP protein is characterized by the loss of residues 413-448, that are not directly involved in PDI binding but their loss could alter the tertiary structure of the protein. Finally, these residues, corresponding to helices 7-9 of the central α-helical domain, belong to a critical domain for TG transfer activity as described by Rava and Hussain (Rava and Hussain, 2007).

In conclusion, we report here two new mutations of *MTTP*, c.619G>T and c.1237-28A>G, resulting respectively in Δ6-MTTP protein, a truncated protein of 233 amino acids and Δ10-MTTP protein, deleted of exon 10. Despite these mutations do not change ER

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localization of mutant MTTP proteins, they abolish their binding with PDI and totally impaired their TG transfer activity.

ACKNOWLEDGEMENTS

The authors thank these children and their parents, and also pediatricians taking care of them (Dr. E. Fournié-Gardini and Dr. A.I. Bertozzi). We also thank J. Bertrand-Michel and V. Roques (Lipidomic Plateau of IFR-BMT/IFR150) for lipidomic analysis, advice and technical assistance. We are grateful to Pr. T. Levade (Toulouse, France) for immortalization of B lymphocytes.

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Table 1. Biochemical diagnosis of abetalipoproteinemia

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Table 3. Genomic mutations of

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FIGURES LEGENDS

Figure 1. Diagnosis of abetalipoproteinemia

(A) Upper gastrointestinal endoscopy performed in patient AM revealed a white aspect of duodenal villi, characteristic of lipid storage. (B) Biopsy was performed in duodenum and processed for hematoxylin/eosin-staining. The arrow points at the presence of lipid droplets within the enterocytes. (C and D) Serum from control or patient (AM) was analyzed using FPLC system. Lipoproteins were separated and total cholesterol (C) and triglycerides (D) were measured. Profiles are shown for control (Ctrl) and patient (AM). Proteins were only showed for patient in C. Note the difference of y-axis (lipid content) between control and patient.

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Figure 2. Sequence analysis of mutant *MTTP* transcription products

(A and C) After mRNA extraction and reverse transcription from control and patient (AM) EBV-immortalized lymphocytes, PCR was performed to amplify exon 6 (A) and exon 10 (C) with 5' and 3' flanking regions. PCR products were analyzed by migration on agarose gel. Arrowhead points at the lower band found in patient compared to the control. (B and D) Upper bands in the control and lower bands in patients were purified and then cloned into pCR2.1 vector before sequencing. In (B), electrophoregrams showed the exon 6 and 5' and 3' flanking regions with exon 5 and 7 in the control. In the patient, the exon 6 is deleted, leading to a frameshift and a premature stop codon. In (D), similarly, electrophoregrams showed the exon 10 and 5' and 3' flanking regions with exon 9 and 11 in the control. In the patient, the exon 10 is deleted. (E) The amino acid sequences corresponding to exons 6 and 10 are shown underlined and in blue in the normal MTTP sequence. // determined the deletion of exons 6 or

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10 in each mutant. For Δ6-MTTP, the frame-shift mutation results in an abnormal sequence described in red and in italic.

Figure 3. MTTP expression and activity in duodenal biopsy

(A) Duodenal biopsy from control (Ctrl) or patient (AM) and Caco-2 cell lysate were analyzed by SDS gel electrophoresis and western blotting with antibodies against MTTP. (B) The triglyceride transfer activity of MTTP was measured on duodenal biopsy from control or patient (AM). Values are expressed as the mean of three independent experiments; standard errors are indicated.

Figure 4. Interaction of WT and mutant MTTP with PDI

(A and B) HeLa cells were transfected or not with WT-MTTP, Δ6-MTTP or Δ10-MTTP tagged with EGFP (A) or 2xMyc (B). Cell lysates (100 µg) were analyzed by SDS gel electrophoresis and western blotting with antibodies against GFP (A), Myc (B) and Rab5 as loading control. Arrowheads point at WT-MTTP, Δ6-MTTP or Δ10-MTTP and arrows point at non specific band (ns). Blots in (B) were scanned and the quantification is shown in (C). Each experiment was repeated at least 3 times, and (C) shows a representative example. (D and E) HeLa cells were transfected with 2xMyc-tagged WT-MTTP, Δ6-MTTP or Δ10-MTTP. Cell lysates (Lys) were subjected to immunoprecipitation with (+) or without (-) the anti-myc antibody (D) or the anti-PDI antibody (E). Analysis was performed by SDS gel electrophoresis and western blotting with antibodies against Myc or PDI. Arrowheads point at immunoprecipitated WT-MTTP, Δ6-MTTP or Δ10-MTTP (D) or PDI (E) and arrows point at non specific band (ns). IgG HC represents heavy chain of anti-PDI antibody used for immunoprecipitation.

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Figure 6. Interaction of WT and mutant
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Figure 5. Localization of WT and mutant MTTP and ER marker

(A) HeLa cells were co-transfected with GFP-tagged WT-MTTP, Δ6-MTTP or Δ10-MTTP and a vector encoding DsRed-ER marker. Cells were then processed for fluorescence analysis. (B) HeLa cells were transfected with GFP-tagged WT-MTTP, Δ6-MTTP or Δ10-MTTP and were then processed for immunofluorescence using anti-PDI antibody. Bar: 10µm.

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Figure 8. Localization of WT and mutant
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HeLa cells were transfected with GFP-tagged WT-
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HeLa cells (A) or HepG2 cells (B) were transfected with GFP-tagged WT-MTTP, Δ6-MTTP or Δ10-MTTP and were then processed for immunofluorescence. Bar: 10µm.¶
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Supplementary Figure S2: Localization of WT and mutant MTTP and Golgi marker¶
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HeLa cells were transfected with GFP-tagged WT-MTTP, Δ6-MTTP or Δ10-MTTP and were then processed for immunofluorescence using anti-Golgin 97 antibody. Bar: 10µm
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(Zamel, et al., 2008)

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MTTP could be used to lower cholesterol levels in patients with hypercholesterolemia (Cuchel, et al., 2007). However, adverse events like liver steatosis or vitamin E deficiency should be prevented.

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MTTP transcription is controlled by hepatocyte nuclear factor-4α (HNF-4α) (Sheena, et al., 2005). Hepatocyte nuclear factor binding element (HNF1A) within the

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MTTP mRNA when mice are fed with high-cholesterol and high-fat diets (Iqbal, et al., 2008). The

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MTTP promoter variant -579T/T is associated with peripheral arterial disease (Schgoer, et al., 2008). The -579G/T polymorphism contributes to fat liver accumulation in 3 infected patients with HCV genotype (Zampino, et al., 2008), but could also have a role in the steatosis of these children.[A1]

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MTTP promoter serves as a novel negative insulin-responsive element (Au, et al., 2008). Forkhead box O1 (FoxO1) mediates insulin-regulated

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MTTP production, that may be a causative factor for VLDL overproduction and hypertriglyceridemia in diabetes (Kamagate, et al., 2008). Regulation of

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MTTP expression and CM production is controlled by inositol-requiring enzyme 1beta (IRE1beta), which degrades

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	AM	PM	Children Normal Range	Father	Mother	Adults Normal Range
Triglycerides	6	1	< 75 mg/dL	77	87	50-150 mg/dL
Total cholesterol	30	30	< 170 mg/dL	166	235	105-240 mg/dL
HDL-cholesterol	29	30	> 40 mg/dL	46	69	40-80 mg/dL
VLDL-cholesterol	10	0	ND	16	17	10-30 mg/dL
LDL-cholesterol	0	0	< 110 mg/dL	105	148	108-162 mg/dL
ApoA-I	0.43	0.48	NA	1.25	1.71	1.1-2.1 g/L
Apo-B	0.02	0.01	< 90 mg/dL	0.68	0.92	0.5-1.35 g/L
Retinol/RBP	0.68	1.06	0.95-1.06	ND	ND	
Tocopherol	0.41	0.16	6-15 mg/L	ND	ND	

NA: no available; ND: not done.

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	AM, born the 21/01/2004	PM, born the 25/07/1999	

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	29/04/2004	11/10/2005	27/07/2010	30/09/1999	29/11/2005	27/07/2010	Normal range
IgG	0.95	7.86	6.48	0.33	8.63	5.47	5.6 - 10.4 g/L
IgA	0.04	0.11	1.11	0.02	1.73	1.67	0.4 - 1.4 g/L
IgM	0.11	0.67	0.73	0.04	0.44	0.36	0.6 - 1.6 g/L

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	exon 6 deletion	exon 10 deletion
AM	c.619G>T	c.1237-28A>G
PM	c.619G>T	c.1237-28A>G
Father	c.619G>T	normal
Mother	normal	c.1237-28A>G

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A severe form of abetalipoproteinemia caused by new splicing mutations of microsomal triglyceride transfer protein

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ABSTRACT

Abetalipoproteinemia is a rare autosomal recessive disease characterized by low lipid levels and by the absence of apoB-containing lipoproteins. It is the consequence of microsomal triglyceride transfer protein (MTTP) deficiency.

We report 2 patients with new *MTTP* mutations. We studied their functional consequences on the triglyceride transfer function using duodenal biopsies. We transfected *MTTP* mutants in HepG2 and HeLa cells to investigate their association with protein disulfide isomerase (*PDI*) and their localization at the endoplasmic reticulum.

These children have a severe abetalipoproteinemia. Both of them had also a mild hypogammaglobulinemia. They are compound heterozygotes with c.619G>T and c.1237-28A>G mutations within *MTTP* gene. mRNA analysis revealed abnormal splicing with deletion of exon 6 and 10, respectively. Deletion of exon 6 (*Δ6-MTTP*) introduced a frame shift in the reading frame and a premature stop codon at position 234. Despite *Δ6-MTTP* and *Δ10-MTTP* mutants were not capable of binding PDI, both MTTP mutant proteins normally localize at the endoplasmic reticulum. However, these two mutations induce a loss of MTTP triglyceride transfer activity.

These two mutations lead to abnormal truncated MTTP proteins, incapable of binding PDI and responsible for the loss of function of MTTP, thereby explaining the severe abetalipoproteinemia phenotype of these children.

Keywords: abetalipoproteinemia, *MTTP*, triglyceride transfer, intestinal HDL, endoplasmic reticulum

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INTRODUCTION

Abetalipoproteinemia (ABL, OMIM 200100) is a rare autosomal recessive disease caused by the deficiency of the microsomal triglyceride transfer protein (MTTP, OMIM 157147). ABL is characterized by the absence of apolipoprotein B (apoB)-containing lipoproteins from plasma. MTTP is a 97 kDa protein, containing 894 amino acids, that forms a heterodimer with the 55 kDa protein disulfide isomerase (PDI). MTTP is mainly found in the endoplasmic reticulum (ER) of hepatocytes and enterocytes (Wetterau, et al., 1992). MTTP is required for the transfer of neutral lipids to nascent apoB-lipoproteins, resulting in the secretion of very low-density lipoproteins (VLDL) in the liver or chylomicrons (CM) in the intestine. When *MTTP* is deficient, nascent apoB is degraded by the proteasome (Benoist and Grand-Perret, 1997). It differs from familial hypobetalipoproteinemia, displaying dysfunctional apoB and leading to the absence of apoB-lipoproteins in the plasma. Familial hypobetalipoproteinemia is a co-dominant disorder and even if heterozygotes can be asymptomatic, they had low levels of total cholesterol (TC), low density lipoprotein (LDL)-cholesterol and apoB (OMIM 107730).

ABL was characterized as the consequence of MTTP absence or dysfunction in 1992 by Wettereau *et al.* (Wetterau, et al., 1992). The genetic defects in *MTTP* gene, located on chromosome 4q22-24, were confirmed one year later (Sharp, et al., 1993; Shoulders, et al., 1993). ABL is a very uncommon disease, which highlights the crucial role of this protein. Indeed, the mttp-deficient mice died at embryonic day 10.5 with abnormal neurological development (exencephalia) (Raabe, et al., 1998). When a specific inducible intestinal deletion of mttp is generated in mice, they presented a phenotype similar to ABL, with steatorrhea, growth arrest and decreased cholesterol absorption (Xie, et al., 2006).

During evolution, MTTP has progressively acquired TG transfer activity in vertebrates whereas invertebrate MTTP is known to only transfer phospholipids (Rava and Hussain,

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2007). Another way to study MTTP functions is to characterize human mutations. This approach could help us to determine its active domains that transfer phospholipids, cholesterol esters or TG, as well as its binding domains to PDI or to apoB. To our knowledge, so far, 40 mutations have been described in 52 patients (Supp. Table S1) (Najah, et al., 2009; Zamel, et al., 2008). Here, we report 2 children with a severe form of ABL that are compound heterozygotes for *MTTP*. The new mutations induce deletion of exon 6 and 10 and translation of truncated proteins.

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MATERIALS AND METHODS

Patients:

We report 2 new cases of ABL, whose diagnosis was performed when AM was a 13 month-old boy and his sister PM was 6 years-old. They were born from Caucasian non-consanguineous parents. We received the agreement of both parents for genetic investigations.

Endoscopy and duodenal biopsies:

Upper gastrointestinal endoscopy was realized under general anesthesia with a Pentax EG-1870K. Duodenal biopsies were fixed in Bouin for pathology or immediately frozen in liquid nitrogen for further investigations (Western blot and MTTP activity assay). For routine microscopy, biopsies were embedded in paraformaldehyde and sections were hematoxylin/eosin-stained.

Intestinal MTTP analyses:

Proteins were extracted from duodenal biopsies to perform a western blot of MTTP. MTTP activity was measured by radiolabelled TG transfer from donor to acceptor vesicles as previously described (Levy, et al., 2002).

Plasma sample analyses:

Blood samples were collected for lipids, lipoproteins, vitamin A and E investigations, and ALT. TG and TC were measured by an enzymatic method (Roche). HDL and VLDL-cholesterol were measured by a dextran/PEG method (Roche). LDL-cholesterol was calculated by Friedwald method. ApoA-I and apoB were determined by immuno-turbidimetry (Roche).

Lipid chromatography:

The FPLC system consisted of a Hitachi L7250 auto-sampler, a L7100 pump system and two detectors: L7400 (Hitachi) and UVD170U (Dionex). 20µL of plasma were injected and

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lipoproteins were separated on SuperoseTM6 10/300GL column (GE Healthcare) with 0.15M NaCl solution at a flow rate of 0.4mL/min. The column effluent was split equally into two lines by a microsplitter 50:50, mixing with cholesterol or triglyceride reagents (Biolabo), thus achieving a simultaneous profile from single injection. The two enzymatic reagents were each pumped at a rate of 0.2mL/min. Both enzymatic reactions proceeded at 37°C in a Teflon reactor coil (15mLx0.4mm id). Proteins were measured directly at 280nm.

Genomic DNA sequencing of *MTTP*:

DNA from patients was extracted from blood samples and then exons and introns of *MTTP* gene (NCBI Reference Sequence: NG_011469.1) were sequenced with Applied ABI Prism.

mRNA sequencing of *MTTP*:

Total RNA was extracted using RNeasy Mini Kit (Qiagen) from either EBV-immortalized B lymphocytes for both children or primary lymphocytes for both children and their parents. Primary lymphocytes were isolated from blood samples using a ficoll technique. A reverse transcription was performed and nested PCR was used to amplify exon 6 and exon 10 with 5' and 3' flanking regions (see primers in Supp. Table S2). PCR products were separated on agarose gel, then extracted with a QIAquick Gel Extraction Kit (Qiagen) and directly inserted into pCR2.1 vector (Invitrogen) before sequencing analyses.

Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence (www.hgvs.org/mutnomen). The initiation codon is codon 1.

Constructs:

WT-MTTP cDNA was a generous gift of Dr M.Hussain (New-York). We produced by PCR *WT-MTTP* and *MTTP* mutants with deletions of patients: *Δ6-MTTP* and *Δ10-MTTP*. PCR fragments (*WT-MTTP*, *Δ6-MTTP* and *Δ10-MTTP*) were digested using BamHI and HindIII restriction enzymes, purified with a QIAquick Gel Extraction Kit (Qiagen) and then inserted

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in pEGFP-N1 or in p2xMyc-N1, generated by replacing the EGFP from pEGFP-N1 with a 2xMyc epitope. pDsRed2-ER vector (Clontech) was used to encode an ER marker.

Cell culture and transfection:

HepG2 (human hepatocellular carcinoma), HeLa (human cervix carcinoma) or Caco-2 (human epithelial colorectal adenocarcinoma) cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 0.1 mM non essential amino acids (Invitrogen), and 100 µg/ml penicillin/streptomycin (Invitrogen) in a humidified atmosphere containing 5% CO_2. HepG2 and HeLa cells were transfected using GenJuice (Novagen, Merck) or Fugene (Roche) respectively.

Immunoprecipitation:

The cells were lysed for 30 min in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol) containing protease and phosphatase inhibitors. Soluble material was then incubated overnight at 4°C with 5 µg of monoclonal anti-Myc antibody (clone 9E10) or polyclonal anti-PDI antibody (DL-11). The antigen-antibody complex was incubated with a mix of protein-G and protein-A Sepharose for 1h and then washed in lysis buffer containing 0.1% Nonidet P-40. The bound proteins were eluted by boiling the beads in Laemmli buffer and analyzed by immunoblotting.

Immunoblotting:

The proteins were separated by SDS-polyacrylamide gel electrophoresis under reducing conditions, transferred onto nitrocellulose membrane, and analyzed by immunoblotting according to standard protocols using indicated antibodies. Anti-MTTP antibody was a generous gift of Drs. J. R. Wetterau and H. Jamil. We used a mouse monoclonal antibody against Myc (clone 9E10) from BD Biosciences, a rabbit polyclonal antibody against PDI (DL-11) from Sigma and a mouse monoclonal antibody against GFP from Roche. HRP-labelled secondary antibodies were from Santa-Cruz or Roche Diagnostics.

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Immunofluorescence experiments:

HepG2 and HeLa cells were grown on collagen I coated or glass coverslips respectively. Cells were fixed in 4% paraformaldehyde, permeabilized with 0.05% saponin, saturated with PBS containing 10% fetal bovine serum and incubated with the anti-PDI rabbit polyclonal antibody (Sigma) or the anti-Golgin 97 mouse monoclonal antibody (Molecular Probes) and then with Cy3-labelled secondary antibody (Jackson Immunoresearch Laboratories). Pictures were captured using a Zeiss LSM 510 META confocal microscope equipped with a 63x Plan-Apochromat objective.

Immunological investigation:

Serum immunoglobulin levels were determined by nephelemetry. B lymphocyte phenotype and function were analyzed as previously described (Peron, et al., 2008).

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RESULTS

Severe form of abetalipoproteinemia:

When AM was 13-months-old, he presented with a failure to thrive. An intestinal malabsorption was suspected on the basis of abdominal distension, abnormal stools (diarrhea or constipation) and hydro-aeric levels on abdominal X-ray. An upper gastrointestinal endoscopy was performed and revealed a white aspect of duodenal villi characteristic of intestinal fat malabsorption and abnormal lipid storage (Figure 1A), as confirmed by histology (large fat inclusions located within enterocytes, Figure 1B). Blood analyses revealed a severe hypocholesterolemia and hypotriglyceridemia without LDL particles and apoB (Table 1). Since parents have normal lipid values as well as apoB and LDL levels, we ruled out a hypo-betalipoproteinemia which is a dominant inheritance disease. Thus ABL was suspected. They also had an increased level of alanine amino-transferase (ALT) and a hyperechogeneic aspect of the liver on ultra-sound scan. Furthermore, both children presented a severe vitamin E deficiency secondary to intestinal fat malabsorption. His sister (PM), 6-years-old at the time of diagnosis, had no rotula reflex (the very early diagnosis of AM prevented the development of peripheral neuropathy). However, both of them have no sign of retinopathy.

Abnormal lipoprotein profiles:

We assessed the blood lipoprotein profiles by FPLC analysis. Both children presented a complete absence of LDL and an almost complete absence VLDL in TC and TG moieties (Figure 1C and 1D). Although HDL-cholesterol levels are only 20% of control, HDL particles remain the only lipid class, with a normal ratio of cholesterol ester to TC (Table 1) reflecting a functional lecithin cholesterol acyl-transferase (LCAT) activity. The HDL pattern differs between patient and control. Patient had 2 peaks of HDL particles. The early peak is specific of the patient while the later one appears at the same time than control, as it is suggested by

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the concordance of protein chromatogram. HDL particles of the early peak seem to be cholesterol-enriched and to have less protein than HDL of the second peak.

Immunoglobulin synthesis abnormalities:

Both of these children had also a congenital hypogammaglobulinemia requiring regular immunoglobulin substitution. They were diagnosed as immunodeficient on serum IgG, IgM and IgA levels that were performed because of a failure to thrive in the first months of age (Table 2). There was no protein loosing enteropathy. Mild hypogammaglobulinemia was discovered, affecting IgM and IgG in both children. IgA was also slightly decreased in the brother AM during the first years of age. AM and PM had normal T and B cell populations (data not shown). B cell phenotype was found normal in PM but absence of memory switched B cells was observed in the younger brother AM. In contrast, *in vitro* B cell immunoglobulin production was found normal (data not shown).

***MTTP* mutations:**

Genomic DNA sequencing of *MTTP* gene showed 2 different mutations: c.619G>T and c.1237-28A>G (Supp. Table S3). The first one c.619G>T was located at the first nucleotide of exon 6 and the second one c.1237-28A>G was located within intron 9. In order to understand the consequences of such mutations, we extracted mRNA from lymphocytes and analyzed *MTTP* transcripts. MTTP is mainly expressed in the liver and in the intestine, but can also be detected from EBV-immortalized B lymphocytes. After reverse transcription and PCR amplification, products were analyzed by gel migration, thereby revealing a decrease in size for both mutations in patient compared to the control (Figure 2A and 2C). As expected, we obtained in control a band at 228 pb and 888 pb for exons 6 and 10 respectively, whereas lower fragments were revealed in patient. Those fragments were detected at the size of ~90pb and ~780pb, suggesting the loss of exons 6 and 10 respectively. Sequencing confirmed that these two mutations are responsible for abnormal splicing of exons 6 and 10

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respectively (Figure 2B and 2D). The first genomic DNA mutation c.619G>T induces a frank deletion of exon 6 and a shift in the open reading frame leading to a premature stop codon at position 234 and resulting in an abnormal truncated protein consisting of 233 amino acids (~25kDa). The second DNA mutation c.1237-28A>G induces a frank deletion of exon 10 (36 amino acids), with no shift in the reading frame, leading to a protein of 858 amino acids (~94kDa) (Figure 2E).

These mutations were also confirmed with sequencing analyses from primary lymphocytes from parents and children, meaning that abnormal splicing of *MTTP* was a consequence of genomic mutations and not of EBV immortalization.

As children are compound heterozygotes, these results confirm the genetic diagnosis of ABL.

Loss of function of MTTP protein:

Duodenal biopsies were performed and samples were analyzed for MTTP expression and activity. In patient, we detected a band with a slight decrease of the apparent MTTP size compared to control or Caco-2 cells (Figure 3A), which probably corresponds to Δ10-MTTP with the loss of 36 amino acids (3kDa) due to exon 10 deletion. We observed no band at a size of 25kDa, corresponding to the expected size of Δ6-MTTP (data not shown), suggesting that Δ6-MTTP might be degraded by the proteasome. MTTP activity was also measured on these samples and showed a complete loss of TG transfer activity (5-8%/mg protein in control *versus* 0.08-0.8%/mg protein in AM, Figure 3B).

We further investigated the ability of both Δ6-MTTP and Δ10-MTTP mutants to interact with PDI and their subcellular localization by first generating constructs encoding GFP- or 2xMyc-tagged WT-MTTP, Δ6-MTTP or Δ10-MTTP. Tagged MTTP mutants as well as WT-MTTP were expressed in HeLa cells and analyzed by immunoblotting. We observed that Δ6-MTTP and Δ10-MTTP were detected, as well as WT form (Figure 4A and 4B). This

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is in contrast to the mutant MTTP analysis in duodenal biopsy since Δ6-MTTP could not be detected. The overexpression level of tagged proteins might overcome the degradation machinery, thereby allowing the detection of a part of Δ6-MTTP and Δ10-MTTP. However, Δ10-MTTP seemed to be lower expressed compared to WT-MTTP, either expressed as GFP- or 2xMyc-Δ10-MTTP (quantification in Figure 4C), probably because a part of abnormal proteins was degraded by proteasome.

Since MTTP is localized in the ER through its binding with PDI, we tested the association of MTTP mutants with PDI. 2xMyc-tagged MTTP mutant and WT proteins were expressed in both HeLa and HepG2 cells and immunoprecipitation was performed using anti-myc antibody (Figure 4D) or anti-PDI antibody (Figure 4E). As expected, we found that WT-MTTP interacted with PDI (Figure 4D and 4E, upper panel). By contrast, neither Δ6-MTTP nor Δ10-MTTP was able to associate with PDI (Figure 4D and 4E, middle and lower panels).

We then investigated the cellular localization of WT-MTTP as well as Δ6-MTTP and Δ10-MTTP. After expression in both HeLa and HepG2 cells, WT-MTTP, Δ6-MTTP and Δ10-MTTP displayed an identical pattern, showing a reticular localization throughout the cytosol, reminiscent of ER staining (Supp. Figure S1). However, in HepG2 cells but not in HeLa cells, Δ6-MTTP also displays a non specific localization both in the nucleus and throughout the cytosol (Supp. Figure S1B, see inset in Δ6-MTTP-GFP), much like GFP alone. This suggests that in HepG2 cells, Δ6-MTTP-GFP protein can be degraded, as we observed by immunoblotting in duodenal biopsies. Surprisingly, WT-MTTP as well as Δ6-MTTP and Δ10-MTTP co-localized with ER markers such as ER-DsRed, a red marker consisting of a fusion of the DsRed and the ER retention sequence, KDEL (Figure 5A) and PDI (Figure 5B). By contrast, no co-localization was observed with cis-Golgi marker such as Golgin 97 (Supp. Figure S2) or trans-Golgi marker such as TGN46 (data not shown).

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Altogether, these data showed that MTTP mutants lost TG transfer activity. They are still localized in the ER despite their inability to interact with PDI.

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DISCUSSION

MTTP is mainly expressed in enterocytes and hepatocytes where it participates in the synthesis of chylomicrons and VLDL respectively. These patients presented fat storage in the small intestine (Figure 1A and 1B) and in the liver (as suggested by increased ALT levels and hyperechogeneic aspect), as a consequence of MTTP dysfunction.

Lipids are almost exclusively transported in blood into HDL lipoproteins in ABL patients (Figures 1C and 1D and Table 1). These particles are mainly synthesized in peripheral tissues to ensure the reverse cholesterol transport to the liver. But, HDL are also produced at the basolateral membrane of enterocytes, via ATP Binding Cassette-A1 (ABCA1) efflux of cholesterol to apoA-I (Levy, et al., 2007). Cholesterol is transported by enterocytes in two different pathways: the apoB-dependent and the apoB-independent pathways resulting respectively in the production of CM or HDL lipoproteins (Iqbal, et al., 2003). Some differences exist between these two pathways. Cholesterol ester is only secreted within CM while free cholesterol is secreted by both pathways. The apoB-dependent pathway is also regulated by MTTP and contributes to cholesterol absorption during post-prandial states. MTTP is not required for cholesterol secretion by the apoB-independent pathway that may be more important during fasting states. In the human epithelial Caco-2 cell line, Liver X Receptor/Retinoid X Receptor activation increases *ABCA1* gene expression and basolateral efflux of cholesterol in intestinal HDL (Murthy, et al., 2002). In ABL patients, neurological impairment (ataxia and peripheral neuropathy) and retinopathy are the consequences of vitamin E deficiency. Like cholesterol, these two routes also exist for vitamin E absorption by enterocytes (Anwar, et al., 2007). In *MTTP*-deficient enterocytes, the HDL pathway is used to deliver vitamin E. Vitamin E secretion within HDL lipoproteins is not increased by MTTP inhibition (Anwar, et al., 2007). This pathway is not as important as chylomicron synthesis but, in ABL patients, intestinal HDL represent the only route for lipids or vitamin E from

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intestine to the liver (Anwar, et al., 2007; Anwar, et al., 2006). These ABL patients have a specific class of HDL particles that have a lower density and a lower protein content.

MTTP is also expressed in natural killer T (NKT) cells. Inhibition of MTTP in fetal thymocyte organ culture results in a complete loss of NKT cells (Dougan, et al., 2007). In these antigen presenting cells, MTTP loads lipids onto nascent CD1d and regulates presentation of glycolipid antigens (Dougan, et al., 2005). MTTP deficiency could impair the recycling of CD1d from lysosome to the plasma membrane (Sagiv, et al., 2007). Interestingly, both patients present with a hypogammaglobulinemia that was mild but however required immunoglobulin substitution. We only found an absence of memory switched B cells in one patient while both of them had a normal *in vitro* immunoglobulin production (Table 2). Since such an association between mild B lymphocyte immunodeficiency and abetalipoproteinemia has not been previously reported in ABL patients, it is unlikely that MTTP defect is directly involved in hypogammaglobulinemia. However, this point should be further investigated, looking for a subtle defect in immunoglobulin levels in abetalipoproteinemia patients. Recently, Zeissig *et al*. characterized the loss of CD1 function in ABL patients (Zeissig, et al., 2010). They found a defect of all antigen-presenting CD1 family members in dendritic cells from ABL patients. Similarly to apoB, MTTP deficiency in the ER leads to the degradation of group 1 CD1 by the proteasome pathway, which altered activation of NKT cells.

In these new patients with ABL, we characterized mutations of *MTTP* by analyzing genomic DNA and mRNA products. Sequencing revealed abnormal splicing leading to deletion of exon 6 or 10, as a result of two genomic mutations, c.619G>T and c.1237-28A>G respectively. c.619G>T is not only a missense mutation (207 Val>Phe), but induces as well an abnormal splicing (Mitchell, et al., 1986). The deletion of 140 bp of exon 6 causes a shift in the reading frame and a truncated protein because of a premature stop codon in position 234. Recently, Najah *et al.* (Najah, et al., 2009) found similar results with c.619-3T>G mutation

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located in intron 5 of *MTTP*. We showed that this truncated protein is not detected in duodenal biopsies (data not shown), suggesting that Δ6-MTTP is degraded by the proteasome pathway (Pan, et al., 2007). c.1237-28A>G located in intron 9 leads to the frank deletion of 108 bp of exon 10, with no shift in the reading frame, resulting in the loss of 36 amino acids.

Human MTTP structure contains an N-terminal β-barrel (β^N) (residues 22-297), a central α-helical domain (α) (residues 298-603), and two C-terminal β-sheets (β^C and β^A) (residues 604-894). β^N is conserved in apoB, lipovitellin, and apolipophorin and may be one of the two phospholipid binding sites of MTTP. Helices 4-6, β^C and β^A domains of MTTP are conserved in vertebrates, but not in invertebrates, suggesting that they are involved in TG transfer activity (Rava and Hussain, 2007). β^N mediates the interaction with the N-terminus of apoB; the middle α-helical domain mediates the interaction with both PDI (residues 520-598) and apoB (residues 517-603); and the C-terminal mediates the lipid-binding and transfer catalytic activity of MTTP (Mann, et al., 1999; Read, et al., 2000). Expression of *MTTP* constructs in HepG2 and HeLa cells showed that Δ6-MTTP and Δ10-MTTP proteins are located at ER (Figure 5) but are not associated with PDI (Figure 4D and 4E). MTTP mutants might be retained in the ER as defective or misfolded proteins and a part might be retrotranslocated to the cytosol for proteasomal degradation. The truncated Δ6-MTTP protein only conserved one apoB-binding domain. By contrast, Δ10-MTTP protein is characterized by the loss of residues 413-448, that are not directly involved in PDI binding but their loss could alter the tertiary structure of the protein. Finally, these residues, corresponding to helices 7-9 of the central α-helical domain, belong to a critical domain for TG transfer activity as described by Rava and Hussain (Rava and Hussain, 2007).

In conclusion, we report here two new mutations of *MTTP*, c.619G>T and c.1237-28A>G, resulting respectively in Δ6-MTTP protein, a truncated protein of 233 amino acids and Δ10-MTTP protein, deleted of exon 10. Despite these mutations do not change ER

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localization of mutant MTTP proteins, they abolish their binding with PDI and totally impaired their TG transfer activity.

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FIGURES LEGENDS

Figure 1. Diagnosis of abetalipoproteinemia

(A) Upper gastrointestinal endoscopy performed in patient AM revealed a white aspect of duodenal villi, characteristic of lipid storage. (B) Biopsy was performed in duodenum and processed for hematoxylin/eosin-staining. The arrow points at the presence of lipid droplets within the enterocytes. (C and D) Serum from control or patient (AM) was analyzed using FPLC system. Lipoproteins were separated and total cholesterol (C) and triglycerides (D) were measured. Profiles are shown for control (Ctrl) and patient (AM). Proteins were only showed for patient in C. Note the difference of y-axis (lipid content) between control and patient.

Figure 2. Sequence analysis of mutant *MTTP* transcription products

(A and C) After mRNA extraction and reverse transcription from control and patient (AM) EBV-immortalized lymphocytes, PCR was performed to amplify exon 6 (A) and exon 10 (C) with 5' and 3' flanking regions. PCR products were analyzed by migration on agarose gel. Arrowhead points at the lower band found in patient compared to the control. (B and D) Upper bands in the control and lower bands in patients were purified and then cloned into pCR2.1 vector before sequencing. In (B), electrophoregrams showed the exon 6 and 5' and 3' flanking regions with exon 5 and 7 in the control. In the patient, the exon 6 is deleted, leading to a frameshift and a premature stop codon. In (D), similarly, electrophoregrams showed the exon 10 and 5' and 3' flanking regions with exon 9 and 11 in the control. In the patient, the exon 10 is deleted. (E) The amino acid sequences corresponding to exons 6 and 10 are shown underlined and in blue in the normal MTTP sequence. // determined the deletion of exons 6 or

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10 in each mutant. For Δ6-MTTP, the frame-shift mutation results in an abnormal sequence described in red and in italic.

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Figure 3. MTTP expression and activity in duodenal biopsy

(A) Duodenal biopsy from control (Ctrl) or patient (AM) and Caco-2 cell lysate were analyzed by SDS gel electrophoresis and western blotting with antibodies against MTTP. (B) The triglyceride transfer activity of MTTP was measured on duodenal biopsy from control or patient (AM). Values are expressed as the mean of three independent experiments; standard errors are indicated.

Figure 4. Interaction of WT and mutant MTTP with PDI

(A and B) HeLa cells were transfected or not with WT-MTTP, Δ6-MTTP or Δ10-MTTP tagged with EGFP (A) or 2xMyc (B). Cell lysates (100 µg) were analyzed by SDS gel electrophoresis and western blotting with antibodies against GFP (A), Myc (B) and Rab5 as loading control. Arrowheads point at WT-MTTP, Δ6-MTTP or Δ10-MTTP and arrows point at non specific band (ns). Blots in (B) were scanned and the quantification is shown in (C). Each experiment was repeated at least 3 times, and (C) shows a representative example. (D and E) HeLa cells were transfected with 2xMyc-tagged WT-MTTP, Δ6-MTTP or Δ10-MTTP. Cell lysates (Lys) were subjected to immunoprecipitation with (+) or without (-) the anti-myc antibody (D) or the anti-PDI antibody (E). Analysis was performed by SDS gel electrophoresis and western blotting with antibodies against Myc or PDI. Arrowheads point at immunoprecipitated WT-MTTP, Δ6-MTTP or Δ10-MTTP (D) or PDI (E) and arrows point at non specific band (ns). IgG HC represents heavy chain of anti-PDI antibody used for immunoprecipitation.

Figure 5. Localization of WT and mutant MTTP and ER marker

(A) HeLa cells were co-transfected with GFP-tagged WT-MTTP, Δ6-MTTP or Δ10-MTT*P* and a vector encoding DsRed-ER marker. Cells were then processed for fluorescence

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analysis. (B) HeLa cells were transfected with GFP-tagged WT-MTTP, Δ6-MTTP or Δ10-MTTP and were then processed for immunofluorescence using anti-PDI antibody. Bar: 10µm.

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TABLES

Table 1. Clinical and biological findings in abetalipoproteinemia patients and their parents

	AM	PM	Children Normal Range	Father	Mother	Adults Normal Range
Age (year)	1.3	5.9		40	37	
Weight (kg)	8.2	19		NA	NA	
Height (cm)	75	111		NA	NA	
Triglycerides	6	1	< 75 mg/dL	77	87	50-150 mg/dL
Free cholesterol	7,9	6,6	NA	ND	ND	
Esterified cholesterol	22,1	23,4	NA	ND	ND	
Total cholesterol	30	30	< 170 mg/dL	166	235	105-240 mg/dL
HDL-cholesterol	29	30	> 40 mg/dL	46	69	40-80 mg/dL
VLDL-cholesterol	10	0	ND	16	17	10-30 mg/dL
LDL-cholesterol	0	0	< 110 mg/dL	105	148	108-162 mg/dL
ApoA-I	0.43	0.48	NA	1.25	1.71	1.1-2.1 g/L
Apo-B	0.02	0.01	< 90 mg/dL	0.68	0.92	0.5-1.35 g/L
Retinol/RBP	0.68	1.06	0.95-1.06	ND	ND	
Tocopherol	0.41	0.16	6-15 mg/L	ND	ND	
ALT	60	60	< 36 IU/L	ND	ND	
AST	76	76	< 50 IU/L	ND	ND	

NA: not available; ND: not done.

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Table 2. Immunoglobulin levels

	AM, born the 21/01/2004			PM, born the 25/07/1999			
	29/04/2004	**11/10/2005**	**27/07/2010**	**30/09/1999**	**29/11/2005**	**27/07/2010**	**Normal range**
IgG	0.95	7.86	6.48	0.33	8.63	5.47	5.6 - 10.4 g/L
IgA	0.04	0.11	1.11	0.02	1.73	1.67	0.4 - 1.4 g/L
IgM	0.11	0.67	0.73	0.04	0.44	0.36	0.6 - 1.6 g/L

Immunoglobulin levels of both patients were reported at the diagnosis of hypogammaglobulinemia, just after the diagnosis of abetalipoproteinemia and at the last follow-up.

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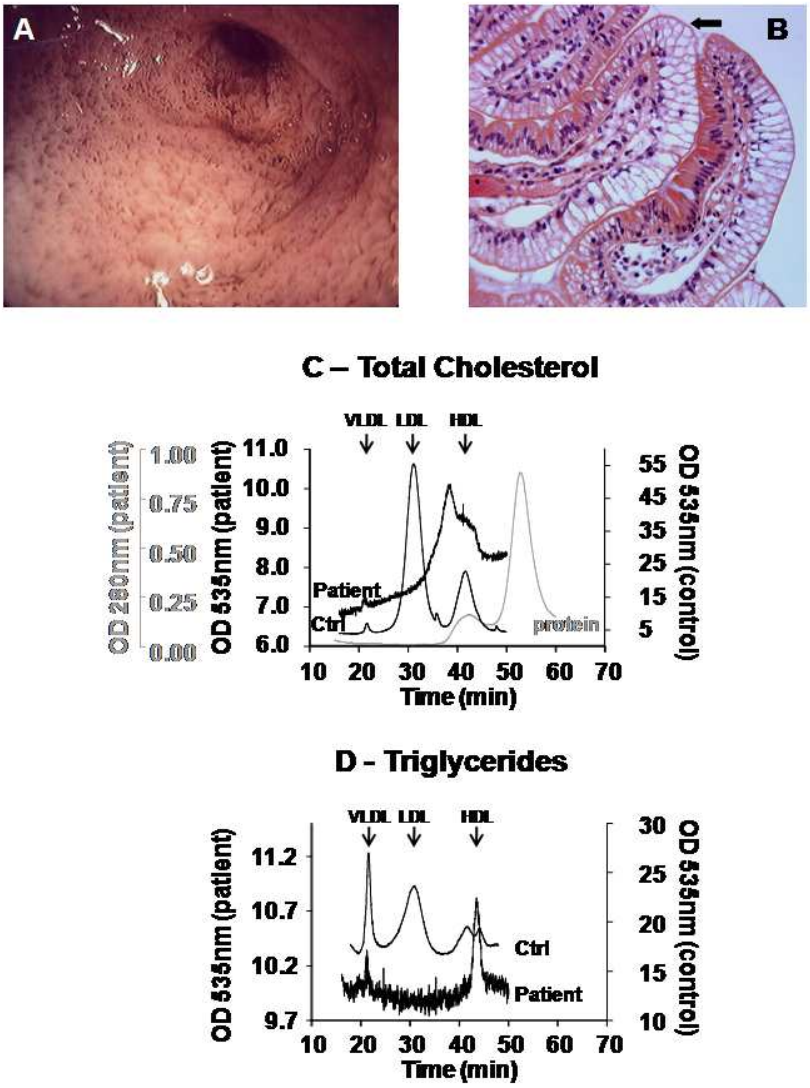
Figure 1



190x254mm (96 x 96 DPI)

Figure 1

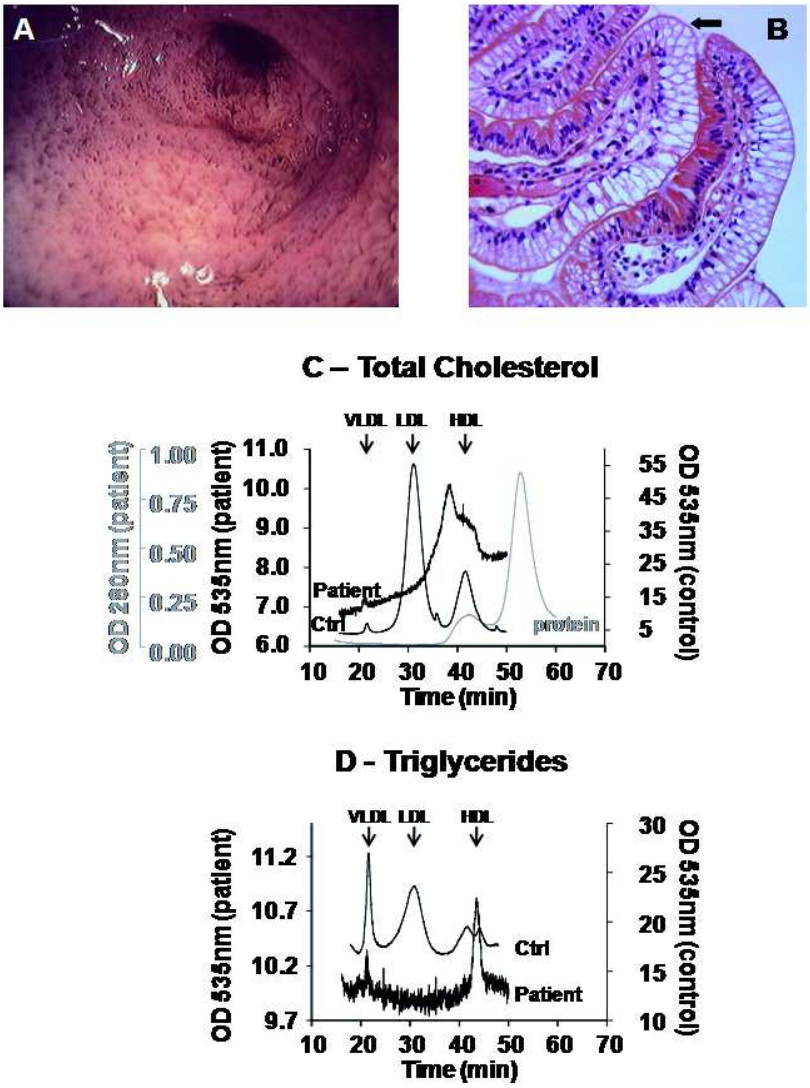


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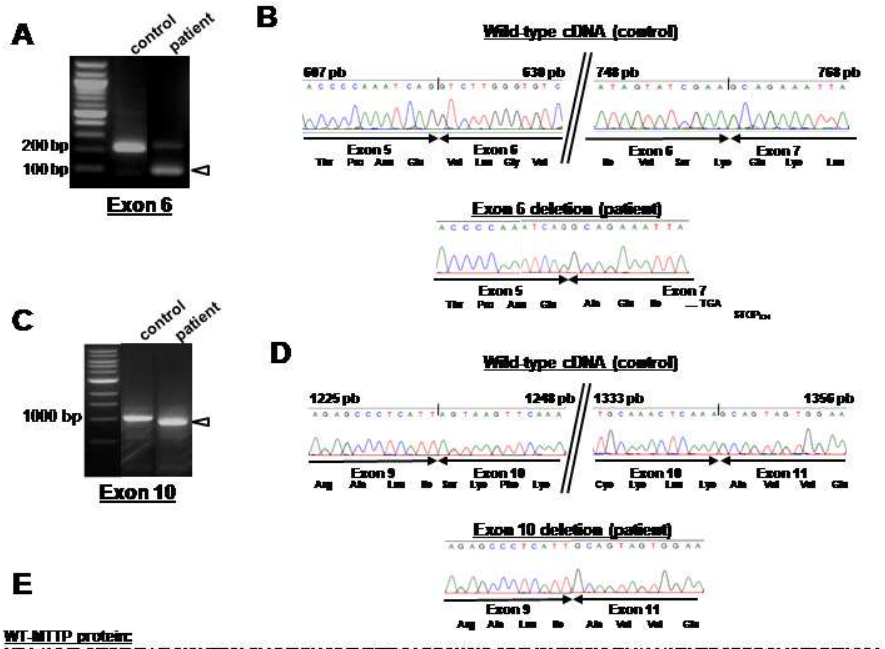
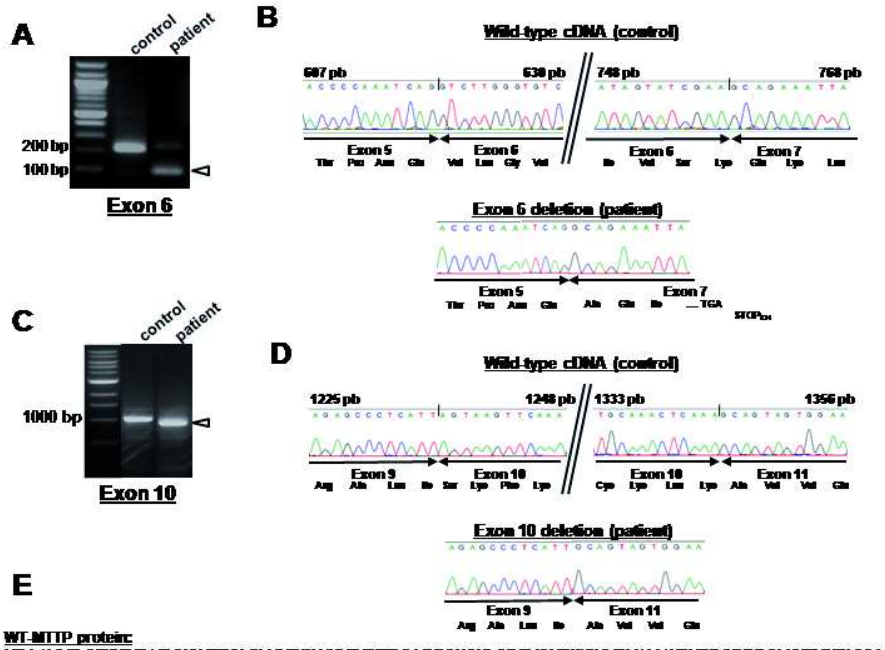
Figure 2



190x254mm (96 x 96 DPI)

Figure 2



WT-MTTP protein:

MLLAVLFLCFSSYSASVKGHTTGLSLNNIDRLYKLTYSTEVLLDRGKGKLQDSVGYRSSNVDVALLWRNPDGDDDQLIQITMKDVNV
ENVNQQRGEKSIFKGKSPSKIMGKENILEALQRPTLLHLIHGKVKEFYSYQNEAVAIENIKRGLASLFQTQLSSGTTNEVDISGNCKVTY
QAHQDKVIKIKALDSCKIARSGFTTPNQMLGVSSKATSVTTYKIEDSFVIAVLAEETHNFGLNFLQTIKGKIVSKQKLELKTTEAGPRLMI
SGKQAAAIIKAVDSKYTAIPIVGQVFQSHCKGCPSLSELWRSTRKYLQPDNLSKAEAVRNFLAFIQHLRTAKKEEILQILKMENKEVLPQL
VDAVTSAQTSDSLEAILDFLDFKSDSSIILQERFLYACGFASHPNEELLRALISKQKGSGSSDIETVNITGTLVIKKLCQNEGCKLKA
VVEAKKLILGGLEKAEKKEDTRMYLLALKNALLPEGPSLLKYAEAGEGPISHLATTALQRYDLPFITDEVKKTLNRIYHQNRKVHEKTV
RTAAAAILNNNIPSYMIDVKNILLSIGELPQEMNKYMLAIVQDLRFEMPASKIVRRVLKEMVAHNYDRFSIRSGSSSAYTGYIERSPRSAS
TYSLDILYSGSGILRRSNLNIFQYIGKAGLHGSQVVIEAQGLEALIAATPDEGEENLDSYAGMSAILFDVQLRPVTFFNGYSDLMSKIMLS
ASGDPISVVKGLILLIDHSQELQLQSGLKANIEVQGGLAIDISGAMEFSLWYRESKTRVKNIRVTVVITTDITVDSSFVKAGLETSTETEAG
LEFRSTVQFSQYPFLVCMQMDKDEAPFRQFEKKYERLSTGRGYVSQKRKESVLAGCEFPLHQENSEMCKVVFAPQPDSTSSGWF

A6-MTTP protein:

MLLAVLFLCFSSYSASVKGHTTGLSLNNIDRLYKLTYSTEVLLDRGKGKLQDSVGYRSSNVDVALLWRNPDGDDDQLIQITMKDVNV
ENVNQQRGEKSIFKGKSPSKIMGKENILEALQRPTLLHLIHGKVKEFYSYQNEAVAIENIKRGLASLFQTQLSSGTTNEVDISGNCKVTY
QAHQDKVIKIKALDSCKIARSGFTTPNQ # *AEIRAEDMRSRPKDVMKAGCSRNQSS*

A10-MTTP protein:

MLLAVLFLCFSSYSASVKGHTTGLSLNNIDRLYKLTYSTEVLLDRGKGKLQDSVGYRSSNVDVALLWRNPDGDDDQLIQITMKDVNV
ENVNQQRGEKSIFKGKSPSKIMGKENILEALQRPTLLHLIHGKVKEFYSYQNEAVAIENIKRGLASLFQTQLSSGTTNEVDISGNCKVTY
QAHQDKVIKIKALDSCKIARSGFTTPNQVLGVSSKATSVTTYKIEDSFVIAVLAEETHNFGLNFLQTIKGKIVSKQKLELKTTEAGPRLMS
GKQAAAIIKAVDSKYTAIPIVGQVFQSHCKGCPSLSELWRSTRKYLQPDNLSKAEAVRNFLAFIQHLRTAKKEEILQILKMENKEVLPQL
VDAVTSAQTSDSLEAILDFLDFKSDSSIILQERFLYACGFASHPNEELLRALI # AVVEAKKLILGGLEKAEKKEDTRMYLLALKNALLPE
GPSLLKYAEAGEGPISHLATTALQRYDLPFITDEVKKTLNRIYHQNRKVHEKTVRTAAAAILNNNIPSYMIDVKNILLSIGELPQEMNKYMI
LAIVQDLRFEMPASKIVRRVLKEMVAHNYDRFSIRSGSSSAYTGYIERSPRSASTYSLDILYSGSGILRRSNLNIFQYIGKAGLHGSQVVI
EAQGLEALIAATPDEGEENLDSYAGMSAILFDVQLRPVTFFNGYSDLMSKIMLSASGDPISVVKGLILLIDHSQELQLQSGLKANIEVQG
GLAIDISGAMEFSLWYRESKTRVKNIRVTVVITTDITVDSSFVKAGLETSTETEAGLEFRSTVQFSQYPFLVCMQMDKDEAPFRQFEKKY
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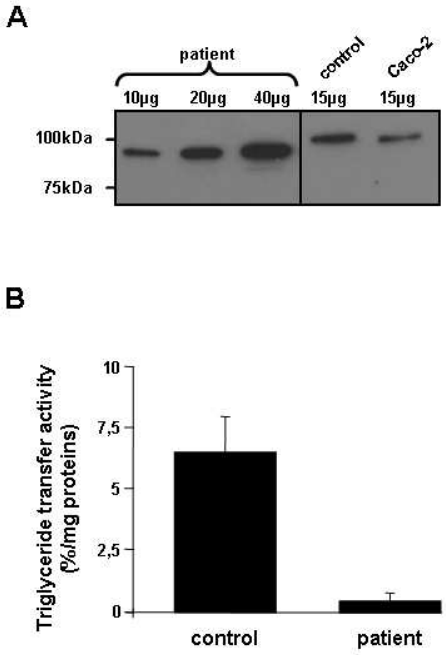
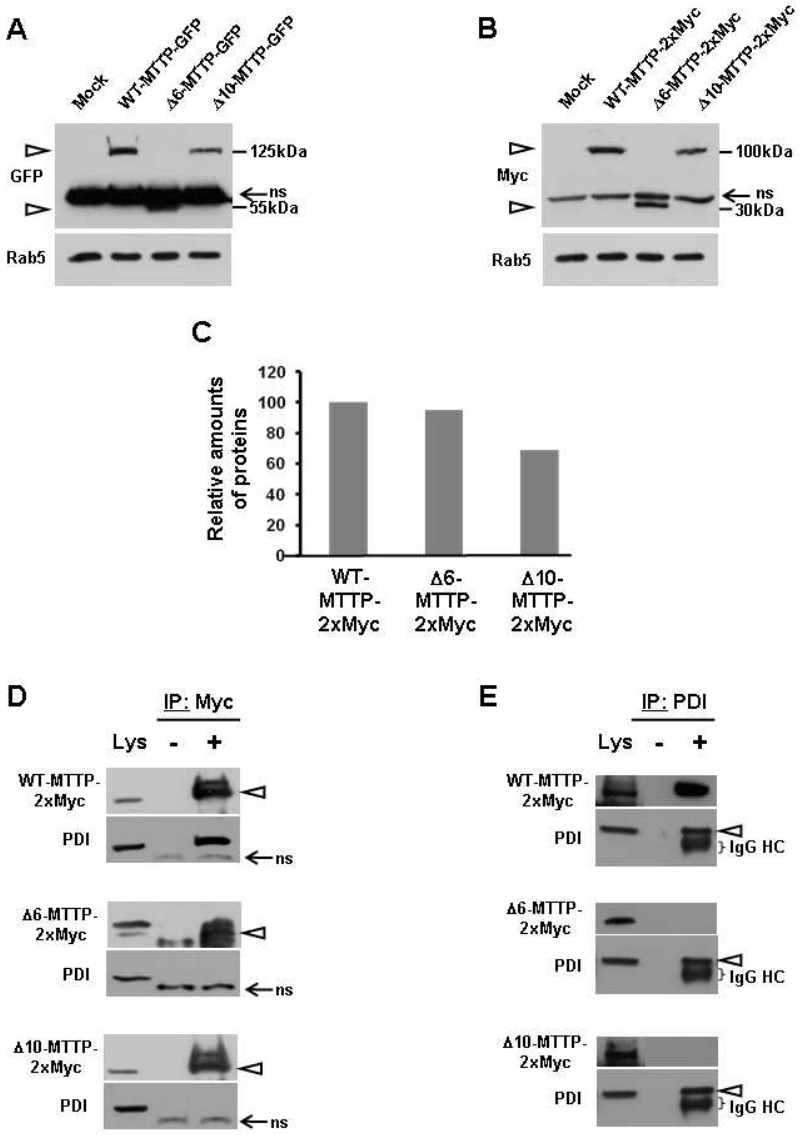
190x254mm (96 x 96 DPI)

Figure 3



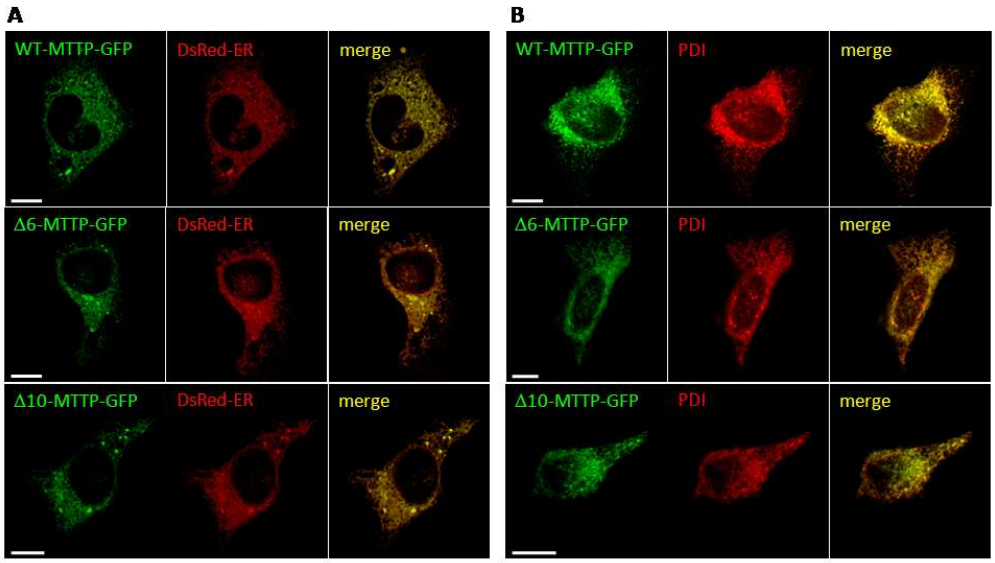
190x254mm (96 x 96 DPI)

Figure 4



190x254mm (96 x 96 DPI)

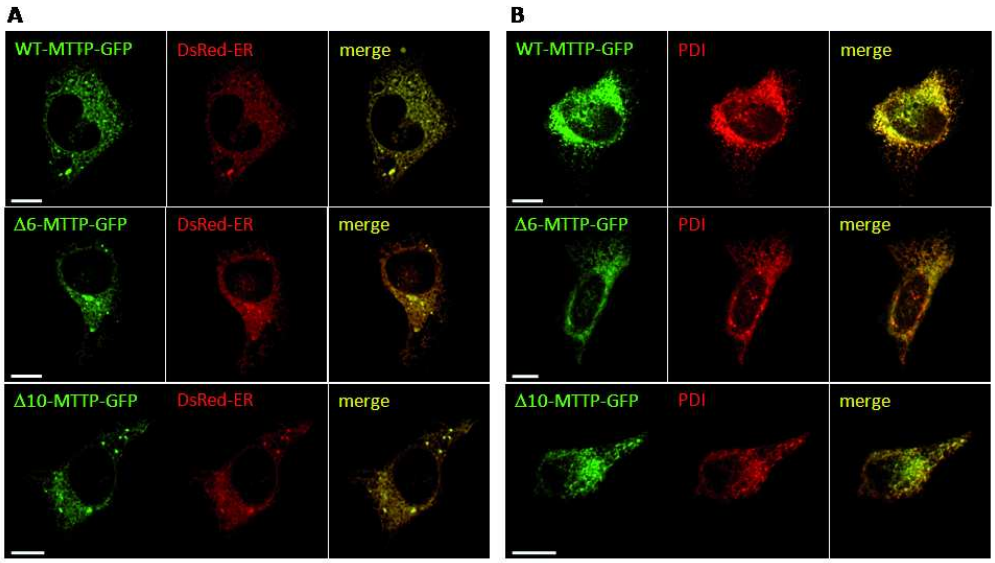
Figure 5



254x190mm (96 x 96 DPI)

Review

Figure 5



254x190mm (96 x 96 DPI)

Review

Supplementary Table S1. Description of *MTTP* mutations reported in the literature

exon/intron mutations	exon/intron mutations	consequency	number of patients	references
intron 9, c.1237-1G>A, maternal unidisomy	intron 9, c.1237-1G>A, maternal unidisomy	abnormal splicing site, exon 10 deletion	1	Yang, et al., 1999
exon 13, c.1820delG	exon 13, c.1820delG	abnormal reading frame	1	Wang, et al., 1999
exon 16, c.2237G>A	exon 18, c.2524A>T	p.G746E / p.K842X	1	Wang, et al., 1999
exon 12, c.1619G>A	exon 12, c.1619G>A	p.R540H	1	Wang, et al., 1999
exon 18, c.2593G>T	exon 18, c.2593G>T	p.G865X	7	Benayoun, et al., 2007 Ricci, et al., 1995 Wang, et al., 1999
exon 10, c.1342A>T	intron 12, 1769+1G>A	p.K448X / abnormal splicing site	1	Wang, et al., 1999
exon 12, c.1769G>T	exon 12, c.1769G>T	p.S590I	2	Al Shali, et al., 2003 Wang, et al., 1999
exon 11, c.1389delA	exon 11, c.1389delA	abnormal reading frame	1	Ohashi, et al., 2000
exon 4, c.419insA	exon 4, c.419insA	abnormal reading frame	3	Al Shali, et al., 2003 Berthier, et al., 2004 Narcisi, et al., 1995 Ohashi, et al., 2000
exon 13, c.1783C>T	intron 15, c.2218-2A>G	p.R595X, abnormal splicing site	1	Ohashi, et al., 2000
exon 16, c.2338A>T	exon 16, c.2338A>T	p.D780Y	1	Ohashi, et al., 2000
exon 9, c.1228delCCCinsT	exon 9, c.1228delCCCinsT	abnormal reading frame	1	Di Leo, et al., 2005
intron 9, c.1237-1G>A	NA	abnormal splicing site, exon 10 deletion	1	Di Leo, et al., 2005
exon 9, c.1151 A>C	exon14, c.1982 G>C	p.D384A / p.G661A	1	Di Leo, et al., 2005
exon 15, c.2212delT	exon 15, c.2212delT	abnormal reading frame	3	Benayoun, et al., 2007 Narcisi, et al., 1995
intron 1, c.148-2A>G	intron 1, c.148-2A>G	abnormal splicing site	2	Benayoun, et al., 2007
exon 3, c.307A>T	exon 3, c.307A>T	p.K103X	1	Benayoun, et al., 2007
large deletion of 481 kb	large deletion of 481 kb	loss of MTP and 8 other genes	1	Benayoun, et al., 2007
exon 15, c.2076-39_2303+52del319	exon 15, c.2076-39_2303+52del319	exon 15 deletion	1	Vongsuvanh, et al., 2007
exon 2, c.215delC	exon 2, c.215delC	abnormal reading frame	1	Sharp, et al., 1993
exon 13, c.1783C>T	exon 13, c.1783C>T	p.R595X	2	Chardon, et al., 2009 Sharp, et al., 1993
exon 17, c.2346insACTG	exon 17, c.2346insACTG	abnormal reading frame	1	Heath, et al., 1997
exon1, c.59del17	exon1, c.59del17	abnormal reading frame	1	Chardon, et al., 2009
exon 5, c.582C>A	exon 5, c.582C>A	p.Cys194X	2	Chardon, et al., 2009
intron 5, c.619-3T>G	intron 5, c.619-3T>G	abnormal splicing site, exon 6 deletion	1	Najah, et al., 2009
exon 8, c.923G>A	exon 8, c.923G>A	p.W308X	1	Najah, et al., 2009
exon 10, c.1237-1344del107	exon 12, c.1619G>A	exon 10 deletion / p.R540H	1	Rehberg JBC 1996
exon 9, c.1147delA	exon 9, c.1147delA	abnormal reading frame	1	Narcisi, et al., 1995
intron 10, c.1344+5del7	intron 10, c.1344+5del7	abnormal splicing site	1	Narcisi, et al., 1995
exon 4, c.419insA	exon 11, 1401insA	abnormal reading frame	1	Narcisi, et al., 1995

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intron 13, c.1867+1G>A	intron 14, c.1990-1G>A	abnormal splicing site / abnormal splicing site	1	Narcisi, et al., 1995
exon 4, c.419insA	intron 13, c.1867+5G>A	abnormal reading frame / abnormal splicing site	1	Narcisi, et al., 1995
intron 13, c.1867+5G>A	intron 13, c.1867+5G>A	abnormal splicing site	2	Narcisi, et al., 1995 Shoulders, et al., 1993
intron 1, 147+2T>C	exon 4, c.419insA	abnormal splicing site / abnormal reading frame	1	Dische, et al., 1970
intron 1, 147+1G>C	exon 12, c.1692T>C	abnormal splicing site / p.I564T	1	Sakamoto, et al., 2006
exon 6, c.619G>T	intron 9, c.1237-28A>G	abnormal splicing site / abnormal splicing site	2	this report

Supplementary Table S2. PCR primers

	sens	antisens	amplicon (bp)
exon 10	5' GGAGAGGTTTCTCTATGCCT 3'	5' GCCAGTTGCTGACAGCATTT 3'	997
nested exon 10	5' ATCCCAATGAAGAACTCCTGAGA 3'	5' CAAAGAGGATGGCTGACATACC 3'	888
exon 6	5' GACCTACCAGGCTCATCAAGACA 3'	5' GATGTCTGGAAAGCAGGCTGC 3'	294
nested exon 6	5' GGCCTTGGATTCATGCAAAATAGCG 3'	5' GCCTGCTTCGGTTGTCTTCAGC 3'	228

Supplementary Table S3. Genomic mutations of *MTTP*

***MTTP* locus**	**Allele 1**	**Allele 2**
AM	c.619G>T	c.1237-28A>G
PM	c.619G>T	c.1237-28A>G
Father	c.619G>T	normal
Mother	normal	c.1237-28A>G
Translation product	**exon 6 deletion**	**exon 10 deletion**

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Supplementary Figure S1: Localization of WT and mutant MTTP

HeLa cells (A) or HepG2 cells (B) were transfected with GFP-tagged WT-MTTP, Δ6-MTTP or Δ10-MTTP and were then processed for immunofluorescence. Bar: 10µm.

Supplementary Figure S2: Localization of WT and mutant MTTP and Golgi marker

HeLa cells were transfected with GFP-tagged WT-MTTP, Δ6-MTTP or Δ10-MTTP and were then processed for immunofluorescence using anti-Golgin 97 antibody. Bar: 10µm

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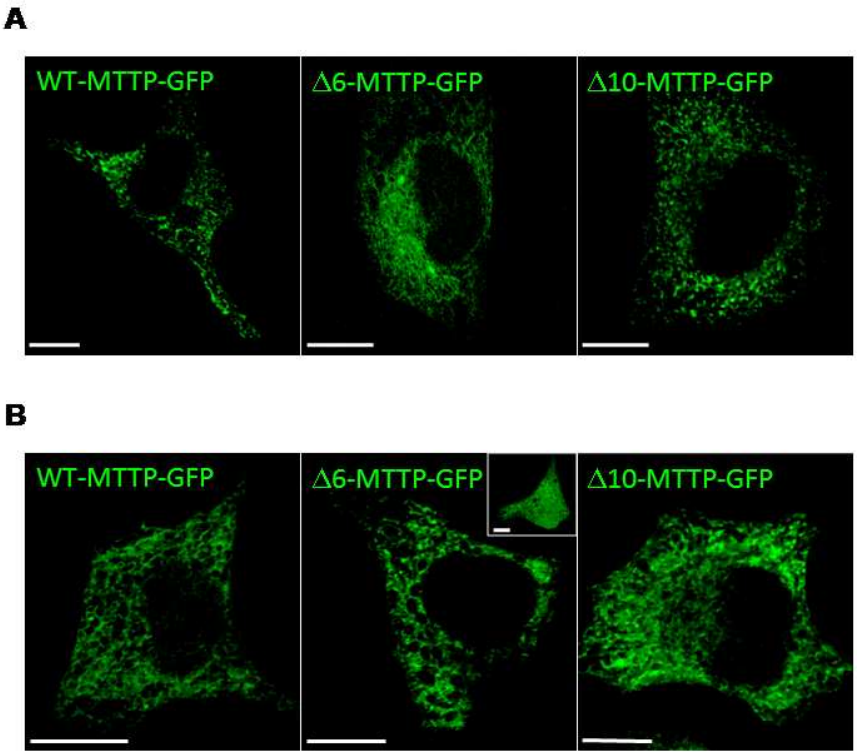
Supplementary Figure S1



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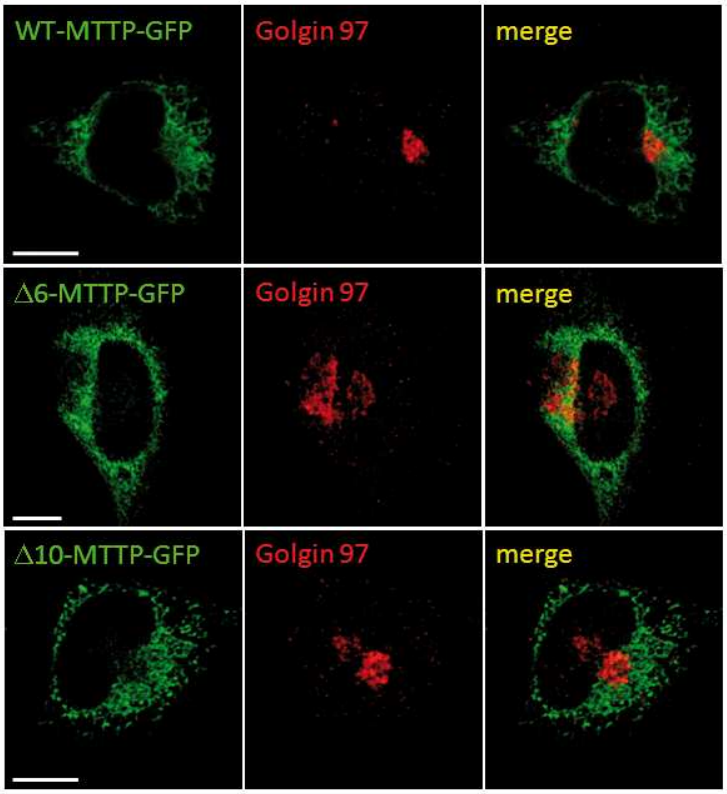
Supplementary Figure S2



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