Novel mutations in the microsomal triglyceride transfer protein gene causing abetalipoproteinemia

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Abstract **Abetalipoproteinemia (ABL) is an inherited disease characterized by the virtual absence of apolipoprotein B (apoB)-containing lipoproteins from plasma. Only limited numbers of families have been screened for mutations in the microsomal triglyceride transfer protein (MTP) gene. To clarify the genetic basis of clinical diversity of ABL, mutations of the MTP gene have been screened in 4 unrelated patients with ABL. Three novel mutations have been identified: a frameshift mutation caused by a single adenine deletion at position 1389 of the cDNA, and a missense mutation, Asn780Tyr, each in homozygous forms; and a splice site mutation, 2218-2A→G, in a compound heterozygous form. The frameshift and splice site mutations are predicted to encode truncated forms of MTP. When transiently expressed in Cos-1 cells, the Asn780Tyr mutant MTP bound protein disulfide isomerase (PDI) but displayed negligible MTP activity. It is of interest that the patient having the Asn780Tyr mutation, a 27-year-old male, has none of the manifestations characteristic of classic ABL even though his plasma apoB and vitamin E were virtually undetectable. These results indicated that defects of the MTP gene are the proximal cause of ABL.**—Ohashi, K., S. Ishibashi, J-i. Osuga, R-i. Tozawa, K. Harada, N. Yahagi, F. Shionoiri, Y. Iizuka, Y. Tamura, R. Nagai, D. R. Illingworth, T. Gotoda, and N. Yamada. **Novel mutations in the microsomal triglyceride transfer protein gene causing abetalipoproteinemia.** *J. Lipid Res.* **2000.** 41: 1199–1204.

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Abetalipoproteinemia (ABL) is an autosomal recessive disorder characterized by the virtual absence of apolipoprotein B (apoB)-containing lipoproteins from plasma (1). Affected individuals develop a wide spectrum of symptoms including retinitis pigmentosa, spinocerebellar degeneration, and a bleeding tendency due to malabsorption of vitamin K. Symptoms observed in some patients with homozygous ABL are indistinguishable from those with homozygous hypobetalipoproteinemia (HBL), an autosomal codominant disorder arising from molecular defects in apoB (2, 3).

Wetterau, Sharp, Shoulders et al. (4–6) have delineated that ABL is caused by molecular abnormalities in microsomal triglyceride transfer protein (MTP). MTP forms a heterodimer with protein disulfide isomerase (PDI), an ubiquitous endoplasmic reticulum (ER) resident protein involved in disulfide bond formation (7) and functions in the assembly of apoB-containing lipoproteins. Without MTP, as in the case of ABL, apoB is unable to be properly lipidated and undergoes rapid presecretory degradation (8–11). On the basis of its homology with vitellogenin (VTG), an ancient transport and storage lipoprotein found in egg-laying vertebrates, the MTP molecule is predicted to consist of three major structural domains: N-terminal β-barrel (residues 22–297), middle α-helical (residues 298–603), and C-terminal domains (residues 604–894) (12, 13). The N-terminal β-barrel domain mediates the interaction with the N terminus of apoB (13); the middle α-helical domain mediates the interaction with both PDI and apoB (14, 15); and the C terminal mediates the lipid-binding and transfer catalytic activity of MTP. The first two domains are globular in shape and closely related in structure to VTG (13). Amino acids 666–736 of MTP are homologous to amino acids 389–458 of cholesteryl ester transfer protein (CETP) (16).

To date, 14 separate mutations have been described in the MTP gene and/or cDNA from patients with ABL (5, 6,

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12, 17, 18). Most of these are either major truncations or large deletions. Only one encodes a missense mutation, Arg540His, in a compound heterozygous form (14). At the present time it remains uncertain whether MTP deficiency is the sole cause of ABL and it is possible that other defects in the assembly and secretion of apoB-containing lipoproteins cause expression of the clinical phenotype of ABL.

In the present study we have investigated a cohort of 2 Japanese and 2 American patients with ABL for defects in MTP. Mutations of the MTP gene, including 1 missense mutation, were identified in both alleles of all individuals. The missense mutation is the second to be reported and was located in the middle of the C-terminal domain, the region that contains a putative lipid-binding and transfer domain.

MATERIALS AND METHODS

Clinical data

Patient 1: A 32-year-old Japanese female (150 cm, 42 kg) was identified during routine cholesterol screening as having extremely low cholesterol levels (**Table 1**). Her parents were consanguineous; their laboratory data were not available. Her sister was normolipemic with low plasma apoB levels (Table 1). Except for notable intolerance for fat-rich meals, her development was uneventful. Neurological examination revealed absent ankle and knee jerks and positive Romberg's sign. But she had normal nerve conduction velocities. Fine mottling was noted in the retina. Marked acanthocytosis with a hemoglobin level of 12.8 g/dL was found. The duodenal mucosa appeared white endoscopically and the cytoplasm of the enterocytes was vacuolated.

Patient 2: A 27-year-old Japanese male (173 cm, 66 kg) with no history of steatorrhea or other medical problems was found to be extremely hypolipemic during a routine medical examination (Table 1). His parents were consanguineous; their laboratory data were not available. His general health had been excellent. Neither neurological nor ophthalmologic abnormalities were found. Acanthocytosis and mild fatty liver were detected; otherwise, his laboratory data were normal.

Patients 3 and 4: Clinical characteristics of patients 3 and 4 were described previously (19); patients 3 and 4 were cases 1 and 2 in the original article, respectively. In brief, patient 3 had shown intermittent diarrhea and steatorrhea since birth and the diagnosis of ABL was made at age 15, at which time he presented with loss of deep tendon reflexes, an ataxic gait, and a positive Romberg's sign. Patient 4 was diagnosed at 7 months of age, when he was evaluated for failure to thrive. Both patients have been managed by restricting dietary intake of long-chain fats and supplementation of fat-soluble vitamins A, E, and K. There was no evidence of consanguinity in either family. Plasma lipid profiles of these patients and their parents are shown in Table 1.

Informed consent was obtained from the subjects, and the procedures described in this study were approved by ethics committees of the University of Tokyo (Tokyo, Japan) and the Oregon Health Sciences University (Portland, OR).

PCR amplification and sequencing of the MTP gene

Genomic DNA was extracted from either blood leukocytes (patients 1 and 2) or skin fibroblasts in culture (patients 3 and 4). The promoter, 189 bp of 5′ flanking sequences, and all 18 exons and their associated exon–intron junctions of the MTP gene were amplified by polymerase chain reaction (PCR) according to Narcisi et al. (12). PCR products were directly sequenced by the dideoxy chain-termination method on an ABI 373 DNA sequencer (Perkin-Elmer Cetus, Applied Biosystems, Foster City, CA). Each mutation was verified by sequencing the PCR products from 6 independent amplifications. Compound heterozygosity of patient 4 was established by sequencing the subcloned PCR fragments.

PCR-restriction fragment length polymorphism

For easy screening of the Asp780Tyr missense mutation, a pair of mismatched primers was designed so that the PCR product from the mutant allele specifically contains an *Ssp*I restriction site. The primer sequences were as follows: forward primer, 5′-ACACAACTCAAATGGAATTATCTACAGCAG-3′; reverse primer, 5′-TAAATGTATAATGCATAAACACTTACCAAT-3′.

Construction of the mutant MTP cDNA

Site-directed mutagenesis was performed by recombinant PCR (20). The template was pTARGET/MTP, which contain a cDNA sequence encompassing the entire coding region of MTP from the plasmid pSV7D/MTP (a gift from C. C. Shoulders and J. Scott, MRC Molecular Medicine Group of Hammersmith Hospital, London, UK) (10) in the mammalian expression vector pTARGET (Promega, Madison, WI). The fragment spanning nu-

TABLE 1. Clinical characteristics of patients with abetalipoproteinemia

Family (Country)	Age/Sex	Cholesterol	Triglyceride	HDL-cholesterol	ApoB	Vitamin E
				mg/dL		*mg/L*
Family 1, Japan						
Proband	32/F	42	0.2	36	0.9	<1
Sister	34/F	140	43	65	46	12
Family 2, Japan						
Proband	27/M	34	2.6	23	0.6	<1
Family 3, America						
Proband	15/M	25	5	—	0	1–2
Father		208	98	—	114	—
Mother		315	96	—	154	—
Family 4, America						
Proband	0.6/M	30	5	—	0	1.1
Father		227	210	—	107	—
Mother		138	83	—	61	—

Ages of the probands are at the diagnosis. Clinical data of families 3 and 4 are adopted from ref. 19.

cleotides 1818–2342 was amplified by primer set 1: forward primer, MPCR1, 5′-CCGTTTCTCCAGGAGTGGATC-3′; reverse primer, 16M3′, 5′-CTATATTCACTCGGGTTTTAGACT-3′. The fragment spanning nucleotides 2318–2751 was amplified by primer set 2: forward primer, 16M5′, 5′-AGTCTAAAACCCGAGTGAAATATAG-3′; reverse primer, cDNA11, 5′-ACTTAGTCATGCCACATTGT-3′. The two PCR products were mixed to form a heteroduplex and a second PCR was performed with the primers MPCR1 and cDNA11, yielding a fragment spanning nucleotides 1818–2751. The fragment was restricted with *Pfl*MI and was cloned back into the partially digested pTARGET/MTP.

Transient expression of recombinant MTPs in Cos-1 cells

Plasmids encoding wild-type or mutant MTP were transfected into Cos-1 cells by electroporation as previously described (12). Approximately 48 h after transfection, the cells were washed three times in ice-cold phosphate-buffered saline (PBS) and harvested in homogenization buffer (5) (50 mM Tris [pH 7.4], 50 mM KCl, 5 mM EDTA, leupeptin [5 μg/mL], 2 mM phenylmethylsulfonyl fluoride). Soluble microsomal content was isolated as described (4). HepG2 cells were used as a positive control.

Assay of MTP activity

MTP activity was measured according to Wetterau et al. (4) and Leiper et al. (10). Lipid transfer activity is expressed as the percentage of the total $[^{14}C]$triglyceride transferred from donor small unilamellar vesicles (SUVs) to acceptor SUVs in a 1-h assay as a function of the amount of protein.

Immunoblot analyses

Samples were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel (3–15%) electrophoresis in a reducing condition. After transfer to a polyvinylidene difluoride (PVDF) membrane, immunoblot analyses were performed with a kit (ECL; Amersham, Arlington Heights, IL), using a rabbit anti-human MTP antibody (a gift from C. C. Shoulders and J. Scott, MRC Molecular Medicine Group of Hammersmith Hospital) (12).

Immunoprecipitation with anti-PDI antibody

After transfection, the cells were harvested and homogenized in 15/40 buffer (15 mM Tris [pH 7.4], 40 mM NaCl, 1 mM EDTA, 0.02% NaN$_3$) (4). The homogenates were solubilized with sodium deoxycholate as described (4), and ultracentrifuged at 105,000 g for 1 h. The supernatants (~1 mL) were mixed with 5 μl of protein G–Sepharose 4 Fast Flow beads (Amersham Pharmacia Biotech, Piscataway, NJ)) to which 2 μg of the anti-bovine PDI antibody (rabbit polyclonal IgG; Upstate Biotechnology, Lake Placid, NY) or 5 μl of nonimmune rabbit serum had been absorbed. The mixture was incubated at 4°C for 4 h on a rotary shaker and the beads were extensively washed with 15/40 buffer. The resulting immunoprecipitates were further subjected to immunoblot analyses with the anti-MTP antibody as described above.

RESULTS

Four unrelated ABL individuals have been screened for mutations in the MTP gene. Three mutations in a homozygous form (patients 1–3) and two mutations in a compound heterozygous form (patient 4) were identified (**Fig. 1**). Of these, three are novel mutations.

Frameshift mutations

Sequence analysis of the PCR products amplified from DNA of patient 1 revealed a single adenine deletion in exon 11, within a run of five adenines between nucleotides 1385 and 1389 of the MTP cDNA. This deletion causes a frameshift after the codon for amino acid 462 and generates a premature termination codon (UGA) after coding 14 amino acids in the new reading frame. Patient 1 was homozygous for the deletion.

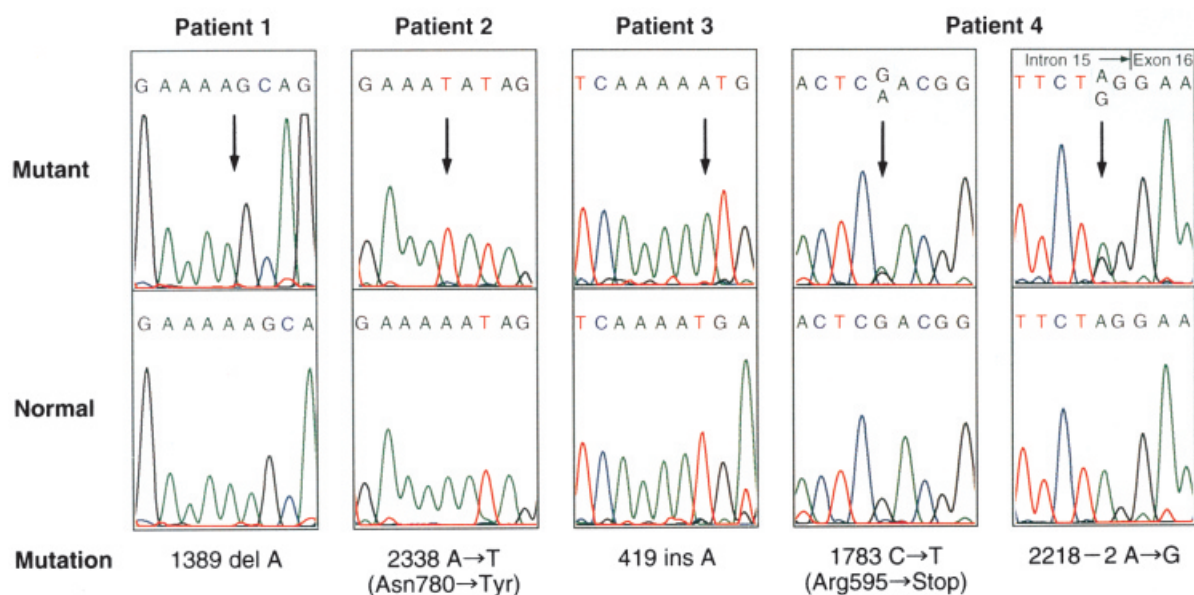Patient 3 was found to be homozygous for an insertion of a single adenine in exon 4, between positions 419 and

Fig. 1. Identification of the mutations in the MTP gene from abetalipoproteinemic subjects. All sequences were determined on both strands by direct sequencing of the amplified DNA and the regions across a mutation were sequenced on the separately amplified DNAs. Note that the chromatogram of the 1783C→T mutation in patient 4 shows the sequence of the antisense strand. del, Deletion of a specified nucleotide; ins, insertion of a specified base. Mutations are indicated by arrows.

420 of MTP cDNA. This insertion is located within a run of four adenines. The mutation results in the generation of a new reading frame after amino acid 140 and causes a premature stop codon (UGA) one amino acid into the new reading frame.

Nonsense mutation

Patient 4 was found to have a C-to-T transition in exon 13, at nucleotide 1783 in one allele of the MTP gene. This changes an arginine codon (CGA) to a premature stop codon (TGA). A truncated translation product of 594 amino acids is predicted.

Splice site mutation

The second mutation in patient 4 was shown to be a single A-to-G substitution at the second to last position of the 3′ acceptor splice site of intron 15. This substitution alters the invariant AG dinucleotide consensus sequence at a splice acceptor site, from 5′-AG:GAACTT-3′ to 5′-GG:GAACTT-3′. Alteration of this invariant dinucleotide sequence is associated with aberrant splicing that could cause either exon skipping, activation of a nearby cryptic splice site, or the production of an incompletely spliced mRNA (21). The first would give rise to an mRNA that lacks exon 16, nucleotide 2218–2342, resulting in a new reading frame after the codon for amino acid 739 and a premature stop codon. Failure to splice intron 15 would give rise to an mRNA encoding 751 amino acids, 12 arising from the unspliced intron.

Missense mutation

Patient 2 was homozygous for each of the reported polymorphic sites (12, 14) and the only sequence abnormality present involved a single A-to-T mismatch at nucleotide 2338 of the cDNA, in exon 16. This substitution results in the conversion of an asparagine to a tyrosine at position 780 of the protein, which is in the middle of its carboxyl-terminal domain (amino acids 603–894). None of 50 alleles from unrelated normal Japanese subjects carried the mutation, ruling out the possibility that it is a common polymorphism.

Functional characterization of the mutant MTP

To examine whether the Asn780Tyr missense mutation leads to a nonfunctional protein, we expressed the mutant cDNA in Cos-1 cells and measured MTP activity (**Fig. 2**). Even though comparable amounts of MTP protein were present in the cell lysates, the cells expressing the Asn780Tyr mutant had negligible levels of MTP activity compared with Cos-1 cells expressing the wild-type MTP, or HepG2 cells. After subtracting the background levels of activity present in the mock-transfected Cos-1 cells, it was calculated that the mutant MTP displayed negligible MTP activities compared with wild-type MTP. These results indicate that the Asn780Tyr mutant MTP is functionally defective. It is important to note that the mutant MTP was recovered as soluble protein. Because binding with PDI is believed to be essential for solubility of the protein, the interaction between the mutant and PDI seemed to be in-
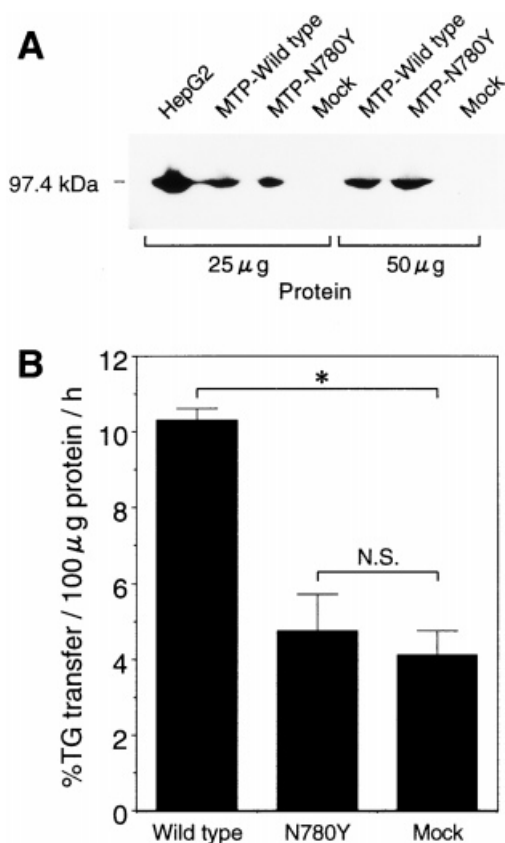

Fig. 2. Transient expression of normal and mutant MTP in Cos-1 cells. Expression constructs containing either normal or mutant (Asn780Tyr) MTP cDNA were transfected into Cos-1 cells and expressed for 48 h. Cell homogenates were used for immunoblot analyses (A) and for measurement of triglyceride transfer activities (B). Transfer activities are expressed as the percentage of the total [^{14}C]triglyceride transferred from donor to acceptor membranes in 1 h. Columns and bars represent means $\pm$ SD (n = 3). * $P < 0.0001$ by ANOVA. N.S., Not significant.

tact. To confirm the proper association between the Asn780Tyr mutant and PDI, immunoprecipitation experiments were carried out with anti-PDI antibody. As shown in **Fig. 3**, both the wild-type and the mutant MTP were coimmunoprecipitated by anti-PDI antibody, supporting the notion that the binding with PDI was intact in the mutant protein.

DISCUSSION

The results presented here support the view that mutations of the MTP gene are the primary cause of ABL in both Japanese and American patients. Two mutations identified herein, the exon 4 insertion and the exon 13 nonsense mutation, have been described in other families (12). The exon 4 insertion mutation was previously found in a white American family of British origin, an English and an Anglo/Irish family. Because a similar family background was observed in patient 3 (19), the families with the exon 4 insertion may have common ancestors. The other reported patient who shares the exon 13 mutation
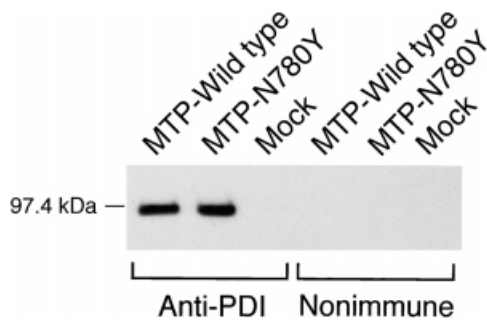
Fig. 3. The mutant MTP is associated with PDI. Expression constructs containing either normal or mutant (Asn780Tyr) MTP cDNA were transfected into Cos-1 cells and expressed for 36 h. Cell lysates were immunoprecipitated with either anti-PDI antibody or nonimmune serum. The immunoprecipitates were subjected to immunoblot analyses for MTP. Both the wild-type and the mutant protein were detected in anti-PDI immunoprecipitates, but not in the nonimmune precipitates. This result indicated that the Asn780Tyr mutant was coimmunoprecipitated by anti-PDI antibody, supporting the notion that the binding with PDI was intact in the mutant protein.

was homozygous for the change, whereas our patient was a compound heterozygote. It is plausible that the patients with the mutation come from common ancestors (12).

Three mutations have been newly identified: one frameshift, one splice site, and one missense. Because both frameshift and splice site mutations theoretically produce severely truncated proteins, it is reasonable to consider that these mutant MTP proteins are functionally defective. Clinically, it is noteworthy that the sister of patient 1 and the mother of patient 4, who were supposed to be obligatory heterozygotes having one allele of the nonfunctional MTP protein, were relatively hypocholesterolemic and had low plasma apoB levels (Table 1). These observations are apparently against the notion that ABL is an autosomal recessive disease (1). Raabe et al. (22) reported that heterozygotes of MTP knockout mice had reduced plasma apoB levels, supporting the possibility that human MTP deficiency is inherited in a codominant manner at least in some families.

On the other hand, it is possible that the MTP with a missense mutation, Asn780Tyr, is functional. To examine this possibility, we performed expression experiments; the results showed that triglyceride transfer activity was markedly reduced in the cells expressing the Asn780Tyr mutant MTP compared with those expressing the wild-type MTP, even though equivalent amounts of protein were produced from the two expression vectors. In view of the fact that this mutation was not found in 50 alleles from unrelated normal Japanese subjects, we conclude that Asn780Tyr is a functionally defective missense mutation. It is noteworthy that this is the first report of a patient who is homozygous for a missense mutation that is also the second missense mutation reported to date.

The other missense mutation identified so far changes Arg-540 to histidine (14). Ricci et al. (17) reported another subtle mutation, a 30-amino acid truncation of the carboxyl terminus of the 894-amino acid protein. In both cases, the mutations disrupted the proper association with PDI, which is thought to be essential for solubility of the

protein, and the mutant proteins became insoluble. In contrast, the Asn780Tyr mutant MTP was recovered as soluble protein in our expression experiments and was coimmunoprecipitated with PDI by anti-PDI antibody. These findings indicate that the association with PDI was maintained in the Asn780Tyr mutant.

Residue 780 is in the middle of the carboxyl terminus of the protein, the region that contains a putative lipid-binding domain. Asparagine and tyrosine are both neutral amino acids, but the latter contains a hydrophobic aromatic ring. The change in hydrophobicity in this region might affect the tertiary structure of the carboxyl terminus of MTP, thus disrupting its lipid-binding capabilities. To date, no missense mutation affecting lipid binding/transfer has been reported. We tried to demonstrate whether the mutant protein bound lipids by employing the method described by Jamil et al. (23). However, we failed to detect significant triglyceride binding that was specific for MTP, probably because the amounts of the expressed MTP proteins were not large enough in our experimental system in which Cos-1 cells were used. Further studies are needed to determine the lipid-binding capacity of the mutant MTP.

From a clinical standpoint, patient 2, who was homozygous for the Asn780Tyr mutation, is of particular interest because he was free from any of the accepted clinical manifestations characteristic of ABL despite having undetectable levels of plasma vitamin E (Table 1). Usually ABL patients develop neurological symptoms within the first or second decade of life (1). It seems reasonable to assume that the missense mutations, like the one found in patient 2, may encode a protein with residual lipid transfer activity in vivo and may therefore account for his milder or atypical phenotype. The patient's plasma concentrations of vitamin E were as low as those seen in other ABL patients, yet the vitamin E content in organs, particularly in the retina and nervous system, may be higher, masking the development of neurological symptoms. Alternatively, his vitamin E consumption might be relatively high, again delaying the development of the neurological symptoms.

In conclusion, we present three novel mutations in the MTP gene leading to ABL. These results provide further insight into the genetic basis of the phenotypic diversity of ABL.

NOTE ADDED IN PROOF

While this manuscript was under revision, six novel mutations including two missense mutations, Ser590Ile and Gly746Glu, were reported in Canadian subjects with ABL (24). No expression studies were provided concerning the missense mutations.

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