

## Original Article

# Identification of a Novel *HADHB* Gene Mutation in an Iranian Patient with Mitochondrial Trifunctional Protein Deficiency

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## Abstract

**Introduction:** Mitochondrial trifunctional protein (MTP) is a hetero-octamer composed of eight parts (subunits): four  $\alpha$ -subunits containing LCEH (long-chain 2,3-enoyl-CoA hydratase) and LCHAD (long-chain 3-hydroxyacyl CoA dehydrogenase) activity, and four  $\beta$ -subunits that possess LCKT (long-chain 3-ketoacyl-CoA thiolase) activity which catalyzes three out of four steps in  $\beta$ -oxidation spiral of long-chain fatty acid. Its deficiency is an autosomal recessive disorder that causes a clinical spectrum of diseases.

**Materials and Methods:** A blood spot was collected from the patient's original newborn screening card with parental informed consent. A newborn screening test and quantity plasma acylcarnitine profile analysis by MS/MS were performed. After isolation of DNA and Amplification of all exons of the *HADHA* and *HADHB*, directly Sequence analyses of all exons and the flanking introns both of genes were performed.

**Result:** Here, we report a novel mutation in a patient with MTP deficiency diagnosed with newborn screening test and quantity plasma acylcarnitine profile analysis by MS/MS and then confirmed by enzyme analysis in cultured fibroblasts and direct sequencing of the *HADHA* and *HADHB* genes. Molecular analysis of causative genes showed a missense mutation (p.Q385P) c.1154A > C in exon 14 of *HADHB* gene.

**Conclusions:** Since this mutation was not found in 50 normal control cases; so it was concluded that c.1154A > C mutation was a causative mutation. Phenotype analysis of this mutation predicted pathogenesis which reduces the stability of the MTP protein complex.

**Keywords:** Deficiency, fatty acid oxidation, *HADHA*, *HADHB*, mitochondrial trifunctional protein (MTP), novel mutation

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## Introduction

Fatty acid oxidation is the main source of energy for heart and muscles. Mitochondrial Trifunctional Protein (M-TFP) is bound to the inner mitochondrial membrane which catalyzes three out of four steps in  $\beta$ -oxidation of long-chain fatty. This protein is a hetero-octamer composed of eight parts (subunits): four  $\alpha$ -subunits containing LCEH (long-chain 2,3-enoyl-CoA hydratase) and LCHAD (long-chain 3-hydroxyacyl CoA dehydrogenase) activity, and four  $\beta$ -subunits that possess LCKT (long-chain 3-ketoacyl-CoA thiolase) activity.<sup>1</sup> M-TFP deficiency is classified into 2 phenotypes: the more prevalent isolated LCHAD deficiency (OMIM: 609016) with defects of the  $\alpha$ -subunits encoded by the *HADHA* (OMIM: 600890) (hydroxyacyl-CoA dehydrogenase  $\alpha$ -subunit) gene and the less common pattern of complete M-TFP deficiency (OMIM: 609015).<sup>2</sup> Generally, all 3 enzyme activities of the M-TFP complex are undetectable in M-TFP deficiency due to defects of both *HADHA* and *HADHB* (hydroxyacyl-CoA dehydrogenase  $\beta$ -subunit) genes (OMIM: 143450).<sup>3</sup> *HADHA* and *HADHB* genes are located in the same region on chromosome 2p23 (Respectively ENSG00000084754, ENSG00000138029). Mitochondrial trifunctional protein deficiency is an autosomal recessive disorder

that causes a clinical spectrum of diseases.<sup>4</sup> Symptoms of mitochondrial trifunctional protein deficiency may present in infancy or later in life. Features that occur during infancy comprise lack of energy (lethargy), feeding difficulties, hypoketotic hypoglycemia provoked by prolonged fasting, weak muscle tone (hypotonia), lactic acidemia, acute renal failure, and fulminant liver failure. That patients who present in infancy are also at high risk for breathing difficulties, serious heart problems, coma, and sudden unexplained infant death. Some patients survive into adolescence and adulthood with hypotonia, muscle aches, damaged skeletal muscle tissue (Rhabdomyolysis) and impaired sensation in the extremities (peripheral neuropathy).<sup>5</sup> The most common defect of mitochondrial trifunctional protein is isolated LCHAD deficiency (LCHADD), and it occurs when the activity of the enzyme "long chain 3-hydroxyacyl-CoA dehydrogenase" (LCHAD) is reduced with substantial preservation of the other two MTP enzymatic activities.<sup>6</sup>

The incidence of LCHAD deficiency is unknown and this disorder can occur in anyone of any ethnic background.<sup>7</sup> Based on an American population, it is estimated at approximately 1 in 75,000, with a calculated carrier frequency of 1 in 138.<sup>8</sup> In Europe, the prevalence is probably much lower, approximately 1 in 100,000.<sup>8</sup> More than 60% of patients with LCHADD have the E474Q (c.1528G > C) mutation in the gene encoding the alpha-subunit of the mitochondrial trifunctional protein. E474Q mutation replaces a glutamic acid with the glutamine at position 474 in the active site of LCHAD that is located in the C-terminal domain.<sup>9</sup> The other defect of mitochondrial trifunctional protein is complete M-TFP deficiency in which the activities of all three enzymes of the complex are disrupted. Complete M-TFP

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deficiency (TFPD) is a rare disorder and far less common than the LCHADD.<sup>5</sup> Despite the clinical and phenotypic similarities between these two disorders, there are some distinct differences in data from screening patients by tandem mass spectrometry and biochemical and genetic analysis can distinguish between LCHADD and TFPD when compared to each other.<sup>10</sup> This article describes the clinical and biochemical findings of a patient with M-TFP deficiency and one novel mutation of the *HADHB* genes.

## Materials and Methods

The male patient was the second born to consanguine parents in Iran. His parents were asymptomatic. They were heterozygous in terms of mutation but without any pretense of disease. In fact, based on all the studies, individuals heterozygous for this disease do not have any clinical symptoms. The newborn patient suffered from asphyxiation while nursing in the first month of his birth. He eventually died of multi-organ failure at 25 days of age. His clinical symptoms included cardiomyopathy, hepatopathy and hypoketotic hypoglycemia. A blood spot from the patient was collected from the patient's original newborn screening card with parental informed consent. All ethical considerations such as parental and all case-control consent form to maintain patient data were included in this study. A questionnaire was distributed to all controls and patient's family. Acylcarnitines were extracted with methanol and then derivatized with butanolic HCl. Butylated acylcarnitines were analyzed by tandem mass spectrometry. Urine organic acids and plasma amino acids were analyzed. For DNA mutation analysis, a blood spot (5 mL) was collected from the patient and his parents. After isolation of DNA by standard salting out method from peripheral blood leukocytes, the quality of extracted DNA was confirmed using gel electrophoresis. Then, the 20 exons and adjacent intronic consensus splice sites of the *HADHA* and 16 exon of the *HADHB* gene were amplified by polymerase chain reaction. Sixteen sets of primers for *HADHB* gene and 20 set for *HADHA* gene were designed by the authors and then all exons were amplified by polymerase chain reaction (PCR).

PCR was completed under the following conditions: 100 ng genomic DNA, 200  $\mu$ M dNTP, 1.5 mM MgCl<sub>2</sub>, 2.5 units SuperTaq polymerase (Genfanavar, Iran), and 25 pmol of each primer. PCR was performed in 25  $\mu$ L volumes and 35 cycles.

PCR conditions were as follows: an initial denaturation step at 95°C extended to 2 min, followed by 35 cycles of 95°C for 30 s, 60°C for 45 s, and 72°C for 45, and ending with one cycle at 72°C for 5 min. The PCR products were examined by electrophoresis on 1.5% agarose gels and then purification and direct sequencing were performed. Direct sequence analysis of all exons and the flanking introns of both genes were performed by means of the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) on an ABI Prism 3700 automated genetic analyzer (Applied Biosystems). The BLASTN program was used to compare the results with the reported gene sequences. Peripheral bloods (5 mL) from 50 unrelated controls without MTPD or LCHADD and fatty acid oxidation disorder were collected in EDTA tubes. DNA was extracted from whole blood samples by standard salting out protocol. PCR was carried out for exon 14 of *HADHB*, under the same conditions as performed for the patient's sample. Then, direct sequencing of *HADHB* exon 14 was performed as done for the patient. Sequencing reactions were

performed with reverse primers. Finally, to predict the possible effects of the observed mutations, an online software was used. Approved software of SIFT, Mutation Taster, Polyphen-2 and PANTHER were used to predict disease-causing mutation. Also, To predict the effects of mutations on protein stability based on the sequence, I-Mutant and MuStab software were used. To verify the effect of this mutation on tertiary structure of the protein, the Swiss model software was used and three-dimensional structure of proteins was rebuilt by Swiss model.

## Results

Acylcarnitine analysis of blood spots showed prominent accumulation of long chain hydroxylated acylcarnitines, as follows: 3-OH-palmitoylcarnitine (C16-OH), 0.76  $\mu$ mol/L (RR < 0.08  $\mu$ mol/L); 3-OH-oleylcarnitine (C18:1-OH), 0.34  $\mu$ mol/L (RR < 0.1) (Figure 1a). In addition, the level of tetradecenoylcarnitine (C14:1), 0.32  $\mu$ mol/L (RR < 0.25) was mildly elevated. The concentration of free carnitine was normal: 34  $\mu$ mol/L (method with derivitisation; RR 20 – 70  $\mu$ mol/L) (Figure 1a). Therefore, a disorder of long chain hydroxylated fatty acid oxidation (LCHAD/TFP-deficiency, Long Chain Hydroxylated Acyl-CoA-Dehydrogenase/Trifunctional Protein deficiency) was suspected.

The results of electromyography and skeletal muscle biopsy pathologic analysis in this study were not available. Since the newborn had a serious problems and died earlier, it was impossible to obtain this data and the parents were not available; so that this Electroretinogram could be performed on them. DNA analysis using genomic DNA revealed a novel homozygous missense mutation in the *HADHB* gene of the patient with MTP deficiency belonging to the second phenotype. The patient had a homozygous missense mutation "c.1154A > C - cDNA.1258A > C - g.41718A > C" mutations in the *HADHB* gene (Figure 1b). The c.1154A > C transition resulted in an amino acid substitution of Glu to PRO at codon 385 (p.Q385P) in exon 14 (reference sequence from NM\_000183.2). The patient's parents were heterozygous for this mutation.

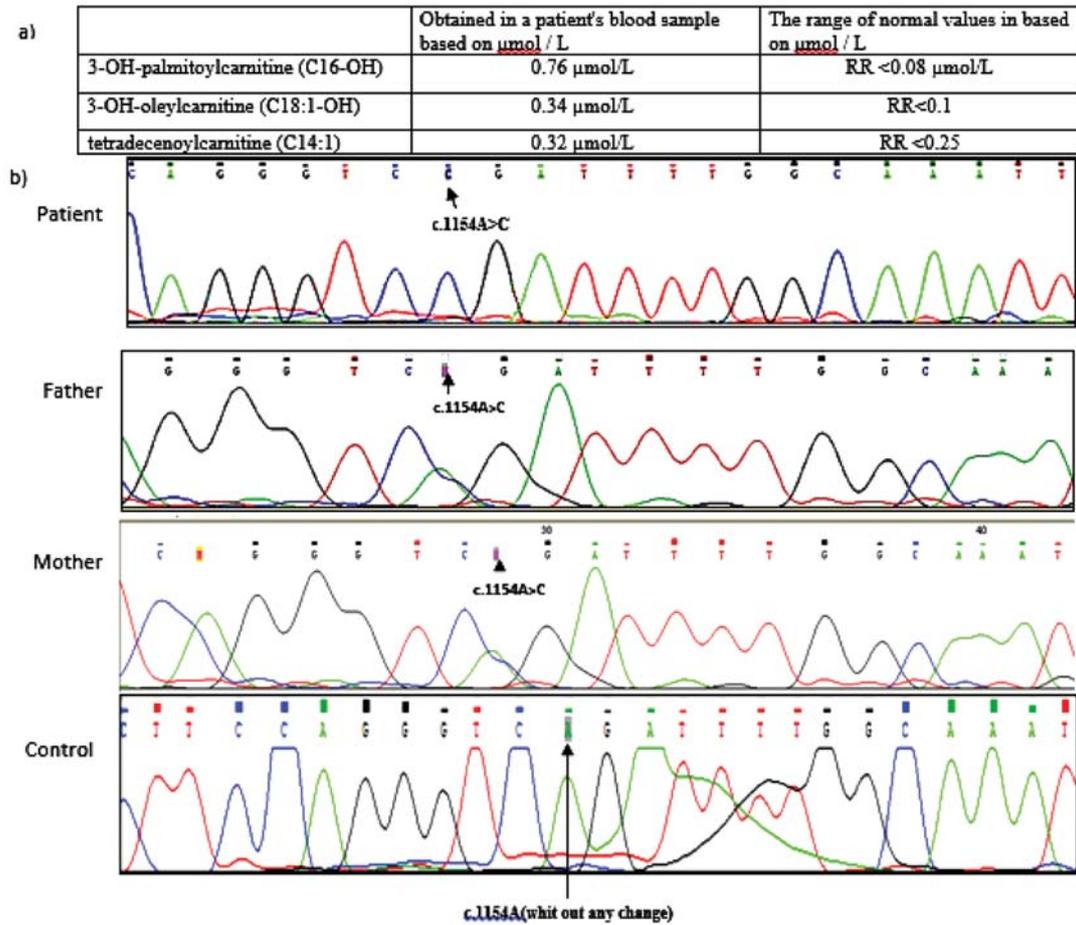
It means that, this defect in *HADHB* gene originated from the parents. This mutation has accrued in C-terminal domain that encodes the thiolase enzyme (Figure 2a).

Molecular analysis of the patient revealed that the *HADHA* gene was normal. The Q385P mutation was screened for its presence in 50 normal unrelated Iranian individuals, but no case was found.

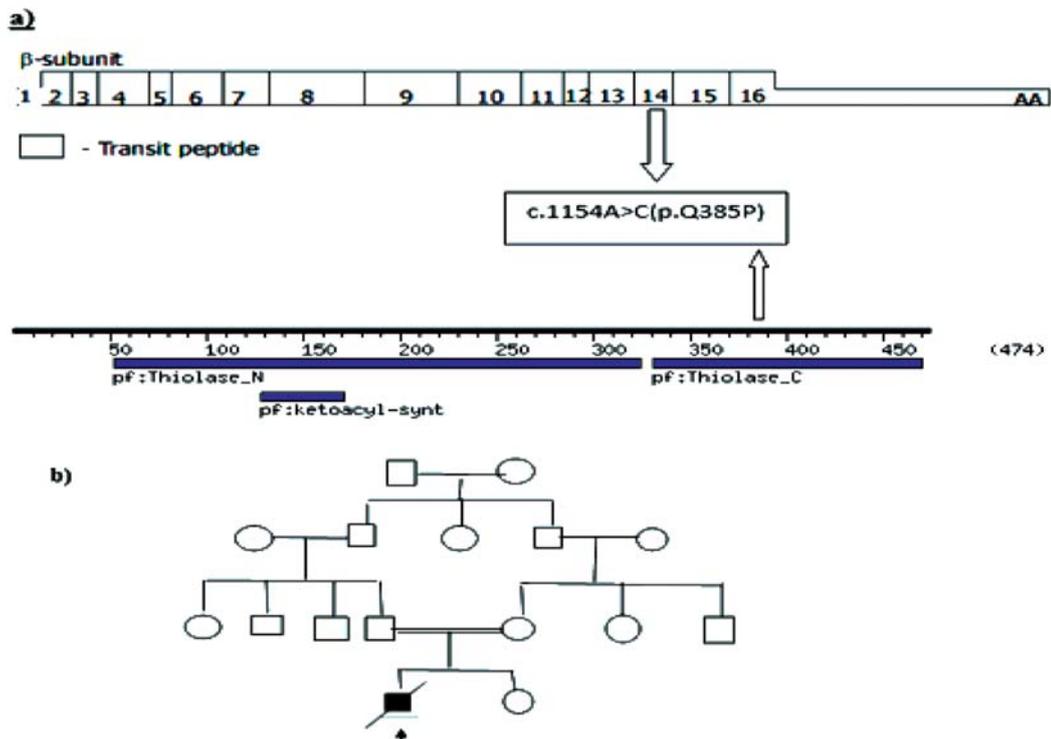
## Discussion

M-TFP deficiency is a very rare disorder and is classified into three clinical manifestations: 1) lethal phenotype which begins in the neonatal period and is quite severe; 2) hepatic phenotype which develops in infancy and is of intermediate severity, and 3) myopathy phenotype which begins in late-adolescence and is mild.<sup>11</sup>

In the deficiency, symptoms occur in organs such as cardiac muscle, the brain cells, liver and kidney based on which organs use larger amounts of energy produced by beta oxidation metabolism.<sup>12</sup> The first case with LCTH was reported in 2006.<sup>13</sup> We report a patient with thiolase deficiency caused by *HADHB* mutation.



**Figure 1.** a) Blood acylcarnitine analyses in proband; b) Sequence chromatographs of the mutated part of the *HADHB* gene in proband revealed a novel missense mutation c.1154A>C (p.Q385P) as compared to wild type. The patient's parents were carrier for this mutation. Arrows denote the mutations.



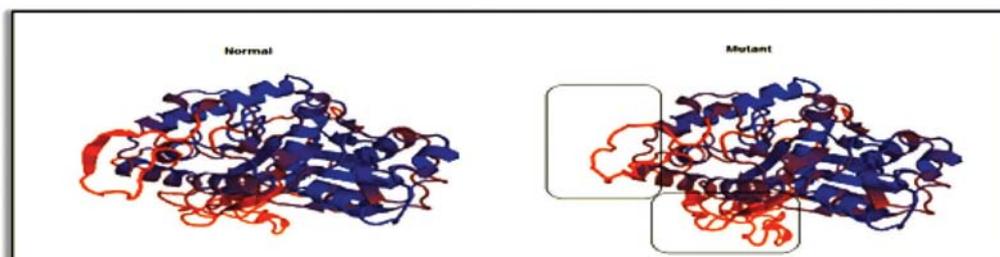
**Figure 2.** a) Schematic picture of the *HADHB* gene structure and consisting exons; b) Pedigree of the MTFP-affected family. The proband is a 25 days old boy affected by MTFP (arrow in the pedigree).

**Table 1.** HADHB mutations found in different populations.

No.	Mutation (c.DNA)*	Mutation Classification	Amino acid change	Predict protein*	Ethnicity	No. of reported families	Reference
1	c.358dupT/c.1364T>G	Compound heterozygote	p.A120Cfs8/p.V455G	Influent protein stability and function	Korean	1	(1)
2	c.1364T > G	Homozygous missense	p.V455G	Represent of protein	Japanese Korean	5	(1), (16)
3	c.919A>G/c.1165A>G	Compound heterozygote	p.N307D/p.N389D	Destabilizing the structure of the protein	Korean	2	(1), (2)
4	c.340A>G/c.919A>G	Compound heterozygote	p.N114D/p.N307D	Destabilizing the structure of the protein	Korean	2	(1), (2)
5	c.520C>T/c.1331G>A	Compound heterozygote	p.R141C/p.R411K	Destabilizing the structure of the protein	Japanese	1	(17)
6	c.1175C>T	Homozygous missense	p.A392V	Decreased or absent activity of protein	Japanese	2	(18)
7	c.210-1G/ c.686G-T	Compound heterozygote	intron4/R229L	Decreased or absent activity of protein	Korean	2	(19)
8	c.788A>G	Homozygous missense	p.D263G	Destabilizing the structure of the protein	Japanese	1	(20)
	c.182G>A/c.740G>A	Compound heterozygosity	p.R61H/p.R247H.	Destabilizing the structure of the protein	Japanese	1	
9	c.1331 G >A/c.777 ins T	Compound heterozygosity	p.R411K/ G226-P237 del	Destabilizing the structure of the protein	Japanese	1	(5)
	c.1331 G >A	Homozygous missense	p.R411K	Destabilizing the structure of the protein	Japanese	1	
10	c.739C > T/ c.817 del G	Compound heterozygosity	R214C/Truncation at c.259)	Destabilizing the structure of the protein	Japanese	1	(16)
	c.181C>T	Homozygous missense	p.R28C	Destabilizing the structure of the protein	Japanese	1	
11	c.685C>T	Homozygous missense	p.R196X	Destabilizing the structure of the protein	Japanese	1	(21)
	c.182G>	Homozygous missense	p.R28H	Destabilizing the structure of the protein	Japanese	1	
	c.A527C>G	Homozygous missense	p.S143X	Destabilizing the structure of the protein	Japanese	1	
	c.788A>G	Homozygous missense	p.D230G	Destabilizing the structure of the protein	Japanese	1	
	c.362T>C	Homozygous missense	p.L88P	Destabilizing the structure of the protein	Japanese	1	
	c.693delC/ 881C>G	Compound heterozygosity	p.198 frameshift/ p.P261R	Destabilizing the structure of the protein	Japanese	1	
	c.725A>G/	Homozygous missense	p.D209G	Destabilizing the structure of the protein	Japanese	1	
	c.839G>A	Homozygous missense	p.G247D	Destabilizing the structure of the protein	Japanese	1	
	c.182G>A/ 740G4A	Compound heterozygosity	p.R28H/p.R214H	Destabilizing the structure of the protein	Japanese	2	
	c.181C>T /c.349A>G	Compound heterozygosity	p.R28C/p.R84G	Destabilizing the structure of the protein	Japanese	2	
	c.607C>T/c.881C>T	Compound heterozygosity	p.R170X/p.P261L	Destabilizing the structure of the protein	Japanese	2	
c.176G>T/C.740G>A	Compound heterozygosity	p.G26D/p.R214H	Destabilizing the structure of the protein	Japanese	1		
c.397A>G/c.881C>G	Compound heterozygosity	p.T100P/p.P261L	Destabilizing the structure of the protein	Japanese	1		

**a)**

Nucleic acid change	Amino acid change	exon	Consequence	Mutation taster prediction	SIFT	Polyphen-2	MuStab	PANTHER	I-Mutant3.0
c.1154A>C	p.Q385P	14	missense	pathogenic	damaging	damaging	decreased stability	deleterious	decreased stability

**b)**

**Figure 3.** Damaging novel mutation identified in *HADHB* gene in patient with MTP; **a)** Anticipated results of SIFT, PolyPhen-2 predict a damaging and PANTHER indicate destructive pathogenic mutation in exon 14 of the *HADHB* gene and analysis software MutationTaster, MuStab and I-Mutant3.0 indicate pathogenesis and reduce the stability of the MTP protein complex; **b)** Comparison of spatial structure of the normal protein *HADHB* and protein contains the p.Q385P substitution using the software Swiss model. Modified points marked with a black rectangle. This mutation has accrued in C-terminal domain of *HADHB* gene that encodes thiolase enzyme

According to the reports, missense mutations in *HADHB* gene are associated with milder myopathic phenotypes.<sup>14</sup> In this study, we report a novel mutation in exon 14 *HADHB* gene (p.Q385P). In other word, the c.1154A > C mutation has never been reported, and is thus novel. In patients with defects in MTP, the type of mutations identified in *HADHB* gene, the type of compound heterozygote or homozygote and phenotypic clinical symptoms are varied. In most cases, children have clinically significant symptoms in childhood which often develop in children under three years of age. Most mutations identified in the *HADHB* gene are compound heterozygous or of missense type, which reduces the stability of the protein complex. Also, the patient's visible symptoms in other studies are similar to those reported in ours (Table1).

According to various studies conducted in the world, the prevalence of this disease is under 1%. In case of a disease occurring with very serious problems, it has been shown that the disease is not usually seen in the normal population. In the case of this mutation, it was not identified in 50 normal controls which was foreseeable.

We concluded that c.1154A > C mutation was a causative mutation. Prediction softwares predicted that the mutation mentioned above in the Results section is a functional missense mutation in exon 14 of the *HADHB* gene. The rated output by software SIFT ranges from zero to one, with 0.00 indicating destructive and 1 showing natural variations. If the score is less than 0.05, SIFT detects amino acid substitution as malicious and points higher than 0.05 are tolerable.<sup>15</sup> According to the results shown in Figure 3a, this tool suggests that this alternative is destructive. Polyphen-2 is an automatic operation interpreter for the coding region SNP. 1.000 score in this online tool represents a high probability of damage caused by mutations. The score given by this software is 1.000. According to the rating assigned by

the software PolyPhen-2, Q385P mutation is a very destructive replacement. Based on the results obtained using I-Mutant-0.3, it is estimated that the mutation reduces the stability of the complex structure of the MTP. The program forecasts are divided into three categories: neutral range of  $0.5 > DDG > -0.5$ , a sharp fall in the stability for  $-0.5 < DDG$  and a sharp rise for  $0.5 < DDG$ . According to the results reported in this study, the c.1154A > C mutation causes a sharp decline in throughput MTP protein structural stability. MuStab and I-Mutant use machine learning methods (SVM) to predict changes in protein stability by replacing amino acids. Mutation Taster results also suggest decreased protein stability in the presence of the mutation reported in this study.

By comparing the normal protein space with mutated protein by means of Swiss model, significant changes were observed that have an enormous impact on the normal function of the protein. During the course of evolution, the amino acids that are essential for the activity of a particular protein are preserved. According to our results, substitution of Q385P alters the stability of enzyme structure because of altering total energy of the structure. On the other hand, replacement of proline of 385, could not be tolerated and impairs the function and activity of the MTP complex enzyme. The marginal chain of glutamine is polemic and without load. Glutamine with a polemic chain without load and a hydrophath quotient of -3.5 pertain to rather hydrophilic amino acids. Based on comparing the marginal chain of the amino acid and R groups of non-polemic amino acids, glutamine is more hydrophilic. This is due to the factorial group of amid which can create hydrogen bond with water. Proline consists of an aliphatic marginal chain and an annular configuration. The hydrophath quotient of this amino acid is 1.6. Hydrophath quotient shows the hydrophobicity or the range of hydrophilic feature of an amino acid and its higher value for an amino acid indicates its higher hydrophobicity. As a result, Glutamine is more hydrophilic than proline and proline is

quite hydrophobic.

Hydrophilic amino acids tend to be located around the outer layers of a protein in order to form hydrogen bonds with water molecules. In contrast, amino acids with a non-polar group are not exposed on the surface layer of a protein facing the solution and the hydrophobic marginal chains tend to gather inside the protein and far from water. Water avoidance of hydrophobic layers creates an entropic pushing force needed for bending and configuration of the tertiary structure of the protein.

The reported situated mutation in this study causes the conversion of glutamine to proline and replacement of a non-polar amino acid (replacement of a quite hydrophilic with a hydrophobic amino acid). This conversion of amino acids in hydrophilic layers of the protein can cause instability and ultimately affect the configuration and function of the protein.

On the other hand, the presence of proline can negatively affect the spiral system of alpha helix. The amine group in proline has a serious confirmation and is a part of a very firm chain and thus orbiting around N-C $\alpha$  bond in proline is impossible. Also, the amine group in proline does not have a hydrogen on the  $\alpha$  amino group, so it cannot form a hydrogen bond to stabilize an  $\alpha$  helix or a  $\beta$  sheet. Furthermore, the amino group lacks the hydrogen needed for forming hydrogen bonds with other moieties. Probably, the protein cannot tolerate these changes and so the protein encounters enzymatic malfunctions. According to the findings from the clinical tests, the results of sequencing and lack of the mutation in 50 normal samples studied, the Q385P mutation in MTP complex enzyme is pathogenic. There is no report about the mutation in the NCBI and Ensemble database showed that this mutation is novel. Using the enzyme analysis in cultured fibroblasts that reveal the enzymatic activities of components of MTP, we can confirm that the *HADHB* mutation affects the function and stability of the protein.

Thereby, distinguishing between isolated LCHAD and complete M-TFP deficiency by MS/MS is unclear. So, the diagnosis must be confirmed by mutation analysis of causative genes.

In conclusion, we screened a patient with complete M-TFP deficiency by MS/MS and confirmed the diagnosis with identification of a novel mutation in the *HADHB* gene. We hope that recognition of a genetic mutation will pave the way for a conceivable prenatal molecular diagnosis in family members at risk.

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