Malonyl-CoA decarboxylase deficiency: Long-term follow-up of a patient new clinical features and novel mutations

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Abstract

Background: Malonyl-CoA decarboxylase (MLYCD, EC 4.1.1.9) deficiency is a rare autosomal recessive disorder that is widely diagnosed by neonatal screening. Methods: We report long term follow up of a patient with MLYCD deficiency showing signs of neonatal hypoglycemia, mental retardation, developmental delay and rheumatoid arthritis. Brain MRI revealed patchy, symmetrical hyperintensity of the deep white matter with periventricular white matter and subcortical arcuate fibers being spared. MLCYD gene sequence analysis was done to identify possible mutations. Expression analyses at mRNA and protein levels were also performed. Further, immunocytochemical studies were implemented to check for its subcellular localization. Results: MLYCD gene sequencing identified a novel compound heterozygous mutation (c.22 T>A, p.M1K, c.454 C>A; pH152N) in our patient and a heterozygous mutation in the healthy mother c.22 T>A; pM1K. Reduced expression of RNA and protein levels was observed. Immunocytochemical analysis showed diffused staining across the cytoplasm with apparent signs of intracellular mislocalization to the nucleus. Results also indicated subcellular colocalization of MLCYD with mitochondria was scant compared to control. Conclusion: Our patient was identified with a novel compound heterozygous MLYCD mutation at the N-terminal helical domain. This study indicates that protein mislocalization is a characteristic feature of MLYCD deficiency in our patient.

Keywords: Malonyl CoA decarboxylase; Mitochondrial disorder; Mental retardation; Low fat diet therapy; Rheumatoid arthritis

1. Introduction

Malonyl-CoA, a product of acetyl-CoA carboxylase (ACC) is a metabolic intermediate in lipogenic tissues that include liver and adipose tissue, where it is involved in the de novo fatty acid synthesis and elongation [1]. In nonlipogenic tissues as cardiac and skeletal muscle, cytosolic malonyl-CoA acts as a key regulator of mitochondrial fatty acid β-oxidation by potentially inhibiting carnitine palmitoyl transferase 1 (CPT), thus maintaining the balance between lipid and glycogen metabolism [1]. Malonyl-CoA decarboxylase (MLYCD, E.C. 4.1.1.9), a 55 kDa enzyme catalyzes the conversion of malonyl-CoA to acetyl-CoA and carbon dioxide thus providing a route for disposal of malonyl-CoA from mitochondria and peroxisomes, whereas in the cytosol the malonyl-CoA pool is regulated by the balance of MLYCD and acetyl-CoA carboxylase activities [1]. Earlier studies demonstrated that Malonyl-CoA could substantially inhibit the other isoforms of CPT, CPT1B in the heart [2] and CPT1C in the brain [3].
MLYCD is encoded by the gene *MLYCD* localized on chromosome 16q24 [4,5]. The highest tissue specific expression of human MLYCD mRNA was found in the heart and skeletal muscle and to a lesser extent in liver, kidney and pancreas [6]. Expression of rat MLYCD has been detected in at least the mitochondria and cytosol, and possibly in peroxisomes as well [7]. However, despite of numerous studies, the subcellular localization of MLYCD has always been a controversy with emergence of conflicting study reports [6–8].

Human MLYCD deficiency (OMIM 248360) also known as malonic aciduria is a rare autosomal recessively inherited inborn error of fatty acid metabolism that is characterized by cardiomyopathy, seizures, hypoglycemia, mental retardation, developmental delay and in some cases neonatal death [9–13]. Although central nervous system (CNS) manifestations are characteristic for MLYCD deficiency, little is still known about the role of MLYCD in the brain. In an adult rat brain, MLYCD expression was found in neurons and microglia of the frontal cortex and hippocampus. In the cerebellum MLYCD labelling was detected in several cell types but not in neurons [14]. Recent studies by Forese et al. show that the crystal structure of MLYCD constitutes two domains, an N-terminal helical domain that is involved in oligomerization and a C-terminal catalytic domain [15]. The elucidated structure gives us a better understanding of the various mutations in the *MLYCD* gene causing the deficiency [15].

Several cases have been reported in literature [7–12,16–24] regarding the metabolic crisis associated with MLYCD deficiency, albeit very little is known about the long term outcome of such cases through subsequent dietary therapy prescribed [22,24–25]. Here we describe the long term follow up of a patient affected by malonic aciduria upon neonatal onset. Molecular analysis of the *MLYCD* gene showed two novel heterozygous mutations. Concurrently we also present the evidence of mislocalization of MLYCD that we hypothesize could be due to the compound heterozygous mutation within the predicted mitochondrial targeting sequence.

### 2. Case presentation

Our 17 year old patient is the daughter of healthy non-consanguineous Finnish parents, was born at 39 weeks by C-section due to breech presentation. Family history shows one healthy boy and an early miscarriage. Birth weight was 3800 g (height-50 cm, head circumference-37.5 cm) with Apgar score 10. Neonatally, the baby had feeding difficulties, failure to thrive and somnolence on day 3. Patient had hypoglycemia 2.1 and 2.6 mmol/L, respectively and she had two series of focal seizures, which responded to fenobarbitone. Plasma ammonia was normal (36 μmmol/L) and alanine transferases were ALAT (<30 U/L) normal.

Electroencephalogram showed centroparietal focal spikes, but normal background. Brain computer tomography revealed nonspecific findings: hypodensic white matter and brain magnetic resonance imaging was normal.

At the age of 6 months, she had a generalized developmental delay, hypotonia, mild motoric asymmetry, and potential loss of acquired skills. Urinary organic acids screening showed increased excretion of malonic acid (110 mmol/mol creatinine; reference <20 mmol/mol creatinine) and methyl malonic acid (5 mmol/mol creatinine; reference <5 mmol/mol creatinine). Repeated fasting amino acid analysis did not show any significant abnormalities and cerebrospinal fluid amino acids were normal. Plasma creatine kinase was normal and did not show any significant difference from the controls.

When she was 18 months old, development was delayed, accompanied by hypotonia, mild dystonia, and a delayed mental development. At 2 years (yrs), her cerebral spinal fluid (CSF) malonate concentrations were considerably higher compared to serum malonate which indicates cerebral production of malonate (Table 1). At the age of 3 and 4 years, the child had developed muscle hypotonia and mild ataxia and able to produce few words but had difficult in understanding abstract matters suggesting a mild mental retardation. The audiometry was normal and had clonic reflexes. She was given dietary therapy from low fat to high-fat diets, as the high fat diet increased malonic aciduria so a moderate low fat diet was continued (Fig. 1A).

At 12 years of age her brain MRI imaging revealed normal spinal fluid spaces and symmetrical hyperintensity of the deep and periventricular white matter. Corpus callosum was thin but there was not a uniform constriction of U-fibres. Subcortical white matter spaces appeared to be constricted. The short echo spectrum showed increased myoinositol, which suggests glial cell damage (Fig. 1B). At 16 years of age, the patient was diagnosed with rheumatoid arthritis, which was HLA-B27 positive, RF negative, and had an increased anti-nuclear antibody 320, but no signs of iritis.

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<td>Methylmalonate and malonate levels in serum and Cerebral spinal fluid (CSF) of patient.</td>
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3. Materials and methods

3.1. Cell Culture and genomic DNA extraction

This study was approved by the Helsinki University ethical committees and informed consent from patient parents. Human skin fibroblasts from the patient, her parents and control were grown in DMEM medium with 10% fetal bovine serum, 2 mM l-glutamine, 0.1 mg/mL streptomycin and 100 U/mL penicillin (Lonza, USA). Genomic DNA (gDNA) was isolated from the cultured fibroblasts using the flexi gene DNA kit from QIAGEN (QIAGEN Venlo, Netherlands). DNA concentration was determined by the absorbance at 280 nm. The primers were designed using Primer3 software based on the reference sequence of MLYCD (MLYCD gene Entrez GeneID 23417 and the mRNA NM_012213).

3.2. PCR amplification and mutation screening

PCR was used to amplify the MLYCD gene from gDNA using standard PCR reaction mixtures for all the exons except exon 1 which is a high GC rich region which was amplified in two overlapping fragments (Exon1a and Exon 1b) with 1.5 M Betaine and 5% DMSO. Exons 2-5 PCR products were purified using the PCR purification kit (ExoSAP-IT®/C210 Affymetrix) (California, USA) and Exon 1 by QIAGEN® Gel extraction kit (QIAGEN®) due to its high GC rich region and sequenced using the ABI BigDye™ terminator sequencing kit (ABI, Foster City, CA).

3.3. Reverse transcription quantitative real-time PCR (RT-qPCR)

Total RNA was prepared by using Trizol (Sigma–Aldrich, Missouri, USA) and QIAGEN™ RNA mini preparation kit (QIAGEN™) according to the manufacturer’s instructions. The quality of the RNA was assessed by gel electrophoresis and its purity was determined from the A260:A280 ratio by using NanoDrop spectrophotometer (NanoDrop Technologies, USA). The RNA was treated with Dnase enzyme (DNase I) to get rid of any traces of DNA according to the manu-
facturer’s instructions. cDNA synthesis was performed by using the 200U Superscript II RT™ (Invitrogen, UK) Oligo (dT)12-18 reagents (Invitrogen) according to the manufacturer’s instructions. RT-qPCR was performed using the Biorad CFX96 system (Biorad, California, USA). Samples were used in triplicates. Relative change in gene expression was calculated by $2^{-\Delta\Delta C_T}$ method. Student $t$-test was performed to determine $P$ value and statistical significance was accepted at $p < 0.05$.

### 3.4. Western blotting

Protein extraction from fibroblasts was performed as described earlier [26]. About 50 μg protein extracts was subjected to SDS–PAGE. Protein concentration was determined by Lowry’s method with BSA as the standard [27]. MLYCD detection was done with rabbit anti-MLYCD antibody (Proteintech, IL, USA) [1:1000] followed by HRP-conjugated donkey anti-rabbit IgG (Santacruz, USA) [1:5000]. Western blots were reprobed using goat anti-Actin antibody (Santacruz, USA) [1:1500] followed by HRP-conjugated sheep anti-goat IgG (Santacruz, USA) [1:5000]. Signal detection was performed using the ECL Western blotting detection reagent kit according to the supplier’s instructions (Perkin Elmer, USA).

### 3.5. Immunocytochemistry

Cell fixation and antibody hybridization were performed as described earlier [8]. Primary antibody used was rabbit anti-MLYCD (Proteintech, IL, USA) [1:50] followed by Alexa Fluorophore 488 goat anti-rabbit IgG (Molecular probes, Invitrogen, UK) as the secondary antibody. Mito tracker™ Red CMXRos was used as a mitochondrial marker (Molecular probes, Invitrogen, UK) according to the manufacturer’s guidelines. DAPI was used to stain nuclei (Molecular probes, Invitrogen, UK). Cover slips were mounted in anti-fade gold reagent (Invitrogen, UK). Digital images were captured with a standard exposure time of 0.5 s, using Olympus IX81 microscope (Olympus, Pennsylvania, USA) and analysis was done by CellIR software (Pennsylvania, USA).

### 4. Results

During the 12 years follow up period, the patient was in good metabolic condition. Patient has avoided high fat diet since the time she had attained 15 months of age. Urinary organic acids screening showed decreased levels of malonic and methylmalonic acid levels on low fat or low protein diet whereas the levels increased after intake of high fat diet (Fig. 1A).

Sequencing analysis of $MLYCD$ gene revealed 2 heterozygous mutations: one in exon 1a (c.22 T>A, p.M1K) region of the second methionine codon within the first start codon and the other in the exon 1b (c.454 C>A, p.H152N) (Fig. 1C). No mutations were found upon sequence analysis of exons 2, 3, 4 and 5. The heterozygous mutation in exon 1a was also detected in mother DNA (Fig. 1C) but not in father and control DNA (Fig. 1C). RT-qPCR was used to compare $MLYCD$ mRNA expression in patient and control fibroblasts. Significant down regulation was observed in the patient $MLYCD$ mRNA expression levels compared to control relative to GAPDH expression (used as a house keeping gene) (Fig. 2A). Similarly Western blotting resulted in low $MLYCD$ expression in patient fibroblasts than in control (Fig. 2B).

To further examine the $MLYCD$ subcellular localization and the effect of the possible targeting mutation, immunocytochemical analysis was carried out on patient and control fibroblasts (Fig. 3A–F). Mitochondrial labelling did not show any major difference between the samples. Anti-MLYCD staining pattern showed a significant difference with the patient fibroblasts exhibiting a diffused staining with occasional dense aggregates while control fibroblasts not showing any abnormal pattern. Interestingly, in patient fibroblasts $MLYCD$ staining was observed not only in the cytoplasmic region but also in the nucleus (including nucleoli) which is not the case with the control fibroblasts where staining was restricted only to the cytoplasmic region (Fig. 3). However, in control fibroblasts the anti-MLYCD staining to mitochondrial marker detected colocalization but very little colocalization was observed in patient fibroblasts.

![Fig. 2. (A) Analysis of MLYCD mRNA by RT-qPCR. mRNA levels were decreased significantly in Pat than in Control cells. Results are the mean ± SD ($n = 3$) (* $p < 0.05$). (B) MLYCD and Actin Western blot analysis: Extracts from MLYCD deficient patient (Pat) fibroblasts and normal fibroblasts (Ctrl) were probed with anti-MLYCD antibody (55 kDa) and anti-Actin (43 kDa).](image-url)
5. Discussion

Extensive studies on MLYCD have been carried out for more than three decades. It is widely accepted that MLYCD has a number of roles to play in the cell, notably amongst them being: (a) is used as a carbon precursor by fatty acid synthase to form long-chain fatty acids (LCFA’s), (b) is required for fatty acid elongation in the endoplasmic reticulum (c) it controls the mitochondrial oxidation of fatty acids through its interaction with CPT1 [6,15]. Earlier studies have provided compelling evidence that malonyl Co-A allosterically binds to CPT1 thereby inhibiting it and subsequently leading to the non-transfer of LCFA’s into the mitochondria. This finding strongly suggests that the interaction of malonyl CoA and CPT1 is a central element of fuel cross talk signaling for various tissues like skeletal muscle, liver and heart [1,28]. Although an enormous amount of work has been done to elucidate the key roles that MLYCD plays in lipid and carbohydrates metabolism, its pathogenesis, metabolic function and subcellular localization still remains obscure [6,8,24]. So far, 34 cases with different MLYCD gene defects comprising point mutations, stop codons and frame-shift mutations have been reported in literature [8–13,16–24] Notably, Wightman et al., have reported that a point mutation in the MLYCD gene lead to protein mislocalization at the subcellular level [8].

Metabolic screening of our patient showed elevated urinary excretion of malonic acid, methylmalonic acids and malonylacylcarntines thus confirming MLYCD deficiency. A high level of malonate was also detected in the patient CSF thereby indicating that it was due to the lack of MLYCD enzyme [29]. Similarly, 11 cases were reported implicating brain abnormalities in MLYCD disorder [8–13]. Our patient’s MRI brain images show a periventricular white matter and thin corpus callosum and prominent dentate nuclei. This may be due to accumulation of organic acid CoA esters and neonatal hypoglycemia causing a neurotoxic effect in the brain [19,23,30]. Previous studies have demonstrated that CPT1c is involved in the metabolism of specific brain lipids and in maintaining energy homeostasis [31,32]. So there is a possibility of disturbed interactions between CPT1c and malonyl CoA thus being the cause for increased levels of malonate in the CSF of our patient. Clinical features in patients with malonic aciduria are variable and no phenotype-genotype relationship has been identified [19,23]. Earlier no MLCYD case was reported with rheumatoid arthritis and she was the first patient who diagnosed.

The genome sequencing of MLYCD revealed five exons and three possible start codons, of which only 2 start codons could be functional as they are primarily located in the open reading frame region. Further analysis unravelled two novel heterozygous mutations in the sequence, the first being identified in the second ATG codon (c.22 T>A; p.M1K) and the other (c.454 C>M; p. H152N) in the later exon 1 region. The second mutation might well be a de novo one as such because sequence analysis of MLYCD in the father did not reveal any mutation. RT-qPCR results indicated that MLYCD mRNA level significantly reduced compared to control. Further, protein expression analysis of

Fig. 3. Immunocytochemistry of MLYCD: MLYCD subcellular localization in fibroblasts. Anti-MLYCD staining, Mitotracker and merging of both the images: Green indicates localization of MLYCD, red indicates mitochondria localization; in the merged image: yellow indicates areas where both MLYCD and mitochondria localized (A) Control cells (Ctrl) Anti-MLYCD staining (B) Mitotracker staining (C) Merging images A and B is showing MLYCD to co-localize with mitochondria along with DAPI in blue (D) Patient cells (Pat) Anti-MLYCD staining (E) Mitotracker staining (F) Merging images D and E is showing partial MLYCD co-localization with mitochondria, MLYCD green also seen in nucleus blue (arrow).
fibroblasts by Western blotting in addition to immunocytochemical staining strongly confirmed MLYCD deficiency. Albeit, the occurrence of a compound heterozygous mutation, Western blotting analysis reveals low MLYCD expression levels, understandably the third methionine codon acts as a start site in transcription. Furthermore, immunocytochemical studies revealed a diffused staining of MLYCD with nuclear staining illustrating a mislocalization of the protein in patient fibroblasts. Subcellular co-localization studies revealed that the MLYCD was more colocalized with mitochondria in control fibroblasts while it was very scant in the case of patient fibroblasts. This discrepancy can be explained by the fact that it might well be due to the two heterozygous mutations detected in the patient. As mentioned earlier, based on the crystal structure of MLCYD [15,33], the two mutations reported in our patient at the N terminal domain (p.M1K and p.H152N) that lie within the predicted mitochondrial targeting sequence might affect the protein localization and it is very likely that these mutations might disrupt protein folding [15]. Several studies indicate that MLYCD is localized to the cytoplasm, peroxisomes, and mitochondria and has two N-terminal regions [29]. However, to date its localization has been a controversial issue because of its canonical sequence in the peroxisomal targeting sequence [4]. Colocalization studies showed that MLYCD was localized to mitochondria in control cells, which were concordant with earlier studies in rat liver showed that MLYCD was expressed in mitochondria and peroxisomes [7]. It suggests that mistargeting could be a partly responsible for variable MLYCD deficiencies as earlier shown by Wightman group [8]. Rightly so, enzyme activity assays of MLYCD did not show any major differences between the controls and patient further associating mistargeting of the protein to disease pathology in our patient. As our results indicate mistargeting of MLYCD to the nucleus, it would be quite tempting to implicate it in the regulation of transcription of some other nuclear factors under such circumstances, but at this stage there is no evidence as such, so it would be premature to conclude if MLYCD has any critical role to play in the nucleus. Further our results are supported by earlier studies that have demonstrated protein mislocalization to be a major cause for a wide range of human diseases that include both neurodegenerative and metabolic disorders [34].

In our patient, treatment with low fat diet showed improved metabolic levels and a better clinical status. Although some mutations [this study, 8] result in unusual compartmental targeting, further studies are required to identify the phenotype-genotype relationship and deeper understanding of underlying pathogenic mechanisms that cause MLYCD deficiency. It is also of primary importance that future studies are necessary to investigate the role of metabolic pathways in brain development associated MLYCD deficiency. Finally it can be concluded that although MLYCD shows to have a normal activity as indicated by the biochemical results, mislocalization to the nucleus might be the major cause for the metabolic disorder, thereby associating it with the disease pathology in our patient.

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Conflict of interest

None.

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References


