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Experimental research

New case of mitochondrial HMG-CoA synthase deficiency. Functional analysis of eight mutations

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ABSTRACT

Mitochondrial HMG-CoA synthase deficiency is a rare inherited metabolic disorder that affects ketone-body synthesis. Acute episodes include vomiting, lethargy, hepatomegaly, hypoglycaemia, dicarboxylic aciduria, and in severe cases, coma. This deficiency may have been under-diagnosed owing to the absence of specific clinical and biochemical markers, limitations in liver biopsy and the lack of an effective method of expression and enzyme assay for verifying the mutations found. To date, eight patients have been reported with nine allelic variants of the *HMGCS2* gene. We present a new method of enzyme expression and a modification of the activity assay that allows, for first time, the functional study of missense mutations found in patients with this deficiency. Four of the missense mutations (p.V54M, p.R188H, p.G212R and p.G388R) did not produce proteins that could have been detected in soluble form by western blot; three produced a total loss of activity (p.Y167C, p.M307T and p.R500H) and one, variant p.F174L, gave an enzyme with a catalytic efficiency of 11.5%. This indicates that the deficiency may occur with partial loss of activity of enzyme. In addition, we describe a new patient with this deficiency, in which we detected the missense allelic variant, c.1162G>A (p.G388R) and the nonsense variant c.1270C>T (p.R424X).

1. Introduction

Mitochondrial 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase (mHS) (EC 2.3.3.10) catalyzes the condensation reaction between the acetyl-CoA and acetoacetyl-CoA in ketone body synthesis. Ketone bodies are produced when glucose is scarce, supplying the body, particularly the brain, with an alternative source of energy [1].

mHS deficiency (MIM 605911) is a rare autosomal recessive genetic disorder, which usually appears in the first year of life after a period of starvation, or in intercurrent illness. Initial symptoms include vomiting, lethargy and hepatomegaly [2], which can be accompanied by respiratory disease and encephalopathy [3]. If the deficiency is not treated it can evolve to coma. The biochemical data are characterized by hypoglycaemia, dicarboxylic aciduria and an increase in the levels of plasmatic free fatty acids. In some cases, metabolic acidosis has been reported [4]. Treatment involves the administration of glucose to correct the hypoglycaemia [2].

The *HMGCS2* gene (GenBank NM_005518.2), which encodes this enzyme, is located on chromosome 1 at 1p12.13 and has 10 exons

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and a size of 21,708 base pairs. Nine allelic variants have been described (7 missense, 1 nonsense and 1 splicing mutation) [3,5–8] in the eight patients diagnosed since the description of the disease in 1997 [2]. However, in a recent crystallographic study of the enzyme, nine novel mutations were identified (8 missense and 1 nonsense) [9]. The low incidence of this illness could be related to the difficulty of its diagnosis: it has no specific clinical symptoms nor characteristic biochemical markers. Moreover, the necessity of a hepatic biopsy and interference with the cytosolic HMG-CoA synthase limit the measurement of activity in tissues [2]. For these reasons, diagnosis is based on genetic analysis. However, the difficulty entailed in measuring the activity of the cloned enzyme has prevented the functional study of the mutations that cause the deficiency.

In this study, a ninth patient with mitochondrial HMG-CoA synthase deficiency is reported. Furthermore, a simple, efficient method for the expression of the enzyme is proposed, together with a modification of the spectrophotometric enzymatic assay proposed by Clinkenbeard [10]. These new procedures allowed us to carry out a functional study of the missense variants described in patients with mHS deficiency (p.V54M, p.Y167C, p.F174L, p.R188H, p.G212R, p.M307T, p.G388R and p.R500H).

2. Patient data

The patient was a 15-month-old boy, the first child of non-consanguineous Caucasian parents. He had no abnormal medical history. He became very unwell during a gastroenteritis illness and in hospital he was found to be hypoglycaemic (<1.6 mmol/L; normal blood glucose level 4 mmol/L), with hepatomegaly, abnormal liver function and elevated transaminases. After treatment with intravenous dextrose he improved. His liver dysfunction also improved. Plasma levels of acylcarnitines were normal. Two urine organic acid samples were analysed. The first showed a trace of ketones, with a marked dicarboxylic/3-hydroxydicarboxylic aciduria. The second sample was similar, with moderate ketonuria. Free fatty acids/3-hydroxybutyrate ratio was elevated >2 (normal value <2). This research was performed in accordance with the Declaration of Helsinki and informed consent for genetic analysis was obtained from the family.

3. Materials and methods

3.1. Mutational analysis

Genomic DNA was extracted from a blood sample with standard procedures. Twenty oligonucleotides to amplify ten exons of the *HMGCS2* gene and their splice junctions were used (eight previously reported [3] and twelve novel; all of them are available upon request). PCR products were purified and sequenced.

3.2. Construction of the expression plasmid pMAL-c2x-mHS

A cDNA fragment encoding mHS without the signal peptide (38–508 (amino acids)) was amplified by PCR using the primers HS2*Eco*F (5′-AAGG*GAATTC*ACAGCCTCTGCTGTC-3′) and HS2*Bam*R (5′-CTTTCCAT*GGATCC*GCAGAACAC-3′) from liver cDNA. These primers contain the *Eco*RI and *Bam*HI restriction sequence respectively. This fragment was subsequently cloned into the expression plasmid pMAL-c2x, which incorporates the codifying sequence of the fusion protein Maltose Binding Protein (MBP). The construct was transformed into the *Escherichia coli* strain XL1-Blue. Finally, positives clones were confirmed by nucleotide sequencing.

3.3. Expression and purification of the mHS

E. coli strain BL21-Codon Plus(DE3)-RIPL expressing MBP-mHS was grown in LB medium containing 0.1 mg/mL ampicillin to an A_{600} of 0.8–1.0 at 37 °C. Optimal protein expression was induced with 0.3 mM IPTG at 20 °C for 18 h. Cells were recovered by centrifugation at 4000× *g* at 4 °C for 15 min. The cells were then resuspended in lysis buffer (20 mM Tris–HCl pH 7.4, 200 mM NaCl, 1 mM EDTA, 1 mg/mL lysozyme, 10 μg/mL DNase, 10 μg/mL RNase, 100 μg/mL PMSF, 1 mM DTT, 0.1% Triton X-100 and 10% glycerol) and disrupted by thermal shock at 37 °C (15 min), −80 °C (45 min) and 37 °C (3 min). The soluble fraction containing the MBP-mHS fusion protein was loaded into an amylase affinity column, which had been washed with 3 column volumes of buffer 20 mM Tris–HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA and 1 mM DTT. The mHS was eluted from the affinity resin using a buffer containing the protease factor Xa.

3.4. Enzyme assay

Protein was quantified by Bradford's method. Mitochondrial HMG-CoA synthase activity was measured by a modification of spectrophotometric method that determines at λ 304 Å the amount of acetoacetyl-CoA consumed [10]. To prevent inactivation of the enzyme by succinylation [11] a previous step, in which the enzyme was incubated with acetyl-CoA 300 μM for 17 min at 30 °C, was added to the protocol. Activity measurements were performed in three independent experiments.

3.5. Mutants

Using PCR-based mutagenesis, all missense mutations reported in patients were induced in the wild-type mHS cDNA. Eight primers pairs were used and these are available on request. DNA sequencing of the new constructs was performed to confirm target mutations. Conditions of expression and enzymatic assay were the same as those used for the wild-type protein.

3.6. Western analysis

The mHS proteins (wild-type and mutants) separated on a 15% SDS-PAGE were transferred to a 0.45 μM nitrocellulose membrane by transfer blot. The membranes were probed with a monoclonal antibody against mHS 425–508 amino acids (1:1000 dilution) (Abnova) and revealed with chemiluminescence method using a peroxidase-conjugated secondary anti-mouse antibody (1:1000 dilution).

3.7. Molecular modelling

Structure coordinates for mHS protein were extracted from Protein Data Bank (www.rcsb.org) entry 2WYA [9] and visualized using the Pymol Molecular Graphics System, Schrödinger, LLC. Model for F174L mutant was generated using the SWISS-MODEL server [12–14] available at http://swissmodel.expasy.org, and its structural quality was checked using the analysis programs provided by the same server (Anolea/Gromos).

4. Results

A novel patient with mHS deficiency was diagnosed with the missense mutation c.1162G>A, which caused the protein change p.G388R [9], and the nonsense mutation c.1270C>T, which caused the stop codon at 424 position [6]. Inheritance was confirmed by analysis of the parents' DNA (Fig. 1A and B).

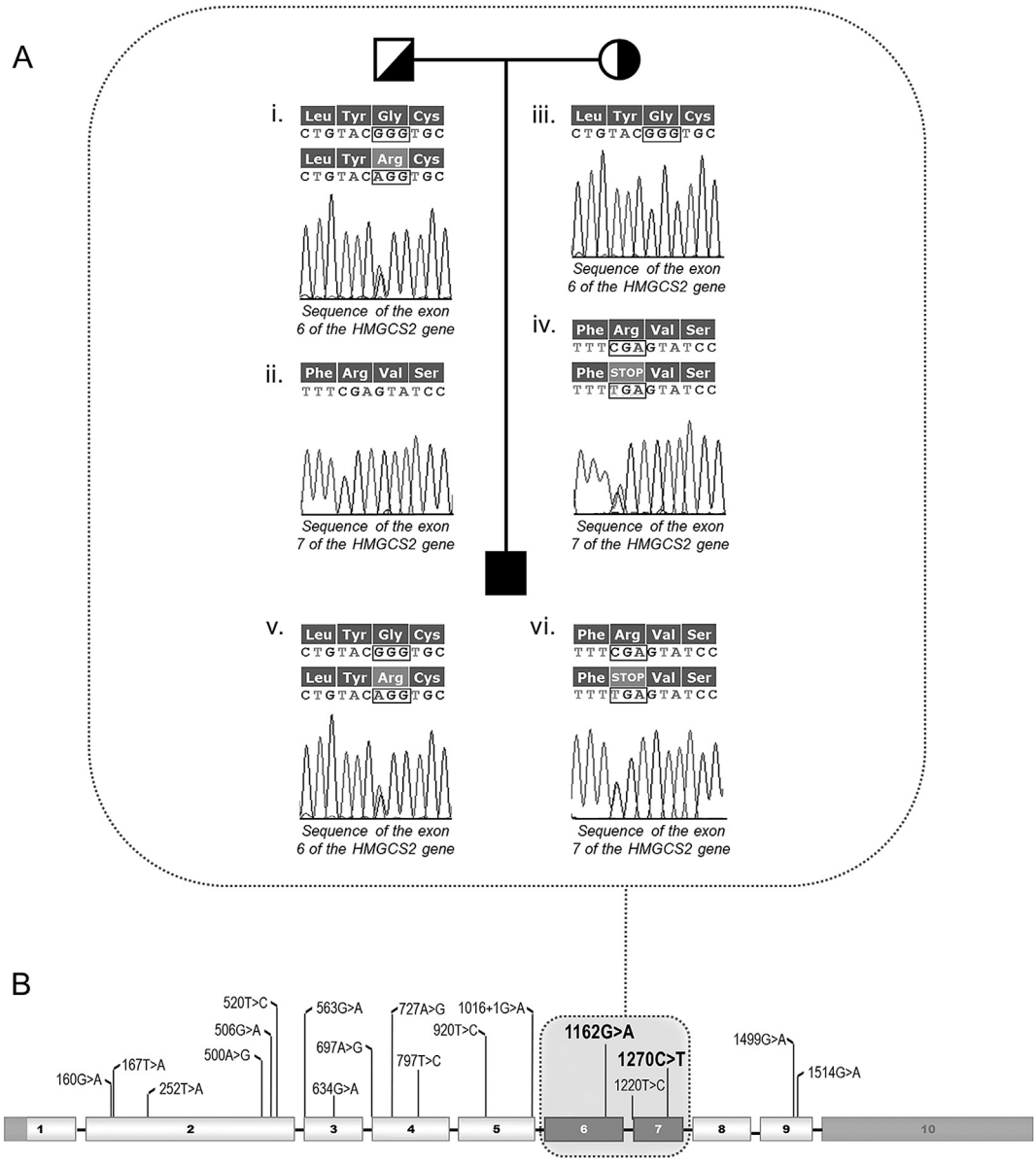

Fig. 1. (A) Pedigree of the affected family and partial electropherograms of exons 6 and 7 of the gene *HMGCS2*. The sequencing analysis performed on genomic DNA from the patient and his parents shows the missense allelic variant c.1162G>A (i and v) and the nonsense variant c.1270C>T (iv and vi). Wild-type electropherograms identified in the carrier parents are also indicated (ii and iii). (B) Scheme of the mutations located in *HMGCS2* gene. The open reading frame of *HMGCS2* gene is marked in light grey. The exons 6 and 7 of the *HMGCS2* gene are highlighted in dark grey.

A novel method for mHS expression in *E. coli* was validated, using the plasmid pMAL-c2x, which yielded a stable and active wild-type mHS, in abundance (0.541 mg/L of culture) and with a high degree of purity. Activity was measured following Clinkenbeard [10] with some modifications. To prevent inactivation of the enzyme by succinyl-CoA [11] different conditions by varying the incubation time and concentration of substrate (acetyl-CoA) were tested (data not shown). Optimal conditions for measuring the cloned enzyme were achieved by adding a previous activation step in which the enzyme was incubated at 30 °C for 17 min in presence of acetyl-CoA 300 μM. Under these conditions, the rate of HMG-CoA synthesis (acetoacetyl-CoA incorporated, nmol/min) is linear and proportional to mHS protein concentration (Fig. 2). The kinetic constants from the wild-type (wt) mHS gave a V_{max} 1.26 ± 0.3 U $\times$ mg^{-1} protein, a Km (acetyl-CoA) 87.3 ± 15 μM and a catalytic efficiency of 14.48 U $\times$ mg^{-1} $\times$ mM^{-1} (Table 1).

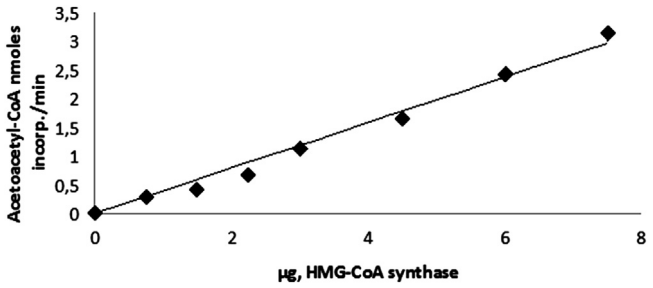

Fig. 2. Dependence of HMG-CoA synthetic rate on enzyme concentration. The rate of HMG-CoA synthesis was determined by a spectrophotometric modified method that determines at λ 304 Å the amount of acetoacetyl-CoA consumed as described under Materials and methods. The amount of mHS enzyme added (μg) was determined by the Bradford method.

Table 1
Functional study of the reported missense mutations in the *HMGCS2* gene from patients with mHS deficiency.

Mutation[a]	Exon	Amino acid change	Enzyme activity			Number of patients/ ethnic origin	Mutant alleles	Reference
			V_{max} (U/mg protein)	Km (μM) (Acetyl-CoA)	Catalytic efficiency (U/mg × mM)			
c.160G>A	2	p.V54M	–	–	–	2 Caucasian	2 (2ht)	Wolf et al. [7]
c.500A>G	2	p.Y167C	n.d.	n.d.	–	2 Caucasian	2 (2ht)	Wolf et al. [7]
c.520T>C	2	p.F174L	0.36 ± 0.1	218 ± 31	1.67	1 Chinese	2	Thompson et al. [2] Bouchard et al. [6]
c.563G>A	3	p.R188H	–	–	–	2 Unknown English	2 (2ht)	Aledo et al. [5]
c.634G>A	3	p.G212R	–	–	–	1 Unknown 1 German	2 (2ht)	Aledo et al. [3] Zschocke et al. [8]
c.920T>C	5	p.M307T	n.d.	n.d.	–	2 Unknown English	2 (2ht)	Aledo et al. [5]
c.1162G>A	6	p.G388R	–	–	–	Unknown and 1 English	2 (ht)	Shafqat et al. [9] This study
c.1499G>A	9	p.R500H	n.d.	n.d.	–	1 Unknown	1 (ht)	Aledo et al. [3]
Wild-type			1.26 ± 0.3	87.33 ± 15	14.48			

Abbreviations: n.d. not detected; ht, heterozygous. Enzyme activity data shown are mean ± S.D of three independent experiments.
[a] cDNA numbering is based on reference sequence GenBank NM_001166107.1; +1 corresponds to the A of the ATG initiation translation codon.

The effect on enzyme function of the 8 missense mutations reported in patients was studied (p.V54M, p.Y167C, p.F174L, p.R188H, p.G212R, p.M307T, p.G388R and p.R500H). The positions of the mutations in the protein structure are shown in Fig. 3A. The mutant proteins of the mHS were obtained by directed mutagenesis. After expression and purification of the mHS mutants, under the same conditions used for the wt mHS, the variants p.V54M, p.R188H, p.G212R and p.G388R (Fig. 3B) do not produce proteins that were expressed in soluble form, and the mutants p.Y167C, p.M307T and p.R500H produced inactive proteins (Fig. 3B) (Table 1). The variant p.F174L showed detectable activity (Fig. 3B) (V_{max} 0.36 ± 0.1 U × mg^{-1} protein, Km 218 ± 31 μM and a catalytic efficiency 1.67 U × mg^{-1} × mM^{-1}) (Table 1).

5. Discussion

Only nine cases of mHS deficiency have been reported, including the one described herein. As other authors suggest [2], the disease may be under-diagnosed. The clinical symptoms are unspecific and they can appear in other metabolic pathologies such as HMG-CoA lyase deficiency [15] or in defects in fatty acid β-oxidation. Confirmatory tests involve activity assay of the deficient enzyme and molecular genetic testing. However, the activity assay in crude liver extract is difficult to perform because of interference from cytosolic HMG-CoA synthase, and genetic studies have been limited by the lack of a method to measure the activity of the cloned mHS enzyme [3,5–8]. Thus, the mutations have only been confirmed from parent study.

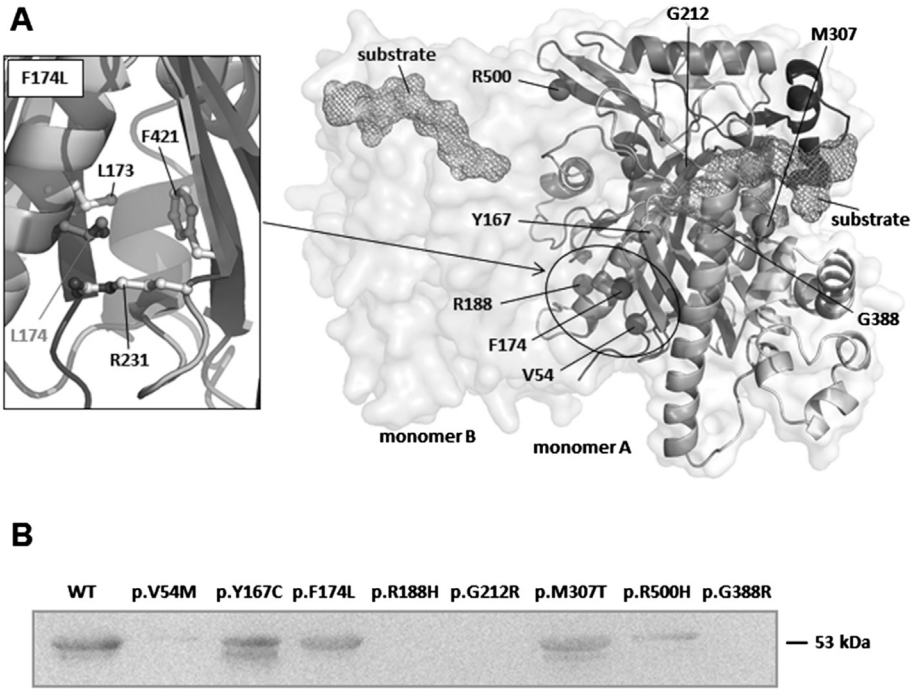

Fig. 3. (A) 3D structure model for human mHS protein. Positions of the eight residues mutated in mHS patients [3,5–8] are indicated by dots. Computational model of p.F174L mutant (inset) indicated that the new Leu residue is able to maintain stable contacts to the surrounding residues (L173, R231 and F421) in a similar manner as it is done by the wild-type Phe residue. (B) Western blot of the human mHS protein (WT) and p.V54M, p.Y167C, p.F174L, p.R188H, p.G212R, p.M307T, p.R500H and p.G388R mutants. Note that the variants p.V54M, p.R188H, p.G212R and p.G388R do not produced soluble proteins.

In the present study, human mHS was cloned in the plasmid pMAL-c2x-mHS in *E. coli*, which proved to be suitably efficient. The activity of the cloned protein was measured by a modification of Clinkenbeard's spectrophotometric method [10]. Thus, an initial activation step was added, in which the mHS was incubated with acetyl-CoA (300 μM) for 17 min at 30 °C. The binding of the substrate acetyl-CoA to the catalytic cysteine is facilitated in this step and displaces succinyl-CoA which inactivates the enzyme [11]. This allowed, for the first time, the enzymatic characterization of human mHS. The kinetic constants were V_{max} 1.26 ± 0.3 U × mg^{-1} protein and Km 87.3 ± 15 μM (Table 1).

This method was successfully applied to the study of the 8 missense mutations reported in mHS patients [3,5–8] (Table 1). The protein expression of four mutant mHS proteins (p.V54M, p.R188H, p.G212R and p.G388R) was not able to be detected by western-blot (Fig. 3B). These mutations caused large differences in size and/or charge of the amino-acids [9], leading to local protein changes, putatively incompatible with correct protein folding. Studies show that between 50% and 80% of the mutations responsible for monogenic diseases cause destabilization in protein folding [16, 17]. In the case of mHS deficiency, 50% of the missense mutations studied appeared to do this (Fig. 3B).

Other mutants resulted in apparently complete protein generation in a stable form, but without measurable enzymatic activity (p.Y167C, p.M307T and p.R500H) (Table 1). This may be attributable to the slight structural modifications generated by changes in the active site or on the dimerization surface, which would interfere with correct substrate processing. Thus, p.M307T is located in the substrate channel, p.R500H on the dimerization surface, and p.Y167C maps to the catalytic center and dimer formation [9] (Fig. 3A). The p.F174L mutant, in contrast, showed low, but detectable, enzymatic activity. A computational model of this mutant (Fig. 3A) indicated that the new Leu residue maintains stable contact with the surrounding residues (L173, R231 and F421) as in the wild-type Phe residue, producing an active enzyme.

In this study a ninth patient with mHS deficiency is reported. He has the missense mutation p.G388R [9] and the nonsense mutation p.R424X [6] which generates a non-functional truncated protein, with the loss of 85 amino-acids at the carboxyl end. Although these mutations caused total lack of activity, the patient presented a moderate clinical outcome, perhaps as a result of the short time lapse between the first appearance of symptoms and the glucose treatment.

To sum up, we report a novel patient with mHS deficiency, together with a modification of the Clinkenbeard assay, which allowed us to measure the HMG-CoA synthase activity of the cloned variants. This method confirms that the missense mutations studied caused the disease.

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