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ORIGINAL INVESTIGATION

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Genetic basis of mitochondrial HMG-CoA synthase deficiency

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Abstract Deficiency of mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (mHMGS) is a recessive disorder of ketogenesis that has been previously diagnosed in two children with hypoglycaemic hypoketotic coma during fasting periods. Here, we report the results of molecular investigations in a third patient affected by this disease. Sequencing of the entire coding region of the *HMGCS2* gene revealed two missense mutations, G212R and R500H. Mendelian inheritance was confirmed by the analysis of parental samples and neither of the mutations was found on 200 control chromosomes. Functional relevance was confirmed by in vitro expression studies in cytosolic HMGS-deficient cells. Whereas wild-type cDNA of the *HMGCS2* gene reverted the auxotrophy for mevalonate, the cDNAs of the mutants did not. The disease may be recognised by specific clinical and biochemical features but it is difficult to confirm enzymatically since the gene is expressed only in liver and testis. Molecular studies may facilitate or confirm future diagnoses in affected patients.

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Introduction

Mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (mHMGS: EC 4.1.3.5) catalyses the first step of ketogenesis from acetyl-CoA and acetoacetyl-CoA and is considered to be the main control step in ketogenesis (Hegardt 1999). The full-length cDNA was reported by Mascaró et al. in 1995. The human protein is encoded by the *HMGCS2* gene, which spans 20 kb genomic DNA on chromosome 1p13-p12 (Boukaftane et al. 1994) and contains 10 exons (for further information see http://www.ncbi.nlm.nih.gov/LocusLink/LocRpt.cgi?I=3158). mHMGS is expressed mainly in the liver and testis and is absent in other body cells.

mHMGS deficiency (OMIM 600234) is inherited as a recessive trait and has been described in two patients with hypoketotic hypoglycaemic coma after prolonged fasting during gastrointestinal infections in childhood (Thompson et al. 1997; Morris et al. 1998). An article reporting the mutations responsible for the mHMGS deficiency (R424X and F174L) of these two patients has been published during the review process of our manuscript (Bouchard et al. 2001). Here, we report the molecular study of mHMGS deficiency in a third patient who shows different mutations (G212R and R500H) but a similar phenotype.

Materials and methods

Patient

The affected boy presented, at the age of 11 months, acute hypoglycaemic coma and respiratory arrest after a 2-day history of gastroenteritis, vomiting and poor food intake. Physical examination revealed mild hepatomegaly. Blood glucose was 1.2 mmol/l (norm: >3 mmol/l). Apart from elevated transaminases (aspartate aminotransferase 283 U/l, norm: 10–27 U/l; alanine aminotransferase 138 U/l, norm: 5–23 U/l) and lactate dehydrogenase (1502 U/l, norm: 200–500 U/l), there were no other abnormalities in routine clinical chemical analysis. Free fatty acids and ketone bodies were not measured at that time. Urinary organic acid analysis revealed massive dicarboxylic aciduria without adequate ketonuria. Acylcarnitines analyses in dried blood spots were normal. The patient recovered well under intravenous glucose infusion.

Enzyme studies in fibroblasts showed normal beta-oxidation capacity. A monitored fasting test was performed, leading to hypoglycaemia (blood glucose 2.3 mmol/l) 12 h after the last meal. At this time, there was massive elevation of plasma free fatty acids (3290 μmol/l, norm: <300 μmol/l) without concomitant elevation of total plasma ketones (174 μmol/l, norm: <150 μmol/l, in fasting: >1500 μmol/l). Blood lactate, insulin and transaminases were within normal ranges. Urinary organic acids again showed massive dicarboxylic aciduria without adequate ketonuria; acylcarnitines in dried blood spots were normal. The patient was started on carnitine (100 mg/kg body weight) and the family was advised to avoid fasting for more than 8 h and to administer a caloric drink at around midnight. Under this regimen, there were no subsequent hypoglycaemic episodes and, at present, the child (4 years old) is developing normally with no residual neurological impairment.

Molecular studies

The ten exons of HMGCS2 were amplified by the polymerase chain reaction (PCR) with flanking intronic primers and 150 ng genomic DNA. PCR products were separated, purified and sequenced automatically with an Applied Biosystems 373 DNA sequencer with the fluorescent terminator kit (Perkin-Elmer)

Site-directed mutagenesis of the cDNA for human mHMGS and construction of the expression plasmids

Full-length *HMGCS2* cDNA was cloned into the pcDNA.3 expression vector in the *Eco*RI site. The mutations G212R and R500H were introduced into this recombinant plasmid by site-directed mutagenesis (Datta 1995) by using two round of amplifications. In the first round, a megaprimer was amplified by means of a mutated oligomer. In the second round, the later fragment was used as a megaprimer for a new amplification. The fragment obtained in the last PCR was replaced in the wild-type cDNA. Constructs were sequenced in order to confirm the changes introduced. For the G212R mutation, the mutant oligomer (5'-CAGGTGGGGCCA-GAGCTGTGGC-3') and the reverse primer (5'- GGTGATAACC-CATGAGGCTCCG-3') were used in the first PCR. The fragment obtained (megaprimer) was employed in the second PCR amplification with the reverse primer 5'-CCTGGAGAAGTATAACA-ATGTGG-3'. The amplified fragment was digested with *Bstz*17I and *Nde*I and cloned at the same sites in the normal cDNA. For the R500H mutation, the mutant oligomer (5'-GCAGCATCACCG-AAAGTATG-3') and the reverse primer (5'GATTCACGGGGA-GAAGCTCTG-3') were used in the first PCR. The fragment obtained (megaprimer) was employed with the reverse primer 5'-CC-GAAAGTGTGTGTCTCCTGAGG3' in the second PCR. The fragment amplified was digested with *Nhe*I and *Bsu36*I, isolated and replaced in the control cDNA.

Expression studies

Mev-1 cells are Chinese hamster ovary mutant cells defective for cytosolic HMGS (cHMGS) activity and therefore auxotrophs for mevalonate. The cells were cultured in Ham's F-12 medium supplemented with 5% fetal calf serum with 430 μM mevalonate. Mev-1 cells were transfected with expression recombinant plasmids by the calcium phosphate method; 48 h after transfection, cells were selected by G418 (GIBCO-BRL). After 3 weeks, the resistant colonies were trypsinized and the stable transfected cells were seeded in 6-well plaques. After 24 h, the mevalonate was removed from the medium in some of the wells. Cells were stained with methylene blue 30 days after removal of the mevalonate.

Results

The *HMGGS2* gene is not expressed in fibroblasts or blood cells, and a liver biopsy sample was not available from our patient. Genomic DNA was extracted from the patient and both parents from fibroblasts and peripheral blood samples. In order to permit mutation analysis from genomic DNA, we determined intron sequences through genomic PCR amplifications with primers located within the exons (Mascaró et al. 1995) and adjacent splice sites of the gene. Once the intron sequences were known, oligonucleotides were synthesised complementary to boundary intron 5' and 3' regions for the PCR amplification of individual exons. Se-

Table 1 Sequences of the primers used for PCR amplification of individual exons of the human mitochondrial HMG-CoA synthase gene. The position of primers are indicated in relation to the 5' (+) or 3' (–) end of each intron

Name of primer	Sequence of primer	Coordinates
1F	5'-GGAACATGACAGTTAGTTGAC-3'	–14333 nt in promoter
1R	5'-CTAGTTACAAGGTGCTTCTC-3'	+34 nt in intron 1
2F	5'-TGGGAGTTTGACTGTTATCTCC-3'	–28 nt in intron 1
2R	5'-CACCTTTCTTACATAAGGTTCG-3'	+35 in intron 2
3F	5'-TGATGGTAAATATTTCTTG-3'	–32 nt in intron 2
3R	5'-CTCTGCTCCATAGACCAGCC-3'	+140 nt in intron 3
4F	5'-TGTTAAGCCCATGTAGGTGAG-3'	–227 nt in intron3
4R	5'-GATTTTAGCATAGACCCAGGG-3'	+165 nt in intron 4
5F	5'-CTGGGAGGCCTGGAGAAACT-3'	–101 nt in intron 4
5R	5'-CTCCCCAAGAAGAGAACTCAC-3'	+21 nt in intron 5
6F	5'-CTCTCACATGGCTACCTCTG-3'	–32 nt in intron 5
6R	5'ACTTTGTTGACCCTGCAGCC-3'	+78 nt in intron 6
7F	5'-GCAGATCGCCTGAACCTAG-3'	+174 nt in intron 6
7R	5'-GGCAGCCTCAGTAATGGTGGGC-3'	+86 nt in intron 7
8F	5'-ACGTTCCTCCAGGGTGTAACC-3'	–114 nt in intron 7
8R	5'-CTCCCAACCATATCTCGTAAAAG-3'	+122 nt in intron 8
9F	5'-TCACTGTGAAGGGGCAGACATC-3'	–93 nt in intron 8
9R	5'-GCACCTGTCCCCACCTTCTC-3'	+88 nt in intron 9
10F	5'-GCCACACCTCGGGCAAGAAG-3'	–80 nt in intron 9
10R	5'-TGCTCTTTCACAAAGGACCCC-3'	+240 nt in exon 10

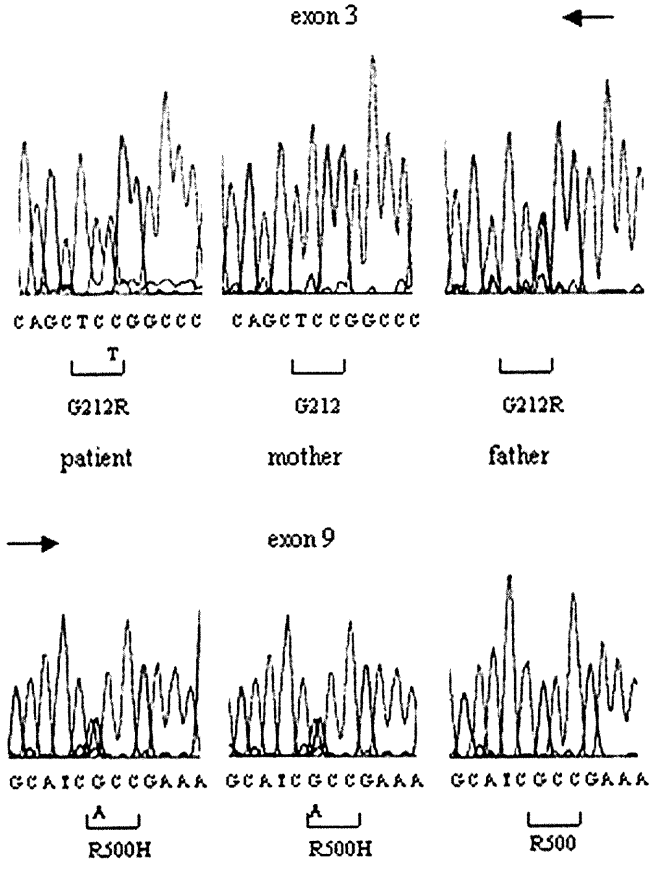

Fig. 1 DNA sequences around the mutations in *HMGCS2* gene from the patient and his parents. The mutated codons and the encoded amino acids are marked (*arrows* 5'–3' sense of the sequences)

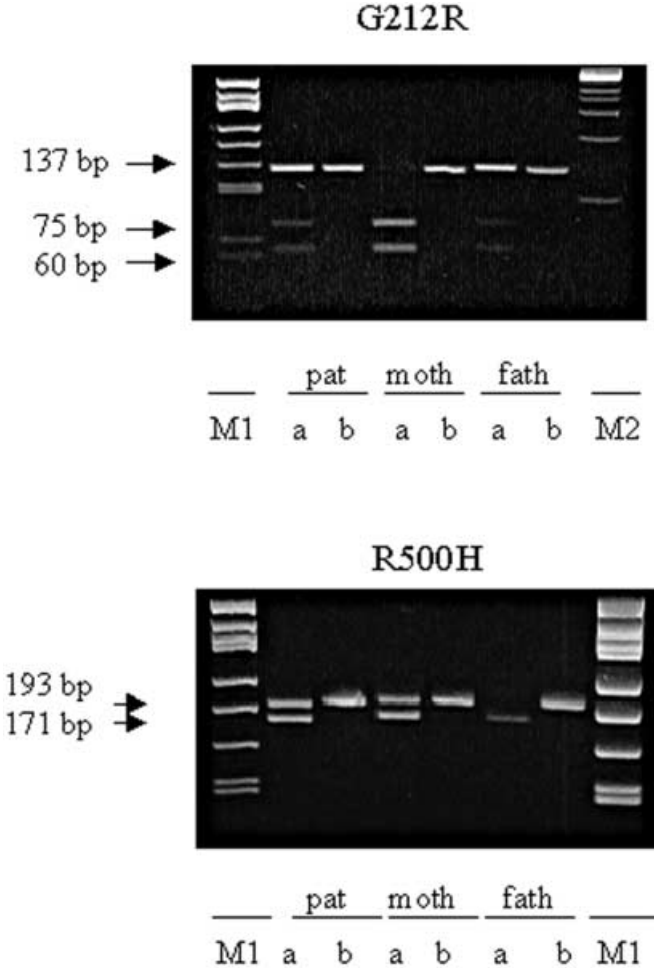

Fig. 2 Restriction enzyme analysis of mutations G212R (*top*) and R500H (*bottom*) in the mitochondrial HMG-CoA synthase gene from the patient and his parents. On each gel, *lane a* represents the fragment digested and *lane b* the fragment not digested. The mutation G212R removes a *Hpa*II site in exon 3, visible as one undigested 137-bp product instead of the normal 75-bp and 60-bp fragments (forward primer, 5'-CAGGTCGTTATGCCATGGTG-3', reverse primer 5'-CTACTAACCTCGCTCCAGGG-3'). The patient (*pat*) and father (*fath*) are heterozygous for G212R, whereas the mother (*moth*) is homozygous normal. The mutation R500H removes a *Taq*I site in exon 9, introduced by PCR amplification with a modified reverse primer adjacent to the mutation (forward primer 5'-TCACTGTGAAGGGGCAGACATC-3'; modified reverse primer GACGCCGGGCATACTTTGGT; the modified nucleotide is underlined). The mutation is visible as one undigested 193-bp product instead of two fragments of 171 bp and 22 bp length. The patient and mother are heterozygous for R500H, whereas the father is homozygous normal. *M1* Size standard GC-015–001*Hpa*II-pBS (Labclinics), *M2* size standard 1 kb plus ladder (Gibco BRL)

quences for the primers in introns 1, 2 and 5 were obtained from genome databases (accession numbers U81851, U81852 and U81855). Genomic primer sequences used for amplification of individual exons are shown in Table 1.

Sequence analysis of the whole coding region of the *HMGCS2* gene revealed two potential disease-causing mutations. A heterozygous G→A transition at nucleotide c.634 was identified in exon 3 of the patient and his father; this was expected to cause the substitution of glycine^{212} by arginine (mutation G212R). In exon 9 of the patient and his mother, a heterozygous G→A transition at nucleotide c.1499 was identified; this was expected to cause the substitution of arginine^{500} by histidine (mutation R500H; Fig. 1). Both mutations were confirmed through restriction enzyme digests of PCR products re-amplified from genomic DNA (Fig. 2). Neither of the two mutations was found on 200 German control chromosomes. In addition to the two mutations, we also detected three novel DNA variants, viz. c.657C→G and c.660T→C in exon 3 and c.891 T→C in exon 4; these do not alter the encoded amino acid and are therefore expected to be silent.

Enzyme studies and expression studies of mHMGS are complicated by the presence of cHMGS in all cells. We therefore used mutant Chinese hamster ovary (CHO)-K1 cells without detectable cHMCS activity (Mev-1 cells), which are thus auxotrophs for mevalonate (Schnitzer-Polokoff et al. 1982) to determine whether the mutations identified in our patient decreased mHMGS activity in vitro. The enzyme activity of the transfected mHMGS can be recognised by the formation of colonies despite the removal of mevalonate from the medium. Previous studies

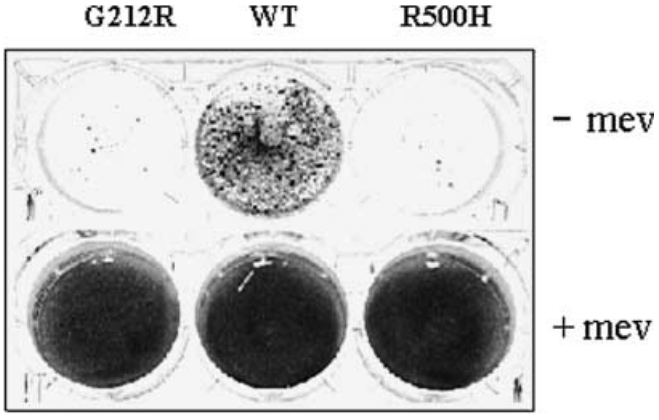

Fig. 3 Lack of mevalonate auxotrophy reversion by mutated (G212R, or R500H) mitochondrial HMG-CoA synthase. Mev-1 cells (auxothophs for mevalonate) cultured in Ham's F-12 medium supplemented with 5% fetal calf serum and 430 μM mevalonate were transfected with the wild-type (*WT*) or mutated (either G212R or R500H) *HMGCS2* cDNAs. They were selected once by neomycin and seeded at 5×10^5 cells in each well. After 24 h, mevalonate (*mev*) was eliminated from the medium in some of the wells (*–mev*, *top*). The cells were stained with 0.1% methylene blue in 50% methanol, 30 days later

in our laboratory have demonstrated that transfection of Mev-1 cells with a plasmid containing rat mHMGS cDNA (Ortiz et al. 1994) and HMGS cDNA from the insect *Blatella germanica* (Buesa et al. 1994) reverts the mevalonate auxotrophy, because the HMG-CoA synthesised is converted to cholesterol.

We transfected Mev-1 cells with pCDNA-3 expression plasmids that contained cDNAs for wild-type or mutant mHMGS. Stably transfected cells were selected with neomycin. When mevalonate was removed from the culture medium, we obtained several colonies of wild-type cDNA stably transfected cells and observed that they did not depend on the presence of mevalonate for growth and division. In contrast, the two cell lines produced after transfection with the individual mutated cDNAs (G212R or R500H) died and no colony appeared after transfection (Fig. 3). This indicates that only cells transfected with wild-type cDNA are able to revert the auxotrophy for mevalonate and none of the two mutated proteins show HMGS activity

Although the clinical and biochemical findings in our patient were not suggestive of a deficiency of mitochondrial HMG-CoA lyase (the enzyme that catalyses the second step of ketogenesis and the last step of leucine oxidation), we ruled out this disorder by sequencing all exons of the *HMGCL* gene. This failed to identify any potential disease-causing mutation.

Discussion

We report here the molecular the basis of mHMGS deficiency. Sequencing of the whole coding region of the *HMGCS2* gene in a patient with this disease revealed two missense mutations, G212R and R500H. Mendelian inheritance was confirmed by analysis of parental samples; neither of the mutations was found in 200 control chromosomes. Functional relevance was confirmed through in vitro expression studies by using mutant Mev-1 cultured cells deficient for cHMGS (Mev-1 cells; Schnitzer-Polokoff et al. 1982). Both mutations alter highly conserved amino acid residues. R500 is conserved in the mHMGSs and cHMGSs of all organisms studied. Ketogenic mitochondrial HMGS contains R500 in rat (Ayté et al. 1990), human (Mascaró et al. 1995), mouse (Boukaftane et al. 1994) and pig (Adams et al. 1997); in addition, R500 is also present in the isoprenoid cytosolic HMGS mRNAs of mammals (rat, human, Syrian hamster), chickens, yeasts (*Schizosaccharomyces pombe*, *Saccharomyces cerevisiae*), plants (*Arabidopsis thalliana*, *Pinus pinea*), and insects (*Blattella germanica*, the two genes described). Glycine 212 is also present in all HMGSs from these organisms, except for the second gene of *B. germanica*, in which it is replaced by glutamic acid (reviewed in Hegardt 1999).

Our patient is only the third child in whom mHMGS deficiency has been clinically identified, although other patients with this disorder may have remained undiagnosed. mHMGS deficiency is easily recognised in acute hypoglycaemic coma by highly elevated serum free fatty acids, little or no elevation of serum ketones, the normal acylcarnitine spectrum and the excretion of large amounts of dicarboxylic acids without adequate ketones in the urine. However, as serum free fatty acids and ketones are not routinely measured, even in many metabolic centres, the organic acid profile may be wrongly interpreted as being normal for a fasting sample (the absence of ketones being the most specific finding) and the normal acylcarnitine profile and normal results of beta-oxidation studies may be misleading rather than helpful.

The hypoglycaemic episode with respiratory arrest was life-threatening in our patient but the disease course after the manifesting episode was remarkably benign in all patients described so far. The fasting tolerance as determined in controlled conditions in our patient was relatively short, with onset of hypoglycaemia 12 h after the last meal. Hypoglycaemia and coma in mHMGS deficiency may be expected to start earlier than in medium-chain acyl-CoA dehydrogenase (MCAD) deficiency, the commonest beta-oxidation deficiency, as ketone body production in the latter condition is not as severely impaired because of the release of acetyl-CoA from the beta-oxidation of long-chain acyl-CoA compounds. Approximately one quarter of children with MCAD deficiency do not survive the first episode (Pollitt and Leonard 1998) but this proportion may be even higher in mHMGS deficiency. The disease is also a potential cause of the sudden infant death syndrome (SIDS). As the enzymatic diagnosis of mHMGS is difficult, if not impossible, molecular studies may, in future, assist in the diagnosis or confirmation in affected patients.The recent publication of mutations in the *HMGCS2* gene in the two previously identified patients (Bouchard et al. 2001) supports this conclusion.

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