Molecular basis and functional characterization of human 3-methylcrotonyl-CoA carboxylase deficiency

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List of Abbreviations

3-HIVA	3- hydroxyisovaleric acid
3-MCG	3- methylcrotonylglycine
ACC	acetyl-CoA carboxylase
bp	base pairs
cDNA	complementary DNA
CG	complementation group
CG1 and CG2,	complementation group 1 and 2, respectively
kDa	kilo daltons
MCC	3-methylcrotonyl-CoA carboxylase
MCCA	gene encoding the MCCα subunit
MCCB	gene encoding the MCCβ subunit
MCD	multiple carboxylase deficiency
mRNA	messenger RNA
NMD	nonsense mediated mRNA decay
ORF	open reading frame
PA	propionic acidemia
PC	pyruvate carboxylase
PCC	propionyl-CoA carboxylase
PCCA	gene encoding the PCCα subunit
PCCB	gene encoding the PCCβ subunit
PCR	polymerase chain reaction
PTC	premature termination codon
RT-PCR	reverse transcriptase PCR
TMS	tandem mass spectrometry

Single and 3-letter codes for amino acids

Alanine	Ala	A
Cysteine	Cys	C
Aspartic Acid	Asp	D
Glutamic Acid	Glu	E
Phenylalanine	Phe	F
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Lysine	Lys	K
Leucine	Leu	L
Methionine	Met	M
Asparagine	Asn	N
Proline	Pro	P
Glutamine	Gln	Q
Arginine	Arg	R
Serine	Ser	S
Threonine	Thr	T
Valine	Val	V
Tryptophan	Trp	W
Tyrosine	Tyr	Y

Abstract

3-Methylcrotonyl-CoA carboxylase (MCC) deficiency is a rare disorder of leucine catabolism inherited as an autosomal recessive trait. The phenotypic expression of the disease is highly variable, ranging from neonatal onset with severe neurological involvement to asymptomatic adults. Most patients, however, are asymptomatic until an episode of acute metabolic decompensation following intercurrent illness leads to the diagnosis. The metabolic phenotype of MCC deficiency includes a characteristic organic aciduria with greatly increased excretion of 3-hydroxyisovaleric acid and 3-methylcrotonylglycine and elevated blood levels of 3-hydroxyisovalerylcarnitine, usually in combination with severe secondary carnitine deficiency due to urinary loss of carnitine esters. Introduction of tandem mass spectrometry (TMS) to newborn screening has revealed an unexpectedly high incidence of MCC deficiency and indicates that this disorder is the most frequent organic aciduria.

MCC is one of the four biotin-dependent carboxylases present in humans. MCC is a mitochondrial enzyme composed of biotin containing α subunits and smaller β subunits, encoded by *MCCA* and *MCCB*, respectively. In order to understand the molecular biology of human MCC, including the molecular defects causing MCC deficiency and their functional consequences, MCC-deficient patients were studied and attempts were made to correlate molecular defects with the phenotypic variability in our patient population.

In this thesis mutation analysis is described in 30 MCC-deficient probands, of whom 10 came to attention because of clinical symptoms, 18 were asymptomatic newborns detected by TMS based newborn screening, and 2 were affected but asymptomatic mothers diagnosed by abnormal metabolites in the neonatal screening samples of their healthy babies. Among these 30 probands, 11 have mutations in *MCCA*, and 19 in *MCCB*. We identified 10 novel *MCCA* and 14 novel *MCCB* mutant alleles including missense, nonsense, frameshift and splice site mutations.

In order to confirm the functional consequences of *MCCA* and *MCCB* missense mutations, we used transient transfection of SV40T-transformed *MCCA* and *MCCB* deficient skin fibroblasts to express 1 *MCCA* and 3 *MCCB* missense alleles. The *MCCB* missense alleles expressed showed no or very low residual activity, whereas the *MCCA* missense allele had 26% residual activity of wild type, thus confirming that 3 out of 4 missense alleles expressed have a deleterious effect on enzyme activity. The apparent severity of MCC mutations contrasts with the variety of clinical phenotypes found in MCC-deficient patients. Our data demonstrate no clear correlation between genotype and phenotype suggesting that factors other than the genotype at the MCC loci have a major influence on the phenotype of MCC deficiency.

To analyse the MCC polypeptides under steady-state condition in fibroblasts of *MCCA* deficient patients, we carried out Western blot analysis. Our results demonstrate that the MCCα protein was absent in 5 patients homozygous or compound heterozygous for nonsense or frameshift mutations that are expected to result in a truncated protein. The MCCα protein was also absent in 2 further compound heterozygous patients in whom only one missense allele could be identified in the RT-PCR products. The second allele could not be detected because presumably this mutant allele is unstable and degraded by the mechanisms of nonsense-mediated mRNA decay. In contrast, Western blot analysis of the MCCα protein was normal in amount and size in 4 patients carrying *MCCA*-p.R385S. This is in agreement with previous studies, which show that the protein product of this allele is stable.

Finally, we provide evidence that the missense mutation *MCCA*-p.R385S has a dominant negative effect on the activity of wild type. Biotin can partially reverse this negative effect and result in biotin responsiveness *in vivo*. This is the first example of biotin responsiveness for an isolated partial deficiency of any of the biotin-dependent carboxylases, suggesting that therapeutic trials with biotin in patients carrying this mutant allele are warranted.

1. Introduction

1.1. Biotin

Biotin was first isolated as a yeast growth factor from egg yolk in 1936 by Kögl and Tönnis (Kögl and Tönnis, 1936). Its structure was determined soon after by du Vigneaud and colleagues (du Vigneaud et al. 1942), and it was first synthesised two years later by Harris and collaborators (Harris et al. 1943).

Biotin ($C_{10}H_{16}O_3N_2S$) is a heterocyclic ring that is attached to an aliphatic side chain terminating in a carboxyl group. Of the eight different isomers, only d-biotin exhibits coenzyme activity and is found in nature. Biotin is widely distributed in plants and animal tissues and is readily synthesized by a variety of microorganisms. This essential micronutrient for mammals is a member of the B vitamin complex and is present in all natural foodstuffs. Some of the rich sources of biotin are egg yolk, liver, milk and soya. Biotin deficiency is extremely rare and occurs only in special dietary situations, such as excessive intake of raw eggs. Raw egg white contains the glycoprotein avidin that binds very tightly biotin, preventing its absorption (Eakin et al. 1940). Biotin deficiency in animals was demonstrated in rats fed raw egg white (Bateman 1916). They developed a syndrome called “egg white injury”, that consisted of dermatitis, hair loss and neuromuscular dysfunction which could be cured by biotin (Parsons et al. 1937). Therefore biotin was called vitamin H (H stands for Haut, the German word for skin).

The steps involved in biotin metabolism in mammals are depicted in figure 1.1. Biotin serves as a prosthetic group of four important carboxylases, *3-methylcrotonyl-CoA carboxylase* (MCC; EC 6.4.1.4), *propionyl-CoA carboxylase* (PCC; EC 6.4.1.3), *pyruvate carboxylase* (PC; EC 6.4.1.1) and *acetyl-CoA carboxylase* (ACC; EC 6.4.1.2), which are involved in central processes of protein, carbohydrate, and fatty acid metabolism (Samols et al. 1988, Wolf 2001). Biotin is attached to the $\in$-amino group of a specific lysine in the inactive apocarboxylases, thus forming active holoenzymes. This holoenzyme formation is catalysed by *holocarboxylase synthetase* (HCS; EC 6.3.4.10) (Wolf and Feldman 1982).

After proteolytic degradation of holocarboxylases from either endogenous or dietary sources biotin remains bound to lysine (biocytin) or to short biotinyl-peptides. Biotin is released from these compounds by *biotinidase* (EC 3.5.1.12) thus enabling recycling of biotin, i.e. its use for the synthesis of new holocarboxylases (Wolf and Feldman 1982).

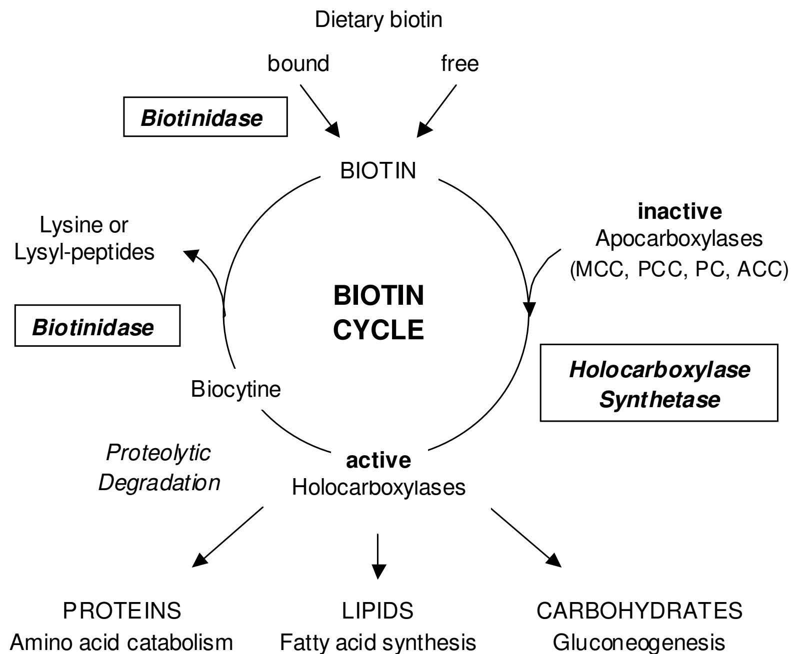


Figure 1.1: ***The biotin cycle.*** MCC, 3-methylcrotonyl-CoA carboxylase; PCC, propionyl-CoA carboxylase; PC, pyruvate carboxylase; ACC, acetyl-CoA carboxylase. Modified from Wolf et al. 1986.

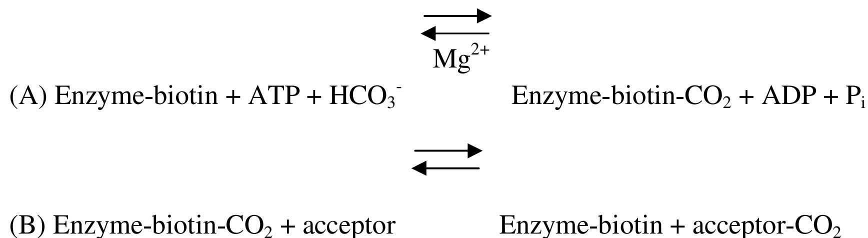
Deficiency of either HCS or biotinidase causes multiple carboxylase deficiency (MCD) leading to typical organic aciduria and severe life threatening illness. In biotinidase deficiency MCD results from progressive development of biotin deficiency due to inability to liberate and recycle biotin, which is lost in urine as biocytin. Common symptoms in patients with MCD are metabolic acidosis, hypotonia, seizures, ataxia, and cutaneous symptoms such as skin rash and hair loss and the occurrence of episodes of massive ketosis which may lead, when not treated, to dehydration, coma and death (Baumgartner and Suormala 1997). The measurement of carboxylase activities in lymphocytes provides direct

evidence of MCD. These activities are low in HCS deficiency but may be normal in biotinidase deficiency, depending on the degree of biotin deficiency. The two disorders can be easily distinguished by assay of biotinidase activity in serum calorimetrically, an assay that is today included in many neonatal screening programs worldwide (Baumgartner and Suormala 1997). Most HCS and all biotinidase deficient patients respond dramatically to pharmacological doses of biotin.

1.2. Biotin-dependent carboxylases

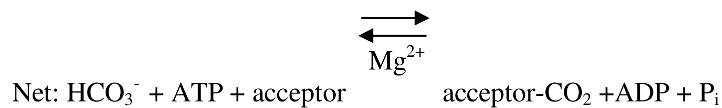
The four biotin-dependent carboxylases are enzymes with diverse roles in intermediary metabolism but common structural features. Members of this family have three structurally conserved functional domains: (i) the biotin carboxyl carrier domain, which carries the biotin prosthetic group; (ii) the biotin carboxylation domain, which catalyses the carboxylation of biotin; (iii) and the carboxyltransferase domain, which catalyses the transfer of a carboxyl group from carboxybiotin to the organic substrate specific for each carboxylase (Samols et al. 1988, Jitrapakdee and Wallace 2003).

In this group of enzymes biotin serves as a covalently bound "CO_2" carrier for reactions in which a carboxyl group is bound into an acceptor (Wolf and Feldman 1982). The reaction processes in two steps. The first partial reaction involves the formation of the carboxybiotinyl enzyme using bicarbonate as the carboxyl donor (the biotin carboxylase reaction; equation A). In the second partial reaction, the carboxyl group is transferred from the enzyme-CO_2 complex to a specific acceptor substrate (the carboxyl-transferase reaction; equation B) (Wolf and Feldman 1982)



(A) Enzyme-biotin + ATP + HCO_3^- $\underset{Mg^{2+}}{\rightleftharpoons}$ Enzyme-biotin-CO_2 + ADP + P_i

(B) Enzyme-biotin-CO_2 + acceptor $\rightleftharpoons$ Enzyme-biotin + acceptor-CO_2



Net: $HCO_3^- + ATP + acceptor \underset{Mg^{2+}}{\rightleftharpoons} acceptor\text{-}CO_2 + ADP + P_i$

MCC, PCC and PC are localized to the mitochondria, while ACC is cytosolic (Samols et al. 1988, Wolf 2001). The genes for all human carboxylases have been cloned and characterized (Abu-Elheiga et al. 1995, Baumgartner et al. 2001, Freytag and Collier 1984, Lamhonwah et al. 1986).

Isolated deficiencies of each of the four biotin-dependent carboxylases have been described in man (Figure 1.2). All isolated deficiencies are characterized by an abnormal, often typical profile of organic acids in urine, which is caused by the accumulation of one or more intermediate compounds and is the clue to diagnosis. The deficiencies of MCC, PCC and PC are inherited as autosomal recessive traits. The diagnosis is confirmed by direct enzyme assay in lymphocytes or cultured fibroblasts. In contrast to MCD, none of the isolated carboxylase deficiencies have so far been shown to respond to biotin therapy.

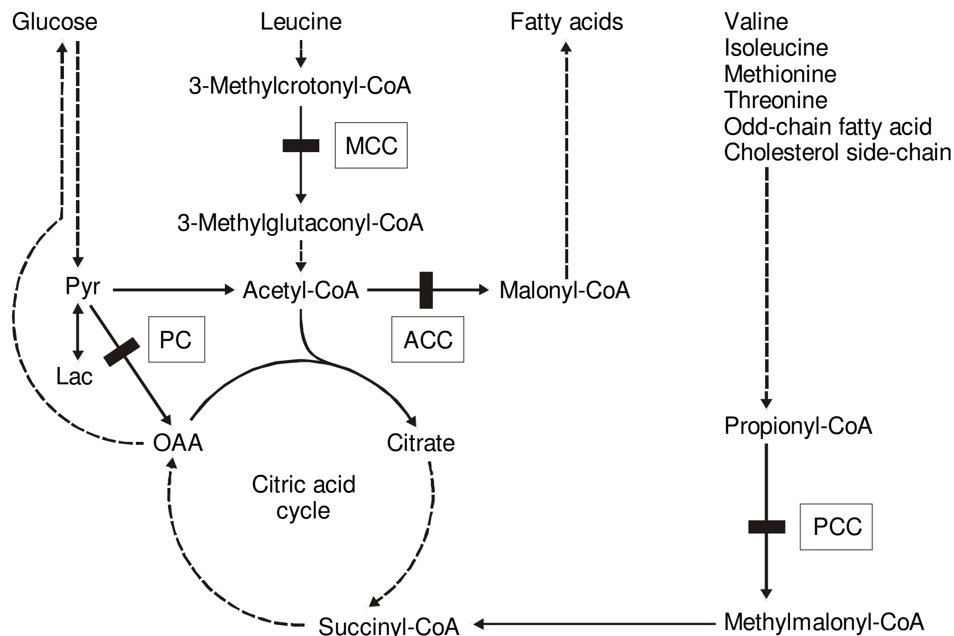


Figure 1.2: ***The carboxylase deficiencies.*** Black bars indicate sites of enzyme defects. Full and dotted lines indicate that one or several enzymes are involved, respectively. Pyr, pyruvate; lac, lactate; OAA, oxaloacetate; MCC, 3-methylcrotonyl-CoA carboxylase; PCC, propionyl-CoA carboxylase; PC, pyruvate carboxylase; ACC, acetyl-CoA carboxylase. Modified from Baumgartner and Suormala, in press.

1.2.1. Acetyl-CoA carboxylase (ACC)

ACC is a cytosolic allosteric enzyme catalysing the first, rate limiting step of fatty acid synthesis by converting acetyl-CoA to malonyl-CoA (Wolf 2001). ACC is activated by citrate or isocitrate, which induce polymerisation to its active form.

The human ACC gene maps to chromosome 17q12, and the cDNA sequence has an open reading frame of 7038 bp that encodes 2346 amino acids. Northern blot analysis revealed an ACC mRNA of 10 kb with high expression in liver and skeletal muscle (Abu-Elheiga et al. 1995).

A single case of isolated ACC deficiency has been reported in the literature (Blom 1981). This patient was a newborn girl with severe brain damage and persistent myopathy.

ACC activity in liver biopsy and cultured skin fibroblasts was severely reduced while the activity of PCC was normal, confirming isolated ACC deficiency.

1.2.2. Pyruvate carboxylase (PC)

PC is an intra mitochondrial allosteric enzyme converting pyruvate to oxalacetate, thus having an important anaplerotic function in providing this intermediate to the citric acid cycle. In addition, it catalyses the first reaction of the gluconeogenetic pathway (Robinson 2001). The active enzyme molecule is formed of four tightly bound identical subunits. Each subunit has one molecule of covalently bound biotin and contains a binding site for acetyl CoA, its allosteric activator (Barden et al. 1975, Scrutton and White 1974).

The human PC gene has been mapped to the long arm of chromosome 11 at 11q13, and has 19 exons spanning over 16 kb of genomic DNA (Freytag and Collier 1984, Lim et al. 1988).

PC deficiency (MIM 266150) can manifest in two main forms (Robinson 2001). In the more severe form, patients present shortly after birth with severe lactic academia associated with hyperammonemia, citrullinemia, and hyperlysinemia; the patients of this group rarely survive to more than three months of age. No PC activity can be detected in fibroblasts. In the less severe form, patients present in the first months of life with mild to moderate lactic acidemia and delayed development. Some residual PC activity can be detected in cultured fibroblasts of these patients. Furthermore, a single case with mild symptoms consisting of episodic acidosis with no psychomotor retardation has been described (Van Coster et al. 1991).

1.2.3. Propionyl-CoA carboxylase (PCC)

In the mitochondrial matrix PCC catalyses the carboxylation of propionyl-CoA to D-methylmalonyl-CoA in the catabolic pathway of the amino acids isoleucine, valine, threonine, and methionine, as well as of odd-chain fatty acids and the side chain of cholesterol (Fenton et al. 2001; Figure 1.2). PCC is composed of two nonidentical $\alpha-$ and

β–subunits, encoded by *PCCA* and *PCCB*, respectively . The active enzyme is thought to be a dodecamer comprised of six heterodimers ($\alpha_6\beta_6$) (Fenton et al. 2001). The cDNA predicts a human α-subunit of 2106 bp encoding a 702 amino acid polypeptide of 72 kDa, which contains the biotin binding site. The β-subunit cDNA contains 1617 bp encoding a 539 amino acid polypeptide of 56 kDa (Lamhonwah et al. 1986). The human *PCCA* and *PCCB* structural genes have been mapped to chromosome 13q32 and chromosome 3q13.3–q22, respectively (Lamhonwah et al. 1986).

Isolated deficiency of PCC causes propionic acidemia (PA; MIM 606054), one of the most frequent inborn errors of organic acid metabolism. Most of the affected individuals present within the first days or weeks of life with vomiting, hypotonia, hyperammonemia, developmental delay and in some cases early death (Wolf et al. 1981). Some patients have presented later either with acute encephalopathy and episodic ketoacidosis or with developmental retardation apparently uncomplicated by attacks of ketosis or acidosis (Mahoney et al. 1971, Surtees et al. 1992). A few patients with almost complete deficiency of PCC activity in fibroblasts remain asymptomatic, and have been identified only during family studies (Wolf et al. 1979, Kuhara et al. 1988). Although PA can be treated by protein restriction and supplementation of carnitine, the outcome for patients is usually poor, mental retardation and movement disorders occurring frequently in survivors.

Because the enzyme is composed of two independently encoded subunits the causative mutations will necessarily occur in one of the two genes. In a mutation update and review of the functional and structural effects of different mutant alleles in PA, Desviat and colleagues reported in 2004 41 mutations in *PCCA* and 54 in *PCCB*, most of them being single base pair substitutions causing an amino acid change. How these mutations affect the enzyme has not been well established and how each mutation accounts for the patients' phenotype is difficult to determine because most of the PA patients are compound heterozygotes.

1.2.4. 3-Methylcrotonyl-CoA carboxylase (MCC)

MCC catalyses the fourth step of the leucine catabolic pathway by carboxylating 3-methylcrotonyl-CoA at carbon-4 to form 3-methylglutaconyl-CoA (Figure 1.3) (Sweetmann and Williams 2001). In contrast to human MCC, bovine MCC is involved also in the catabolism of isovalerate, and has been implicated as a component enzyme of the mevalonate shunt (Lau et al. 1980).

Isolation and purification of MCC from mitochondrial bovine kidney shows an enzyme with an approximate size of 835 kDa that appears to be comprised of six heterodimers ($\alpha_6\beta_6$) (Lau et al. 1980). Similar to PCC, MCC has a larger α–subunit, which covalently binds biotin and contains the biotin carboxylation domains and a smaller β–subunit, which contains the carboxyltransferase domain (Sweetman and Williams 2001). MCC is predominantly localized to the inner membrane of mitochondria and is highly expressed in kidney and liver (Sweetmann and Williams 2001).

Using homology probing and the known cDNAs from *Arabidopsis thaliana* and other plants (Mckean et al. 2000, Weaver et al. 1995), three independent groups have cloned human and murine cDNAs encoding both subunits of MCC, confirmed their identity by biochemical and molecular genetic studies and identified mutations in MCC-deficient patients (Baumgartner et al. 2001, Gallardo et al. 2001, Holzinger et al. 2001).

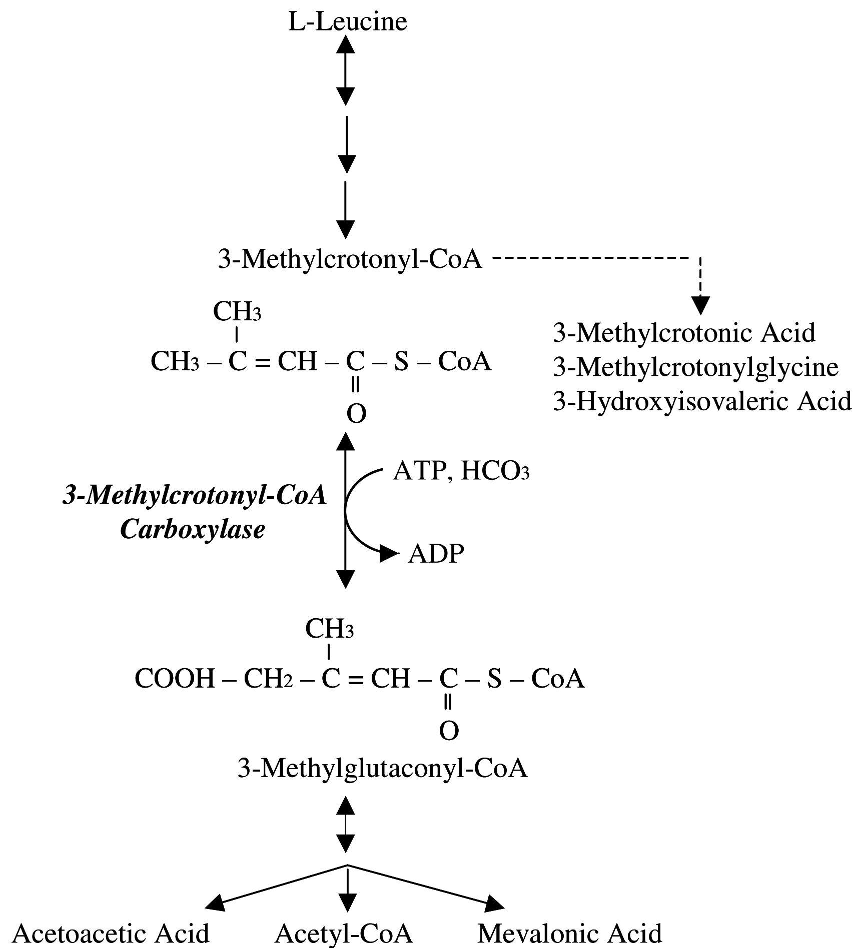


Figure 1.3. The MCC-catalysed reaction and its position in the leucine catabolic pathway. Each arrow represents a enzymatic step. The dashed arrow indicates the metabolites that accumulate due to deficiency of MCC. Modified from Sweetman and Williams 2001

The cDNAs predict a human MCCα of 725 amino acids with a calculated molecular mass of 80 kDa, a mouse MCCα of 717 amino acids with a calculated molecular mass of 79 kDa, and human and mouse MCCβ of 563 amino acids with a calculated molecular mass of 61 kDa. Human MCCα has 84% and 45% identity to MCCα of mouse and *A. thaliana*, respectively. Human MCCβ has 89% and 60% identity to MCCβ of mouse and *A. thaliana,* respectively (Baumgartner et al. 2001). The human *MCCA* and *MCCB* structural genes have been mapped to chromosome 3q25-q27 and 5q12-q13.1 and have 19 and 17 exons,

respectively (Baumgartner et al. 2001, Gallardo et al. 2001, Holzinger et al. 2001). At the beginning of this study, 9 *MCCA* and 13 *MCCB* functionally significant mutant alleles had been reported including missense, nonsense, frameshift and splice site mutations (Baumgartner et al. 2001, Desviat et al. 2003, Gallardo et al. 2001, Holzinger et al. 2001).

1.3. 3-Methylcrotonyl-CoA carboxylase deficiency

Isolated biotin-resistant MCC deficiency (MIM 210200) is an autosomal recessive disorder caused by defects in either the α– or β-subunit of MCC. The diagnosis is confirmed by enzyme assays in lymphocytes and/or cultured skin fibroblasts, which show severely reduced, or absent MCC, but normal PCC and PC activity (Baumgartner 1990).

To date, most patients with confirmed isolated biotin-resistant MCC deficiency have a characteristic organic aciduria with elevated excretion of 3-hydroxyisovaleric acid (3-HIVA) and 3-methylcrotonylglycine (3-MCG). In addition, acyl-CoA derivatives accumulate and are *trans*-esterified to acylcarnitine esters. The major abnormal metabolite, 3-hydroxyisovalerylcarnitine, is found in blood and urine (Sweetman and Williams 2001) and can be easily detected by tandem mass spectrometry (TMS) in dried blood spots. Owing to the enzyme block, 3-methylcrotonyl-CoA accumulates within the mitochondria, and, by an alternative mechanism, 3-MCG and 3-HIVA are formed. Whereas 3-MCG can directly cross mitochondrial membranes, 3-HIVA can do this only after deacylation or conjugation with carnitine. After reaching the cytosol, all these compounds are readily excreted in the urine, leading to secondary carnitine deficiency (Sweetman and Williams 2001).

In 1970 Eldjarn and colleagues identified a new inborn error in the leucine catabolic pathway that was different from maple syrup urine disease and isovaleric acidaemia. They reported on a $4^{1}/_{2}$–month-old girl of consanguineous parents that presented at 2 weeks of age with feeding difficulties. Progressively the signs of retarded motor development, muscular hypotonia and atrophy appeared. Her urine had the odour of cat's urine and she excreted large amounts of two abnormal metabolites, 3-HIVA and 3-MCG. The characteristic odour and the 3-HIVA level decreased significantly when she was put on a

low-leucine diet (150 mg leucine/kg body weight/day). However, this diet did not improve the child's symptoms, even after extra supply of biotin (0.25 mg/day), and she died at the age of nine months. No enzymatic assay was performed. Today it is still not known if this patient had isolated MCC deficiency or a severe form of MCD.

Thereafter several patients with MCC deficiency were reported. However, most of these patients were subsequently found to have MCD (Bartlett et al. 1980, Gompertz and Draffan 1971, Gompertz et al. 1973).

The first well characterized patients with isolated biotin-resistant MCC deficiency were 2 siblings from Vietnam, a boy and a girl, reported by Beemer et al. in 1982. When the boy was almost 5 years old he arrived in the Netherlands and four weeks later started to vomit and was admitted to the hospital in subcoma. Until this time there were no physical abnormalities apart from severe dental caries. Laboratory data showed elevated urinary concentrations of 3-HIVA and 3-MCG. MCC deficiency was confirmed in both patients by direct enzyme assay in leukocytes and cultured fibroblasts showing deficient MCC activity while activities of the 3 other biotin-dependent carboxylases were found to be normal. MCC activity in his young sister was performed and MCC deficiency diagnosis confirmed. The authors speculated that the change from a low protein diet in Vietnam to a high protein diet in the Netherlands might have contributed to the metabolic decompensation in the first patient. Both patients did not respond to biotin treatment and developed normally on a protein-restricted diet (Beemer et al., 1982).

Most patients with isolated MCC deficiency develop normally until they present with an episode of metabolic decompensation frequently following intercurrent illness, usually between the age of 1 and 3 years. Symptoms include hypotonia, hypoglycaemia, hyperammonemia, ketonuria, seizures and Reye-like syndrome (Bartlett et al. 1984, Layward et al. 1989, Pearson et al. 1995). They usually respond to intravenous fluids and cessation of protein feeding, and are asymptomatic between acute episodes. Some affected subjects have been placed on a diet modestly restricted in leucine with oral L-carnitine supplements, but the efficacy of this approach is unproven.

In some cases however, the above mentioned treatment did not improve the patient's condition, and 4 patients had a fatal outcome. All of these patients were the product of a consanguineous union and could have an additional genetic disorder that was not detected. (Bannwart et al. 1992, Baykal et al. 2005, Wiesmann et al. 1998).

A few patients showed a severe form presenting in the early neonatal period with failure to thrive, global developmental delay and neurological complications (Kremer et al, 2002, Lehnert et al. 1996, Murayama et al. 1997, Yap et al. 1998).

Some families with several affected siblings and also affected parents have been reported and showed a high phenotypic variability even within the same family. Jurecki and Packman in 1992 reported a family with four affected siblings, two of whom were asymptomatic, one of whom had mild metabolic abnormalities but normal development, while the last one had severe symptoms and died at the age of 2 years and 2 months. In another family with three affected siblings only one had mild developmental delay at the age of 1 year while the other two were asymptomatic at the age of 6 years and 3 months (Mourmans et al. 1995). Visser and collaborators reported in 2000 a MCC-deficient patient with congestive heart failure and normal psychomotor development. Evaluation of family members revealed an affected brother with no cardiac abnormalities but delayed psychomotor development, whereas the father was also affected but asymptomatic.

Finally, some affected but virtually asymptomatic mothers were diagnosed only after detection of abnormal metabolites in the neonatal screening samples of their healthy babies (Dantas et al. 2005, Gibson et al. 1998, this thesis).

1.4. Newborn screening by tandem mass spectrometry (TMS)

In industrialised countries, genetic disorders have become a significant factor with regard to handicap and mortality, and the development of optimised screening strategies to detect a maximal number of treatable metabolic disorders is a major challenge for preventive medicine. In some countries genetic disorders occur with an incidence of 1:1500 live births due to high rates of consanguineous parents (Joshi et al. 2002). Among other

factors, early diagnosis and treatment are crucial determinants for outcome in many genetic metabolic diseases.

In Switzerland newborn screening using dried blood spots on filter paper (Guthrie card) was introduced in the 1960's. Until 2005 this screening, as in many countries, was based on a one method-one disorder principle and included six different disorders.

TMS provides a one method-many disorders principle which potentially allows simultaneous screening for over 30 rare inborn errors of metabolism (Röschinger et al. 2003). TMS has advanced to the forefront of newborn screening technology by eliminating several technical problems associated with previous screening technology, such as high false positive rates and the expense of individual tests (Chace et al. 1999, Chace et al. 2001, Rashed et al. 1995). The cost of TMS based newborn screening is low given the speed of automated systems that are able to analyse multiple metabolites in up to 500 samples per instrument in a single day. TMS based newborn screening can be used to detect disorders of amino acid, organic acid, and fatty acid metabolism by measuring amino acids and acylcarnitines. Most of the disorders have a birth incidence of less than 1:50'000 (Röschinger et al. 2003)

Among the disorders that may be diagnosed, some cause severe illness or death within the first few days of life, and newborn screening may serve only to suggest a diagnosis that might otherwise have been missed. However, most of the disorders are treatable if they are diagnosed early.

Newborn screening using TMS is currently being utilized in at least seven states in the United States and in other countries including Australia and Germany, and introduction of this technology is being considered nationally as a way to improve the presymptomatic detection of inborn errors of metabolism (Aurey-Blais et al. 2003, Hoffmann et al. 2004, Koeberl et al. 2003, Schulze et al. 2003, Wilcken et al. 2000, Zytkovicz et al. 2001).

Three recent studies of TMS based newborn screening programs in North Carolina, Germany and Australia showed a high incidence of sustained elevation of 3-hydroxyisovalerylcarnitine (C5OH), a marker for MCC deficiency (Koeberl et al. 2003,

Schulze et al. 2003, Wilcken et al. 2003). In these studies the estimated incidence of MCC deficiency was about 1 in 50'000 infants. Although it is possible that some of these infants may never develop clinically significant disease, carnitine deficiency was detected in 4 out of 8 infants upon initial investigation (Koeberl et al. 2003). Based on these studies, MCC deficiency appears to be the most frequent organic aciduria detected in newborn screening programs using TMS.

2. Objectives

Isolated, biotin-resistant 3-methylcrotonyl-CoA carboxylase (MCC) deficiency is an autosomal recessive disorder of leucine catabolism that appears to be the most frequent organic aciduria detected in tandem mass spectrometry based neonatal screening programs with a frequency of about 1:50'000 (Koeberl et al. 2003; Schulze et al.2003; Wilcken et al. 2003). The phenotype is variable, ranging from neonatal onset with severe neurological involvement to asymptomatic adults. MCC is a heteromeric mitochondrial enzyme composed of biotin containing α subunits and smaller β subunits, encoded by *MCCA* and *MCCB*, respectively.

The specific aims of this thesis are:

a. Identification of *MCCA* and *MCCB* mutations

b. Determination of the consequences of these mutations on MCC function

c. Analysis of steady-state levels of normal and abnormal MCCα protein

d. Identification and investigation molecular defects with dominant negative effects on MCC activity

e. To increase the understanding of the variables influencing the phenotypic consequences of MCC deficiency

Finally, knowledge obtained from studies on MCC will serve as an example for other biotin-dependent enzymes and their disorders.

2.1. Outline of the thesis

The material of the experimental section is composed of the following manuscripts, which have been published:

a. ***Dantas MF**, Suormala T, Randolph A, Coelho D, Fowler B, Valle D, Baumgartner MR. 3-Methylcrotonyl-CoA carboxylase deficiency: Mutation analysis in 28 probands, 9 symptomatic and 19 detected by newborn screening. Hum. Mutat. 26(2):164 2005*

b. *Baykal T, Humer Gokcay G, Ince Z, **Dantas MF**, Fowler B, Baumgartner MR, Demir F, Can G, Demirkol M. Consanguineous 3-methylcrotonyl-CoA carboxylase deficiency: early-onset necrotizing encephalopathy with lethal outcome J. Inherit. Metab. Dis. 28(2):229-33 2005*

c. *Pinto LLC, Zen P, Rosa R, Paskulin G, Perla A, Barea L, Baumgartner MR, **Dantas MF**, Fowler B, Giugliani R, Vargas CR, Wajner M, Graziadio C. Isolated Biotin Resistant 3-Methylcrotonyl-Coenzyme A carboxylase deficiency in a child with metabolic stroke. J. Inherit. Metab. Dis. in press*

d. *Baumgartner MR, **Dantas MF**, Suormala T, Almashanu S, Giunta C, Friebel D, Gebhardt B, Fowler B, Hoffmann GF, Baumgartner ER, Valle D. Isolated 3-methylcrotonyl-CoA carboxylase deficiency: evidence for an allele-specific dominant negative effect and responsiveness to biotin therapy. Am. J. Hum. Genet. 75:790-800 2004*

These manuscripts are presented in the **chapters 3 (a, b and c) and 5 (d)**.

Chapter 4 is focused on the consequences of *MCCA* and *MCCB* missense mutations on MCC activity and analysis of steady-state levels of mutant MCCα protein.

In **chapter 6** the question of whether there is any correlation between the molecular defects and the phenotypic variation in our patient population is evaluated.

3. Molecular characterization of MCC-deficient patients by identification of new MCCA and MCCB mutant alleles.

The metabolic unit at the Children's Hospital of Basel has a longstanding interest in inborn errors of biotin metabolism. They are one of the few reference laboratories worldwide providing enzyme activity measurement for the carboxylases. Consequently, they have a large, well-characterized collection of fibroblast cell lines from patients with isolated MCC deficiency, and includes 75 patients from 70 families from several different nationalities and ethnic backgrounds. From each family an individual proband was included in this study. 32 of these probands presented with an acute metabolic decompensation and were sent to Basel for enzymatic confirmation of suspected MCC deficiency. Case reports with biochemical data and clinical symptoms of many of these patients have been reported earlier (Bannwart et al.. 1992, Baumgartner et al.. 2004, Baykal et al.. 2005, Beemer et al.. 1982, Gibson et al.. 1998, Gitzelmann et al.. 1997, Jurecki and Packmann 1992, Kremer et al.. 2002, Lehnert et al.. 1996, Mourmans et al.. 1995, Pinto et al.. in press, Stehen et al.. 1999, Tsai et al.. 1989, Visser et al.. 2000, Wiesmann et al.. 1998). The remaining 38 MCC-deficient probands were asymptomatic newborns detected by TMS based newborn screening, including 4 mothers identified only by detection of abnormal metabolites in the neonatal screening samples of their healthy babies. MCC deficiency seems to be the most frequent organic aciduria detected using TMS based newborn screening. The incidence of MCC deficiency is estimated to be about 1:50'000 live births in North America, Europe and Australia (Koeberl et al.. 2003, Schulze et al.. 2003, Wilcken et al.. 2003).

MCC activity in fibroblasts of these patients was usually less than 2% of he median control value, although a few patients had activities between 2.5% and 17.6%. No correlation between the level of residual activity and clinical phenotype was observed.

This chapter reports mutation analysis, clinical and biochemical data from 28 of these MCC-deficient patients (Baykal et al.. 2005, Dantas et al.. 2005, Pinto et al.. in press).

3.1. 3-Methylcrotonyl-CoA carboxylase deficiency: mutation analysis in 28 probands, 9 symptomatic and 19 detected by newborn screening

Dantas MF, *Suormala T, Randolph A, Coelho D, Fowler B, Valle D, Baumgartner MR.* *Hum. Mutat. 26(2):164 2005*

Abstract

Isolated 3-methylcrotonyl-CoA carboxylase (MCC) deficiency is an autosomal recessive disorder that appears to be the most frequent organic aciduria detected in tandem mass spectrometry (TMS)-based neonatal screening programs. The phenotype is variable, ranging from neonatal onset with severe neurological involvement to asymptomatic adults. MCC is a heteromeric mitochondrial enzyme composed of biotin containing α subunits and smaller β subunits, encoded by *MCCA* and *MCCB*, respectively. We report mutation analysis in 28 MCC-deficient probands, 19 of whom were asymptomatic newborns detected by TMS newborn screening, and nine presented with clinical symptoms. Ten have mutations in *MCCA*, and 18 in *MCCB*. We identified 10 novel *MCCA* and 14 novel *MCCB* mutant alleles including missense, nonsense, frameshift and splice site mutations, and show that three of the missense mutations result in severely decreased MCC activity when expressed in MCC-deficient cell lines. Our data demonstrate no clear correlation between genotype and phenotype suggesting that factors other than the genotype at the MCC loci have a major influence on the phenotype of MCC deficiency.

Introduction

Isolated biotin-resistant 3-methylcrotonyl-CoA carboxylase (MCC, EC 6.4.1.4) deficiency (MIM#s: *MCCA*-210200 and *MCCB*-210210) is a rare metabolic disorder inherited as an autosomal recessive trait (Sweetman and Williams, 2001). MCC catalyzes the fourth step of leucine catabolism converting 3-methylcrotonyl-CoA to 3-methylglutaconyl-CoA in a reversible ATP dependent reaction using bicarbonate as the source of the carboxyl group (Sweetman and Williams, 2001). Patients with MCC deficiency show elevated urinary excretion of 3-hydroxyisovalerate (3-HIVA) and 3-methylcrotonylglycine (3-MCG), usually in combination with severe secondary carnitine deficiency (Sweetman and Williams, 2001). In addition, acyl-CoA derivatives accumulate and are *trans*-esterified to acylcarnitine esters with 3-hydroxyisovalerylcarnitine characteristically present in blood and urine (Sweetman and Williams, 2001). The clinical presentation is extremely variable ranging from severe neurological abnormalities and death in infancy to asymptomatic adults detected in family studies or in mothers due to positive newborn screening findings in their unaffected babies (Bannwart et al.. 1992; Baykal et al.. 2005, Gibson et al.. 1998; Koeberl et al.. 2003; Wiesmann et al.. 1998).

MCC is one of three mitochondrial biotin-dependent carboxylases present in man; the others are propionyl-CoA carboxylase (PCC) and pyruvate carboxylase (Jitrapakdee and Wallace, 2003). The MCC enzyme is composed of nonidentical subunits, MCCα and MCCβ, encoded by *MCCA* (*MCCC1*; MIM# 609010) and *MCCB* (*MCCC2;* MIM# 609014), respectively (Baumgartner et al.. 2001; Gallardo et al.. 2001; Holzinger et al.. 2001). The native enzyme is thought to be a $\alpha 6\beta 6$ heteropolymer (Hector et al.. 1980). The larger MCCα subunit contains the covalently bound biotin prosthetic group and the bicarbonate and ATP binding sites. The MCCβ subunit binds methylcrotonyl-CoA. The *MCCA* structural gene is located on chromosome 3q25-q27 and consists of 19 exons. The corresponding cDNA (*MCCC1* GenBank: AF310972) is 2580 bp long and encodes a protein of 725 amino acids. The *MCCB* gene is located on human chromosome 5q12-q13.1 and consists of 17 exons. The corresponding cDNA (*MCCC2* GenBank: AF 310971) is 2304 bp long and encodes a protein of 563 amino acids (Baumgartner et al.. 2001; Gallardo

et al.. 2001; Holzinger et al.. 2001). To date, 9 *MCCA* and 13 *MCCB* mutant alleles have been reported including missense, nonsense, frameshift and splice site mutations (Baumgartner et al.. 2001; Desviat et al.. 2003; Gallardo et al.. 2001; Holzinger et al.. 2001). One missense allele, *MCCA*-p.R385S, has been shown to have a dominant negative effect that may lead to biochemical abnormalities and clinical symptoms in heterozygous individuals (Baumgartner et al.. 2004).

Introduction of tandem mass spectrometry (TMS) to newborn screening resulted in a large increase in the number of inborn errors that can be detected, including several amino acidemias and organic acidurias. Surprisingly, using this technique, MCC deficiency appears to be the most frequently detected organic aciduria in North America, Europe and Australia, with an overall frequency of approximately 1 in 50'000 (Koeberl et al.. 2003; Schulze et al.. 2003; Wilcken et al.. 2003).

Here, we report mutation analysis in 28 MCC-deficient probands, 19 of which were detected by TMS based newborn screening.

Probands and Methods

Cell lines and enzyme assays

28 subjects in whom MCC deficiency was suspected based on either elevated 3-MCG and 3-HIVA excretion or elevated 3-hydroxyisovalerylcarnitine in blood were included in this study. From 24 probands cultured skin fibroblasts, from 4 probands (018, 019, 025, 026) only RNA and DNA were available. Informed consent to perform enzymatic and molecular genetic studies was obtained from all probands or their parents. Clinical and biochemical data of proband 004 (Gitzelmann et al.. 1987) and proband 030 (Baykal et al.. in press) have been reported earlier. Mutation analysis in probands 004 and 013 has been previously reported (Baumgartner et al.. 2001) with only one mutant allele identified in each. Fibroblasts were cultured in Earl's minimal essential medium supplemented with 10 % fetal calf serum, L-glutamine, and antibiotics as described earlier (Suormala et al.. 2002). The biotin concentration of this standard medium was 6.5 nmol/L. The activities of PCC and MCC were assayed in fibroblast homogenates by measuring the incorporation of 14C-

bicarbonate into acid non-volatile products with established methods (Suormala et al.. 1985). Somatic cell complementation was used to distinguish between α- and β-subunit deficiency in fibroblasts as previously described (Baumgartner et al.. 2001).

Mutation analysis by RT-PCR and genomic PCR

Eighteen probands were grouped by complementation analysis followed by RT-PCR amplification and sequencing of the entire ORF (Open Reading Frame) of either *MCCA* or *MCCB,* as appropriate. In the remaining 10 probands RT-PCR amplification and sequencing of the entire *MCCB* ORF was first performed and, if no coding alterations were detected in *MCCB,* the entire *MCCA* ORF was also analyzed.

Total RNA and genomic DNA were extracted from cultured skin fibroblasts of 24 and from blood of 4 probands using the QIAamp® RNeasy and DNA isolation kits (Qiagen AG, Basel, Switzerland), respectively. The RT-PCR reaction was performed using the 1-Step RT-PCR kit (Qiagen AG, Basel, Switzerland) following the manufacturer's instructions. First-strand *MCCA* and *MCCB* cDNA was amplified as described (Baumgartner et al.. 2001). PCR products were sequenced in a thermocycler and analyzed with an ABI Prism 3100 Avant using the dye-terminator method (Applied Biosystem, Rotkreuz, Switzerland) according to the manufacturer's instructions. To confirm mutations identified in RT-PCR products, a genomic fragment containing the corresponding exon was amplified using flanking intronic primers, and the PCR product was sequenced directly.

In cases where only one of the two alleles could be identified in the standard RT-PCR product, the analysis was either repeated using fibroblasts cultured in the presence of Emetine (100 µg/ml medium; Sigma, Buchs, Switzerland) for 10 h before harvesting the cells to inhibit nonsense-mediated mRNA decay (NMD) (Carter et al.. 1995), or all exons and flanking intronic sequences were sequenced. The sequences of all primers are available upon request.

To survey a European control population for the identified missense mutations, relevant exons and flanking intronic sequences were amplified from genomic DNA, and the

indicated mutations were searched for by restriction digest or, if not possible, by direct sequencing of the PCR product.

Construction of wild type and mutant MCCA and MCCB expression vectors and transfections

Full-length wild type *MCCA* (-51 to +2275) and *MCCB* (-99 to +1824) cDNAs were TA cloned into the pCR Blunt II TOPO vector (Invitrogen, Basel, Switzerland) as described (Baumgartner et al.. 2001). To introduce the *MCCA* A291V missense mutation, an 896 bp *ACC* I restriction fragment from RT-PCR-amplified cDNA of proband 025 was subcloned into the p*MCCA*-TOPO construct. The wild type and mutant *MCCA* and *MCCB* constructs were then transferred into the mammalian expression vector pTracer-CMV2 (Invitrogen, Basel, Switzerland) at the *EcoR* I site. This vector contains the green fluorescent protein (GFP) gene fused to the Zeocin resistance gene. To introduce the *MCCB* H190Y and G352R missense mutations, a 965 bp *BstE* II/*Sfi* I restriction fragment from RTPCR-amplified cDNA of probands 018 and 019, respectively, were subcloned directly into the pTracer-CMV2 vector. All constructs were sequenced in both directions to validate their sequences. The constructs indicated above were incorporated into either immortalized MCCα or MCCβ deficient cell lines by electroporation as described (Baumgartner et al.. 2001), harvested 72 hours later and assayed for MCC and PCC activity.

Results and Discussion

This study provides new information on the nature of mutations in the *MCCA* and *MCCB* genes including expression studies in some cases, and allows a comparison of mutations between symptomatic patients and subjects detected by newborn screening.

Of 28 probands studied, 9 were diagnosed because of clinical symptoms (Table 3.1.1), while 19 were asymptomatic newborns detected by TMS based newborn screening (Table 3.1.2). Carboxylase activities in fibroblast homogenates from 24 probands are shown in Tables 3.1.1 and 3.1.2. MCC activity was below the level of detection (< 2.0 % of the median control value) in 18 probands (five with a defect in *MCCA* and 13 in *MCCB*). In 6 probands (020, 031, 034, 037, 043 and 046) MCC activity varied between 2.5% and 6.3%

of the median control value indicating the presence of residual, albeit low enzyme activity. The activity of PCC was within the control range in all cell lines.

Table 3.1.1. *Genotype and laboratory findings in MCC-deficient patients detected by clinical symptoms.*

Patient	*Affected Gene*[a]	*Allele 1*[b] *(Consequences)*	*Allele 2*[b] *(Consequences)*	*Clinical Phenotype*[c]	*Origin*	*Carboxylase Activities (pmol/min/mg protei)*	
						MCC	*PCC*
013	*MCCA*	c.1263dupG (frameshift)	c.1282-3A>G[e] (splice)	Mild	Swedish	0	1049
036	*MCCA*	c.1527C>A (nonsense)	c.1527C>A (nonsense)	Severe	Turkish	0.4	420
004	*MCCB*	c.517dupT (frameshift)	c.994C>T[e] (nonsense)	Mild	Swiss	20	1153
018	*MCCB*	c.568C>T (missense)	c.568C>T (missense)	Mild	Turkish	-	-
019	*MCCB*	c.1054G>A (splice)	c.1054G>A (splice)	Mild	Turkish	-	-
030	*MCCB*	c.1574+1G>A (splice)	c.1574+1G>A (splice)	Severe	Turkish	0	636
032	*MCCB*	c.127C>T (nonsense)	c.127C>T (nonsense)	Mild	Arabian	5.0	864
042	*MCCB*	c.929C>G (missense)	c.929C>G (missense)	Mild	Australian	0	665
044	*MCCB*	c.463C>T (missense)	c.463C>T (missense)	Mild	Brazilian	4.0	425

[a] *MCCC1 (MCCA)*, MIM# 609010; *MCCC2 (MCCB)*, MIM# 609014.

[b] cDNA variation numbering based on GenBank AF310972 *(MCCA)* and AF310971 *(MCCB)*, with +1 as A of the ATG start codon. Consensus nomenclature according to approved guidelines (http://www.hgvs.org/mutnomen/).

[c] Mild: late onset, good recovery after acute attack, no or mild developmental delay; Severe: onset in infancy, severe neurological involviment with severe developmental delay.

[d] Control values for 3-methylcrotonyl-CoA carboxylase (MCC) and propionyl-CoA carboxylase (PCC) activities in fibroblasts: MCC, median value 319 pmol/min/mg protein (range 125-831); PCC, median value 740 pmol/min/mg protein (range 207-2150); n=73 different control cell lines.

[e] Detected only after pre-incubation of cells with Emetine prior to isolation of RNA.

Table 3.1.2. *Genotype and laboratory findings in MCC-deficient patients detected by TMS-based newborn screening.*

Patient	*Affected Gene*[a]	*Allele 1*[b] *(Consequences)*	*Allele 2*[b] *(Consequences)*	*Origin*	*Carboxylase Activities*[c] *(pmol/min/mg protei)*	
					MCC	*PCC*
020	*MCCA*	c.1155A>C	c.559T>C	Geman	15.4	812
		(missense)	(missense)			
025	*MCCA*	c.872C>T	RNA not detectable	Australian	-	-
		(missense)				
027	*MCCA*	c.1155A>C	RNA not detectable	German	1.1	305
		(missense)				
028	*MCCA*	c.1155A>C	Exon 15 skipping[d]	German	1.1	317
		(missense)	(intragenic deletion)			
031	*MCCA*	c.1155A>C	c.400G>A	German	12.4	519
		(missense)	(missense)			
041	*MCCA*	c.694C>T	RNA not detectable	American	0	595
		(missense)				
043	*MCCA*	c.640_641delGG	c.1930G>T	American	8.1	705
		(frameshift)	(nonsense/skip exon 17)			
046	MCCA	c.2088dupA	c.1526_1527delG[e]	Amercian	20.0	1054
		(frameshift)	(frameshift)			
021	*MCCB*	c.803G>C	c.803G>C	Turkish	0	275
		(splice)	(splice)			
022	*MCCB*	c.464G>A	c.464G>A	Turkish	1.9	742
		(missense)	(missense)			
023	*MCCB*	c.469C>T	c.469C>T	Turkish	0	594
		(nonsense)	(nonsense)			
024	*MCCB*	c.295G>A	c.295G>A	Turkish	1.8	390
		(missense)	(missense)			
026	*MCCB*	c.1690T>C	RNA not detectable	Australian	-	-
		(add 3 aa at C-terminus)				
029	*MCCB*	c.295G>A	c;1574+1G>A[d]	Turkish	0	920
		(misssense)	(splice)			
033[f]	*MCCB*	c.282-1G>C	c.282-1G>C	Turkish	4.6	520
		(splice)	(splice)			

Table 3.1.2. *Continue*

Patient	*Affected Gene*[a]	*Allele 1*[b] *(Consequences)*	*Allele 2*[b] *(Consequences)*	*Origin*	*Carboxylase Activities*[c] *(pmol/min/mg protei)*	
					MCC	*PCC*
034	*MCCB*	c.845A>G (missense)	c.845A>G (missense)	Italian	16.2	542
037[f]	*MCCB*	c.1367C>T (missense)	c.1367C>T (missense)	Taiwanese	9.6	1269
039	*MCCB*	c.517dupT (frameshift)	c.1123G>T (missense)	American	0.7	696
040	*MCCB*	c.214C>T (nonsense)	c.416_427del12ins16 (framseshift)	Turkish	5.1	619

[a]*MCCC1 (MCCA)*, MIM# 609010; *MCC2 (MCCB)*, MIM# 609014.

[b]cDNA variation numbering based on GenBank AF310972 *(MCCA)* and AF310971 *(MCCB)*, with +1 as A of the ATG start codon. Consensus nomenclature according to approved guidelines (http://www.hgvs.org/mutnomen/).

[c]For control values see footnote[d] to Table 1.

[d] Detected only after pre_incubation of cells with Emetine prior to isolation of RNA.

[e]Detected only by sequencing all exons and flanking intronic sequences by genomic PCR.

[f]Asymptomatic mother detected by elevated 3-hydroxyisovaleryl carnitine in her nonaffected newborn.

Mutation analysis

Of the 28 probands investigated, 10 (36%) had mutations in *MCCA* and 18 (64%) in *MCCB* (Tables 3.1.1 and 3.1.2). Thirty-one different mutant alleles and one undefined intragenic deletion were identified, accounting for 52 of 56 possible mutant alleles. Twenty-four of the identified mutant alleles are novel, seven have been previously reported by us and two other groups (Baumgartner et al.. 2001; Gallardo et al.. 2001; Holzinger et al.. 2001). Because DNA of parents was not available in most cases, analysis of family members to rule out the possibility of a partial or complete deletion of the corresponding gene could not be performed.

Ten novel *MCCA* mutant alleles were identified including 4 predicted missense mutations (p.E134K, p.S187P, p.R232W, and p.A291V), 2 nonsense mutations (p.C509X, p.E644X), 1 splice site mutation (c.1682-3A>G) and 3 frameshift mutations (c.640_641delGG, c.1526_1527delG and c.2088dupA) (Table 3.1.3).

Table 3.1.3 *MCCA (MCCC1) mutant alleles and their consequences.*

No. of alleles detected	*Nucleotide change at cDNA level*[a]	*Exon / Intron*	*Amino acid change*	*Consequence (at RNA level)*	*Reference*
1	c.400G>A	exon 5	p.E143K	missense	novel
1	c.559T>C	exon 6	p.S187P	missense	novel
1	c.694C>T	exon 7	p.R232W	missense	novel
1	c.872C>T	exon 8	p.A291V	missense	novel
4	c.1155A>C	exon 11	p.R385S	missense	Baumgartner et al. 2001
1	c.1527C>A	exon 13	p.C509X	nonsense	novel
1	c.1930G>T	exon 17	p.E644X	nonsense (skip exon 17)	novel
1	c.640_641delGG	exon 7	p.G214IfsX5	frameshift	novel
1	c.1263dupG	exon 11	p.Q421AfsX10	frameshift	Baumgartner et al. 2001
1	c.1526_1527delG[c]	exon 13	p.C509SfsX38	frameshift	novel
1	c.2088dupA	exon 19	p.V697SfsX19	frameshift	novel
1	c.1682-3A>G[b]	intron 14	p.N561KfsX10	splice (frameshift / NMD)	novel

[a] cDNA variation numbering based on GenBank AF310972, with +1 as A of the ATG start codon; genomic reference sequence from intronic mutations is GenBank NM_020166.

[b] Detected only after pre-incubation of cells with Emetine prior to isolation of RNA.

[c] Detected only by sequencing all exons and flanking intronic sequences by genomic PCR.

NMD; nonsense-mediated mRNA decay.

We assume deleterious functional consequences for the frameshift mutations c.640_641delGG, c.1526_1527delG and c.2088dupA, the splice site mutation c.1682-3A>G and the nonsense mutations p.C509X and p.E644X because they result in truncated proteins lacking functionally important domains such as bicarbonate and/or biotin binding sites (Baumgartner et al.. 2001; Jitrapakdee and Wallace, 2003). The *MCCA* missense mutations p.E134K, p.S187P, p.R232W and p.A291V all change residues that lie within the biotin carboxylation domain and are highly conserved (Baumgartner et al.. 2001; Jitrapakdee and Wallace, 2003). Moreover, in our expression studies, p.A291V was associated with a reduction of activity to 26% of normal confirming functional significance (Table 3.1.5).

The previously described p.R385S mutant allele found here in four compound heterozygous *MCCA*-deficient subjects of German origin is of particular relevance. An arginine at position 385 is strictly conserved (Jitrapakdee and Wallace, 1999), and the corresponding residue in the biotin carboxylation domain of *E.coli* acetyl-CoA carboxylase has been shown to be part of the bicarbonate binding pocket (Sloane and Waldrop, 2004; Thoden et al.. 2000). Recent studies provided evidence that this missense mutation in the presence of the wild type allele has a dominant negative effect that may lead to biochemical and clinical abnormalities in heterozygous individuals (Baumgartner et al.. 2004). Moreover, in such subjects biotin therapy appears to counteract the dominant negative effect *in vivo* (Baumgartner et al.. 2004). We speculate that the occurrence of *MCCA*-p.R385S together with other mutant alleles that have residual activity may also respond to biotin therapy suggesting that therapeutic trials with biotin in subjects carrying this mutant allele are warranted (e.g. probands 020 and 031).

Fourteen novel mutant alleles were identified within the 18 MCCβ-deficient subjects including five predicted missense mutations (p.R155W, p.H190Y, p.H282R, p.A456V and p.V375F), one missense mutation which causes a substitution of the translational stop codon by adding 3 amino acids to the C-terminus of the β-subunit (p.X564QLE, Fig. 3.1.1), four nonsense mutations (p.R332X, p.Q157X, p.Q43X and p.R72X), three splice site mutations (c.1054G>A, c.1574+1G>A and c.282-1G>C), and 1 frameshift mutation (c.416_427del12ins16) (Table 3.1.4)

We assume functional significance for the frameshift mutation c.416_427del12ins16, the splice site mutations c.1574+1G>A, c.282-1G>C and c.803G>C, the nonsense mutations p.R332X, p.Q157X, p.Q43X and p.R72X, and the point mutation c.1054G>A yielding two transcripts (see below).

The *MCCB* missense mutations p.R155W, p.H190Y, p.H282R, p.V375F, p.G352R and p.A456V all change residues that are highly conserved (Baumgartner et al.. 2001; Desviat et al.. 2003; Jitrapakdee and Wallace, 2003). Furthermore, expression studies clearly demonstrate that the *MCCB*-p.H190Y and -p.G352R mutant alleles produce severely

decreased MCC activity confirming the deleterious functional consequences of these missense mutations (Table 3.1.5).

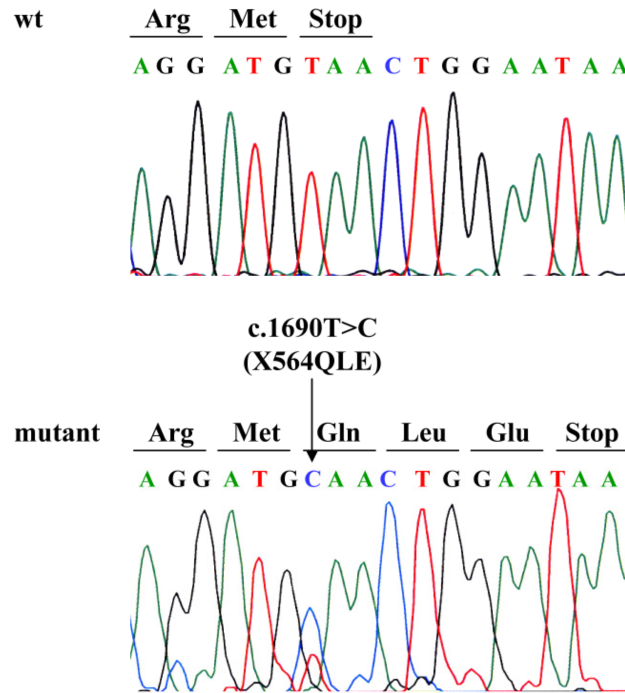


Figure 3.1.1. Electropherograms showing the p.X564QLE missense mutation in the MCCB gene in proband 026. A corresponding control sequence is shown in the upper panel. The lower panel shows the heterozygous T>C substitution, changing the stop codon to a glutamine residue which results in a extension of the polypeptide by 3 amino acids at the C-terminus.

Earlier studies have demonstrated that expression of *MCCB*-p.R155Q resulted in no detectable MCC activity (Baumgartner et al.. 2001). It is highly likely that the missense allele p.R155W also has deleterious consequences since it causes replacement of an arginine by a tryptophan at the very same position. The remaining missense mutation p.X564QLE changes the stop codon to a glutamine codon and consequently extends the encoded putative mutant MCC polypeptide by 3 amino acids at the C-terminus (Fig. 3.1.1). Since the C-terminal part of MCCβ is highly conserved, addition of 3 amino acids will likely interfere with normal functioning of the enzyme. Mutations of a similar type have been reported as causes of other monogenic disorders (Abe et al.. 2003; Marr et al.. 2002).

Table 3.1.4. *MCCB (MCCC2) mutant alleles and their consequences.*

No. of alleles detected	*Nucleotide change at cDNA level*[a]	*Exon / Intron*	*Amino acid change*	*Consequence (at RNA level)*	*Reference*
3	c.295G>A	exon 4	p.E99Q	missense	Baumgartner et al. 2001
2	c.463C>T	exon 5	p.R155W	missense	novel
2	c.464G>A	exon 5	p.R155Q	missense	Baumgartner et al. 2001
2	c.568C>T	exon 6	p.H190Y	missense	novel
2	c.845A>G	exon 9	p.H282R	missense	novel
2	c.929C>G	exon 10	p.P310R	missense	Baumgartner et al. 2001
1	c.1123G>T	exon 12	p.V375F	missense	novel
2	c.1367C>T	exon 14	p.A456V	missense	novel
2	c.127C>T	exon 1	p.Q43X	nonsense	novel
1	c.214C>T	exon 3	p.R72X	nonsense	novel
2	c.469C>T	exon 5	p.Q157X	nonsense	novel
1	c.994C>T[b]	exon 10	p.R332X	Nonsense (NMD)	novel
1	c.416_427del12ins16	exon 5	p.T139_G143>RW VPGEfsX35	frameshift	novel
2	c.517dupT	exon 6	p.S173FfsX25	frameshift	Baumgartner et al. 2001 Gallardo et al. 2001
2	c.282-1G>C	intron 3	(p.G94_S127del)	splice (skip exon 4)	novel
2	c.803G>C (r.785_803del)	exon 8	p.R268T (p.G262_R268delfs X5)	splice	Holzinger et al. 2001
2	c.1054G>A (r.1054G>A + r.1000_1072delins r.999+858_r.999+992)	exon 11	(p.G352R + p.V334_G358delins KFFMKYFLRLDL NSYNSTWQH)	missense / splice (skip exon 11, insert 64 bp from intron 10)	novel
3	c.1574+1G>A[b]	intron 16	(p.F497_V526>Gfs X4)	Splice (skip exon 16)	novel
1	c.1690T>C	exon 17	p.X564QLE	add 3 aa at C-terminus	novel

[a] cDNA variation numbering based on GenBank AF310971, with +1 as A of the ATG start codon; genomic reference sequence from intronic mutations is GenBank NM_022132.

[b] Detected only after pre-incubation of cells with Emetine prior to isolation of RNA.

NMD; nonsense-mediated mRNA decay.

Table 3.1.5. *Expression[a] of MCCA (MCCC1) and MCCB (MCCC2) alleles.*

Experiment	*Allele*	*Enzyme activity[b] (pmol/min/mg protein)*	
		MCC	PCC
I	*MCCA*-wild type	132	202
	MCCA-A291V	35	213
	vector	0	287
II	*MCCB*-wild type	168	402
	MCCB-H190Y	0	364
	MCCB-G352R	18.4	337
	vector	0	307

[a] Transient transfection in SV40T-transformed reference MCCα- or MCCβ-deficient cell lines.

[b] Numbers represent average of duplicates.

In proband 021 genomic PCR of *MCCB* exon 8 revealed a G to C transversion at the last bp of exon 8 resulting in the known point mutation c.803G>C (p.R268T, Fig. 3.1.2A; Holzinger et al.. 2001). The present study demonstrates that this mutation causes a splicing defect in fibroblasts from proband 021. Direct sequencing of RT-PCR amplified cDNA revealed a homozygous frameshift mutation due to deletion of the last 19 bp of exon 8 (Fig. 3.1.2B). We were not able detect the transcript with the c.803G>C change in 2 independent RT-PCR amplifications. The 3'base pair is conserved in about 80% of exons as a G (Shapiro and Senapathy, 1987) and contributes to donor splice recognition. Interestingly, a cytosine at this position appears to disrupt donor splice recognition leading to utilization of a cryptic splice donor site 19 bp upstream. Thus, the deleterious consequences of this missense mutation appear to be entirely due to the splicing defect.

A further unusual finding was the partial replacement of *MCCB* exon 11 by an in frame insertion of a 64 bp sequence from intron 10 in proband 019 (Table 3.1.4). Sequence analysis of *MCCB*-RT-PCR cDNA showed two overlapping sequences. One transcript contained the normal 73 bp of exon 11 including the point mutation c.1054G>A (p.G352R), while the other transcript skipped exon 11, but instead inserted a 64 bp intronic sequence

from intron 10. Genomic PCR of exon 11 indicated that this proband was homozygous for c.1054G>A. We hypothesize that the reduction in utilization of exon 11 associated with the c.1054G>A mutation is due to disruption of an exon splicing enhancer (ESE). Analysis of the *MCCB* ORF identified this motif as a putative exon splice enhancer sequence in an ESE prediction program (Fairbrother et al.. 2004).

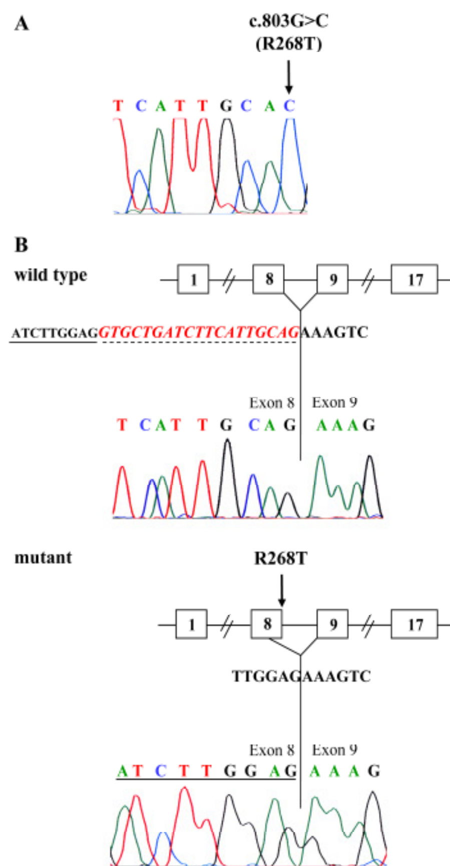


Figure 3.1.2. A: Genomic PCR of MCCB exon 8 in proband 021. The homozygous c.803G>C transversion of the last bp of exon 8 results in the missense mutation p.R268T. However, the 3’ base of an exon also contributes to donor splice site recognition. **B:** RT_PCR of MCCB cDNA in this proband resulted in a slightly smaller product due to utilization of a cryptic splice donor site (solid line). Use of this new splice donor site deletes the last 19 bp of exon 8 (dashed lines), which shifts the reading frame.

Finally, a survey of a European control population of 50 individuals was performed for all 4 *MCCA* (p.E134K, p.S187P, p.R232T and p.A291V) and all 7 *MCCB* missense alleles (p.R155W, p.R155Q, p.H190Y, p.H282R, p.P310R, p.V375F and p.A456V). None of them were identified in this collection of 100 control chromosomes indicating that each of these mutant alleles has a low frequency in this population.

In nine probands (004, 013, 025, 026, 027, 028, 029, 041 and 046) the steady state level of mRNA from the second allele was not detectable (Table 3.1.1 and 3.1.2) suggesting nonsense, framehift or splice site mutations that cause NMD. Contrary to conventional insights, the predominant consequence of nonsense mRNAs is not the synthesis of truncated proteins. Instead most mRNA molecules that contain a nonsense or frameshift mutation are highly unstable and are rapidly degraded by NMD (Frischmeyer and Dietz, 1999). To identify possible mutations that cause NMD, the mutation analysis was repeated using cultured fibroblasts treated with Emetine prior to isolation of RNA. In three probands, this treatment allowed identification of the second mutant allele, i.e. *MCCA*-c.1682-3A>G in proband 013 (Table 3.1.3), *MCCB*-p.R332X in proband 004 and *MCCB*-c.1574+1G>A in proband 029 (Table 3.1.4) confirming that these mutations cause NMD.

In proband 013 sequence analysis of *MCCA*-RT-PCR cDNA after Emetine treatment showed two overlapping sequences. One transcript contained the normal 50 bp of exon 15, while the other was 52 bp long with an insertion of AG. Genomic PCR of exon 15 indicated that the patient was heterozygous for an A>G transition at the –3 position in the splice acceptor site of intron 14 (c.1682-3A>G; TAAAG→TAGAG). A guanine at this position creates a cryptic splice acceptor site 2 bp upstream of the normal weak splice acceptor site. Utilization of this new acceptor splice site in fibroblasts from proband 013 causes insertion of two nucleotides (insAG), which shifts the reading frame. Finally, in proband 028 sequence analysis of *MCCA*-RT-PCR cDNA after Emetine treatment showed clearly two alleles, one containing the already known p.R385S missense mutation, the other skipping exon 15. Genomic PCR of exons 15, 14 and 16 were repeatedly normal suggesting an intragenic deletion. Attempts to define this deletion using long range PCR failed.

Emetine studies could not be performed in five probands (025, 026, 027, 041 and 046) in whom a second mutant allele had not been identified. In these probands genomic PCR of all exons including flanking intronic sequences revealed one additional mutation in proband 046, *MCCA*-p.C509SfsX38 (Table 3.1.3). In the remaining four probands we were not able to identify a second allele despite sequencing of all exons and flanking intronic sequences, suggesting that the steady state of mRNA from the second allele was not detectable as would be the case for a promotor mutation or an intragenic deletion or insertion missed by genomic PCR.

Genotype-phenotype correlation?

Based on small numbers, the present study failed to reveal any obvious genotype-phenotype correlation. No mutations were exclusively present in symptomatic or asymptomatic probands. The majority of the 10 MCCα- and 18 MCCβ-deficient probands in this study carry private mutations with no common mutant allele for either gene.

Combining this study with our earlier report (Baumgartner et al.. 2001), only one *MCCA* and two *MCCB* alleles occurred in more than 2 subjects in our 45 probands. First, the missense mutation *MCCA*-p.R385S, in spite of being the most prevalent so far, i.e. a total of 10 mutant alleles in 8 subjects (this study; Baumgartner et al.. 2001; Baumgartner et al.. 2004; Gallardo et al.. 2001), appears not to be a predictor of a particular phenotype. Second, *MCCB*-p.S173FfsX75, which causes a truncation of the β-subunit, has been detected in proband 004, asymptomatic Swiss compound heterozygote (Gitzelmann et al.. 1987; Baumgartner et al.. 2001), but also in two asymptomatic Amish/Mennonite adult homozygotes (Gibson et al.. 1998; Gallardo et al.. 2001). Third, the missense mutation *MCCB*-p.E99Q has been detected in a Turkish child with fatal outcome and an adult Amish patient with only mild symptoms (Bannwart et al.. 1992; Baumgartner et al.. 2001). Each of these mutations has also been detected in so far asymptomatic newborns (this study). Only time will tell whether these subjects receiving careful management will remain asymptomatic. As a word of caution, it has to be stated that there is no well-defined phenotype for MCC deficiency and therefore there is the possibility that some of the

literature reports of MCC-deficient patients with various neurological problems simply reflect bias of ascertainment.

In total, we have now investigated 45 MCC-deficient probands, 21 of whom were detected by newborn screening. Clinical, biochemical and genetic data strongly support the idea that factors other than the genotype at the MCC loci, such as modifying genes and environmental factors, must have a major influence on the phenotype of MCC deficiency.

3.2. Consanguineous 3-methylcrotonyl-CoA carboxylase deficiency: early-onset necrotizing encephalopathy with lethal outcome

Baykal T, Humer Gokcay G, Ince Z, Dantas MF, Fowler B, Baumgartner MR, Demir F, Can G, Demirkol M. ***J. Inherit. Metab. Dis. 28(2):229-33 2005***

Abstract

A patient with a severe neonatal variant of 3-methylcrotonyl-CoA carboxylase (MCC) deficiency is reported. The first child of healthy consanguineous Turkish parents presented on the second day of life with dehydration, cyanosis, no sucking, generalized muscular hypotonia, encephalopathy, respiratory depression requiring mechanic ventilation, macrocephaly, severe acidosis and hypoglycaemia. Elevated C5-OH-carnitine in dried blood spot by tandem MS and elevated urinary excretion of 3-hydroxyisovaleric acid and 3-methylcrotonylglycine suggested MCC deficiency, confirmed by enzyme analysis in cultured fibroblasts. Cerebral ultrasonography and cranial CT findings revealed progressive changes such as disseminated encephalomalacia, cystic changes, ventricular dilatation and cerebral atrophy. Treatment with high-dose biotin and protein-restricted diet was ineffective and the patient died at the age of 33 days with progressive neurological deterioration. Mutation analysis revealed a homozygous mutation in the splice acceptor site of intron 15 in the MCCβ-subunit. Early-onset severe necrotizing encephalopathy should be included in the differential diagnosis of isolated MCC deficiency.

Introduction

3-Methylcrotonyl-CoA carboxylase (MCC, EC 6.4.1.4) deficiency is an inborn error of leucine catabolism with autosomal recessive inheritance. Isolated MCC deficiency is caused by mutations in either *MCCA* (McKusick 210200) or *MCCB* (McKusick 210210), which encode the a- and b- subunits of MCC, respectively. The clinical course ranges from severe to benign forms.

Patients with MCC deficiency usually have normal growth and development until they present with an acute episode between 6 months and 3 years of age. The episodes frequently follow minor infections and involve feeding difficulty, vomiting, lethargy, apnoea, muscle hypotonia and seizures. Typical laboratory findings of an acute episode are severe hypoglycaemia, hyperammonaemia, elevated hepatic transaminases, mild metabolic acidosis and moderate ketonuria. Detection of elevated 3-hydroxyisovaleric acid (3-HIVA) and 3-methylcrotonylglycine (3-MCG) in urinary organic acids by gas chromatography--mass spectrometry (GC-MS) is diagnostic and may occur without elevation of isovalerylglycine or the distal leucine metabolites (Sweetman and Williams 2001). The analysis of acylcarnitines in dried blood samples by tandem MS consistently shows elevated 3-hydroxyisovalerylcarnitine.

Case Report

The patient was a male infant born post maturely at the gestational age of 44 weeks, with normal spontaneous delivery at home. He was the first child of healthy Turkish parents who were first cousins once removed. The family history is otherwise unremarkable. There was absence of sucking or crying during the first day. He was brought to our hospital at the age of 31 h. Anthropometric evaluation showed a body weight of 3.5 kg (75th centile), height 51 cm (50th centile) and head circumference 38 cm (97th centile). Physical examination revealed dehydration, cyanosis, encephalopathy, respiratory depression and poor capillary circulation. Neonatal reflexes were depressed and hypertonic episodes were observed in spite of prominent hypotonia. He was intubated immediately and put on mechanical ventilation in the neonatal intensive care unit. After cultures had been taken, antibiotics and

intravenous fluids were started, with a presumptive diagnosis of sepsis. Within an hour of hospital admission, cardiac arrest occurred with re-establishment of circulation after 2^3 min of resuscitation. Biochemical evaluation showed metabolic acidosis (pH 7.3, BE _15.3 mEq/L, Pco217.5mm/Hg), hypoglycaemia (glucose 36 mg/dl) and hyperlactataemia (5.7 mmol/L, normal < 2). Urinary organic acid determination by GC-MS revealed elevated lactic acid, 3-HIVA, 3-MCG and 3-hydroxybutyric acid. Acylcarnitines and amino acids by tandem MS disclosed elevated C5-OH-carnitine, normal free carnitine and normal amino acids profile in dried blood spots. Plasma biotinidase activity was normal (4.59 nmol/min per ml, normal 4.2^8.4). Sepsis work-up, blood and CSF cultures were negative. Cerebral ultrasonography showed cerebral oedema on the second day of admission and mannitol therapy was given for a short duration. EEG demonstrated disseminated amplitude depression. Doppler ultrasonography (US) (on the 7th day of admission) showed decreased cerebral blood flow, progression of oedema and ventricular dilatation.

At the age of 3 weeks, depression of the cranial bones was observed and repeated cerebral US showed cystic changes and leukodystrophy. Cranial CT was planned because of the progression in cerebral US findings. Disseminated encephalomalacia, multiple cysts, ventricular dilatation and cerebral atrophy were observed. The infant was treated with protein-restricted diet (1 g/kg per day), high-dose biotin (30--60 mg/day) and carnitine (100 mg/kg per day) supplementation. There was no clinical or bio-chemical improvement with this treatment; the baby could not come off the ventilator and he was lost at the age of 33 days

Material and Methods

Urinary organic acid determination was performed by GC-MS. The activities of biotin-dependent carboxylases were determined in fibroblasts, cultured in media containing different amounts of biotin, by measuring the incorporation of [14C]bicarbonate into acid nonvolatile products as previously described (Suormala et al. 1985). Mutational analysis was performed by amplification of cDNA from fibroblast RNA followed by direct

sequencing and confirmation of identified mutations on genomic DNA as described (Baumgartner et al. 2001).

Results

Table 3.2.1 shows the urinary excretion of 3-HIVA and 3-MCG detected with different biotin dosages and dietary protein intake. There was no clinical or biochemical response to therapy. While MCC activity was undetectable, the other two biotin-dependent carboxylases had activities in the normal range in cultured fibroblasts (Table 3.2.2). RT-PCR amplification and direct sequencing of *MCCB* cDNA resulted in a smaller product than wild-type owing to exon 16 skipping. Amplification of *MCCB* exon 16 from genomic DNA revealed a homozygous mutation in the splice donor site of intron 16 (IVS16+1G>A).

Table 3.2.1. *Urinary excretion of 3-hydroxyisovaleric acid ("-HIVA) and 3-Methylcrotonylglycine (3-MCG) under biotin and dietary therapy.*

	Urinary organic acid excretion			
Age (days)	*3-HIVA (mol/mol creatinine)*	*3-MCG (mol/mol creatinine)*	*Biotin suppementation (mg/day)*	*Dietary therapy*[a]
2	0.153	0.044	-	Breast milk
18	6.703	13.653	30	Breast milk
21	5.267	9.389	40	Protein restriction: 0 g protein/kg per/day Energy: 440kJ/kg per day
29	1.084	1.073	60	Protein restriction: 1 g protein/kg per/day Energy: 440kJ/kg per day

[a]Given with a nasogastric tube

Table 3.2.2. *Activities of biotin-dependent carboxylases in cultured fibroblats.*

	Carboxylase activity (pmol / min per mg protein) at given biotin concentration			
	0.1 nmol/L NB[a]	*10 nmol/L FCS*[b]	*10 µmol/L NB*	*10µmol/L FCS*
Propionyl-CoA carboxylase				
Patient	216	637	346	647
Control (n = 31, range)	183-1451	287-2150	201-1513	302-1600
Methylcrotonyl-CoA carboxylase				
Patient	ND[c]	ND	0.6	ND
Control (n = 31, range)	52-642	160-696	120-776	130-647
Pyruvate carboxylase				
Patient	183	489	360	476
Control (n = 31, range)	207-2373	229-2660	240-1845	225-2753

[a]NB, newborn calf serum

[b]FCS, fetal calf serum

[c]ND, not detectable

Discussion

Patients with MCC deficiency show variable clinical phenotypes ranging from asymptomatic adults to severe neonatal phenotypes, leading to coma and death in early infancy. This patient was born post maturely with macrocephaly. He was apathic soon after birth, did not cry and rejected feeding. The severity of clinical and biochemical abnormalities including lactic acidosis raised the suspicion of holocarboxylase synthetase deficiency, but the absence of 3-hydroxypropionic aciduria points to isolated MCC deficiency even in such severe cases. Very few patients with early-onset MCC deficiency have been reported (Bannwart et al. 1992; Lehnert et al. 1996). They had seizures,

hypotonia, failure to thrive, hypoglycaemia and metabolic acidosis in the neonatal period. Our patient had the earliest presentation of all MCC cases reported to date. We recognize, however, that our patient is the product of a consanguineous union and could have an additional genetic disorder that was not detected, despite comprehensive evaluation.

Cranial MRI changes in isolated MCC deficiency are reported as marked brain atrophy in a 15-year-old patient and multiple foci of leukodystrophy in a 14-month-old patient (Kremer et al. 2002; Murayama et al. 1997). The striking cranial imaging findings of our patient included severe encephalomalacia, leukodystrophy, cystic lesions, ventricular dilatation and cerebral atrophy, indicating a rapid progression within the newborn period. During the last week of his life, this necrotizing encephalomalacia, even manifested as depression of cranial bones, was evident on physical examination.

MCC is a heteromeric mitochondrial enzyme composed of biotin-containing α-subunits and smaller β-subunits (Baumgartner et al. 2001). Mutation analysis revealed a homozygous mutation in the splice donor site of intron 16 in *MCCB*. The functional consequences of this splice site mutation can be assumed to be deleterious, as suggested by undetectable MCC activity in cultured fibroblasts. The patient with severe phenotype and early onset described by Bannwart and colleagues was homozygous for another mutation in *MCCB*, the missense mutation E99Q, and was reported as proband 002 (Baumgartner et al. 2001). According to our current knowledge, most patients carry private mutations (Baumgartner et al. 2001).

These observations add early-onset severe necrotizing encephalopathy to the spectrum of clinical abnormalities in isolated MCC deficiency.

3.3. Isolated biotin resistant 3-methylcrotonyl-coenzyme A carboxylase deficiency in a child with metabolic stroke.

*Pinto LLC, Zen P, Rosa R, Paskulin G, Perla A, Barea L, Baumgartner MR, **Dantas MF**, Fowler B, Giugliani R, Vargas CR, Wajner M, Graziadio C. J. Inherit. Metab. Dis. in press*

Isolated biotin resistant 3-Methylcrotonyl-CoA carboxylase (MCC; EC 6.4.1.4) deficiency (McKusic 210200 and 210210) is an autosomal recessive disorder of leucine catabolism (Baumgartner et al.. 2001). The clinical phenotype is highly variable. We report a 3-year-old boy with isolated MCC deficiency that had a unexpectly severe presentation with seizures and a history of a cerebral ischemic episode.

The patient is the second child of healthy non-consanguineous Brazilian parents, and was born at term after an uneventful pregnancy and delivery. His birth weight was 2800 g (P10), length 47 cm (P3), and head circumference 34 cm (P25-50). At the age of 5 months he presented hypotonia and developed seizures unresponsive even to high doses of anticonvulsants. Psychomotor retardation was observed at 8 months. CT and MRI scans of the brain showed a hypodense perisilvian right lesion corresponding to an ischemic event. On admission with 10 months, he had persistent seizures, failure to thrive and feeding problems and the physical exam showed a left hemiplegia. At 12 months his development was that of a child of 4 months and he was referred for genetic evaluation of epilepsy. At this time his weight was 9875 g (P3-10), his length 79 cm (P50) and his head circumference 44 cm (P2). He had a broad forehead and clinodactyly of 5th fingers. Generalized hypotonia and mild left hemiplegia were observed. Electroencephalogram (EEG) showed generalized spikes and polyspikes, and burst suppression in the background activity. Echocardiography was normal.

Laboratory investigation revealed normal values for white cells, blood gases, lactate, plasma glucose and transaminases. Organic acid analysis (by GC/MS) revealed increased excretion of 3-hydroxyisovaleric acid (3-HIVA) and 3-methylcrotonylglycine (3-MCG). Plasma biotinidase was normal. Enzyme studies were performed. Carboxylase activities in

fibroblast homogenates were determinate by measuring the incorporation of 14C-bicarbonate into acid nonvolatile products. MCC activity was 4 pmol/min./mg protein (normal: 160-969) and propionyl-CoA carboxylase (PCC) activity was 424 pmol/min/mg protein (normal: 287-2150). Thus these results showed markedly decreased activity of MCC while the activity of PCC was in the normal range allowing the diagnosis of isolated 3-methylcrotonyl-CoA carboxylase deficiency. To detect mutant alleles we performed RT-PCR amplification of *MCCA* and *MCCB* cDNAs. Direct sequencing of the products revealed that the patient is homozygous for a missense mutation in exon 5 of *MCCB* that replaces an arginine by a tryptophan (*MCCB*-p.R155W). The result was confirmed by amplification of *MCCB* exon 5 by genomic PCR (Dantas et al. 2005).

He was treated with low protein diet (1g/kg/d) for 4 months and L-carnitine (100 mg/kg/d) as soon the diagnosis was made. After 2 years receiving L-carnitine, physical and psychomotor developments were improved. By the age of 2 years and 10 months he was starting to walk and speaking no more than 3 words. At present he is still receiving L-carnitine 100 mg/kg/d.

Another patient with isolated MCC deficiency associated with metabolic stroke has been reported by Steen et al.. (1999). Our patient is a second example of this confirming that a “metabolic stroke” can occur in MCC deficiency. Moreover, this case underlines the importance of prompt screening for organic acidurias in acute focal brain disease.

4. Consequences of **MCCA** *and* **MCCB** *missense mutations on MCC activity, and analysis of steady-state levels of mutant MCCα protein.*

The spectrum of mutations of MCC-deficient patients shows a majority of missense mutations in both *MCCA* and *MCCB* genes, but also a large number of nonsense, frameshift and splice site mutations. We assume functional significance for splice and nonsense mutations, and for deletions and insertions that change the reading frame. In order to confirm the pathogenic effect of *MCCA* and *MCCB* missense mutations, we show the functional consequences of 1 *MCCA* and 3 *MCCB* missense alleles using transient transfection of mutant constructs into SV40T-transformed reference *MCCA* or *MCCB* deficient cell lines followed by measurement of MCC activity. In addition, Western blotting was used to analyze the effect of different mutations on the stability of the MCCα protein. The apparent severity of MCC mutations contrasts with the variety of clinical phenotypes found in MCC-deficient patients, suggesting that factors other than the genotype at the MCC loci have a major influence on the resulting phenotype.

Material and Methods

Construction of wild type and mutant MCCA and MCCB expression vectors

The full-length wild type *MCCA* (-51 to +2275), wild type *MCCB* (-99 to +1824), and *MCCA* and *MCCB* containing the missense mutations to be analysed, were amplified from control and MCC-deficient patient RNA and TOPO TA cloned into pCR 2.1 TOPO vector (Invitrogen Corp. Basel, Switzerland). The *MCCA*-p.A291V, *MCCB*-p.H190Y, *MCCB*-p.G352R and *MCCB*-p.X564QLE mutant constructs were then transferred into the mammalian expression vector pTracer-CMV2 (Invitrogen Corp. Basel, Switzerland) at the *EcoR* I site. This vector contains a green fluorescent protein gene fused to the Zeocin resistance gene. All constructs were sequenced in both directions to validate the sequences.

Transfections

The constructs indicated above were electroporated into either SV40T-transformed *MCCA* (homozygous for *MCCA*-p.Q421fs+1) or *MCCB* (homozygous for *MCCB*-p.S173L) deficient cell lines as described previously (Baumgartner et al. 2001, Dantas et al. 2005). Briefly, 20-30 µg of plasmid DNA were transfected into $1.2x10^{-7}$ cells using a Gene Pulser II electroporator (Bio-Rad, Reinach, Switzerland) set at 250 V and 950 µF. The cells were harvested 72 hours after transfection and assayed for MCC and PCC activities. Transfection with vector alone was used as a negative control. MCC activity obtained after transfection with vector containing the corresponding wild type MCC allele was used as a positive control.

Western Blot analysis of MCCα protein

Proteins were extracted from fibroblast homogenates of patients with *MCCA* mutations using M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL, USA). Protein content was measured using the BCA protein assay (Pierce, Rockford, IL, USA). Equal amounts of protein (20 µg/well) were applied on a 10% Tris-HCl precast gel, and electrophoresis was performed at 100 V for 90 minutes (Bio-Rad, Reinach, Switzerland). Proteins were transferred onto a nitrocellulose membrane (Bio-Rad, Reinach, Switzerland). Membranes were incubated in blocking buffer (Tris buffered saline pH 7.4, 0.5% non fat dry milk) for 30 minutes, and then incubated with MCCα antiserum (kindly supplied by Prof. D. Valle) at room temperature for 1 hour. After several washings, membranes were incubated with peroxidase-conjugated Anti-Rabbit IgG (Jackson Immuno Research Laboratories, USA). Immunoblots were visualized by chemiluminescence using Super Signal (Pierce, Rockford, IL, USA).

Results and Discussion

Functional characterization of MCCA and MCCB missense alleles

The molecular effect of missense mutations is difficult to predict, whereas the pathogenicity of nonsense, frameshift or splice mutations is generally assumed as they are

predicted to result in truncated proteins. In order to confirm that a missense mutation is responsible for MCC deficiency, we have used transient transfection of transformed *MCCA* and *MCCB* deficient cell lines to investigate the effect of 1 *MCCA* (p.A291V) and 3 *MCCB* (p.H190Y, p.G352R, p.X564QLE) missense mutations.

As shown in Table 1 MCC activity was not detectable or severely decreased in the three MCCβ missense alleles expressed, whereas the *MCCA*-p.A291V mutant allele showed 26% of wild-type activity (Figure 4.1). Regarding *MCCB* alleles, the missense mutations p.G352R and p.X564QLE result in low levels of activity (11% and 9%, respectively) while the missense allele p.H190Y showed no MCC activity (Figure 4.1). These results provide evidence that three of the above mentioned missense alleles are pathogenic and cause MCC deficiency. Transfection efficiency, assessed by scoring a subset of cells in each transfection for the presence of the coexpressed GFP, ranged from 10 to 20% in these experiments.

The mutant allele *MCCB*-p.G352R involves a glycine at position 352 of the MCCβ protein which is highly conserved and maps to a region probably responsible for binding of 3-methylcrotonyl-CoA (Baumgartner et al. 2001, Desviat et al. 2003). Substitution at this position by an arginine may destabilize the region and reduce substrate binding accounting for the loss of function. The missense mutation *MCCB*-p.X564QLE changes the stop codon to a glutamine codon and consequently extends the encoded putative mutant MCC polypeptide by 3 amino acids at the C-terminus (Dantas et al. 2005). Since the C-terminal part of MCCβ is highly conserved, addition of 3 amino acids will likely interfere with normal function of the enzyme. Mutations of a similar type have been reported as causes of other monogenic disorders (Abe et al., 2003; Marr et al., 2002). Regarding the missense allele *MCCB*-H190Y the histidine residue at the position 190 is not strictly conserved, however the tyrosine mutant residue is not present in the homologous proteins (Baumgartner et al. 2001, Desviat et al. 2003).

Regarding the *MCCA*-p.A291V missense allele, the alanine at position 291 of the MCCα protein is highly conserved. A change to a valine at this position can be considered

as a mild change since both amino acids are aromatic and non-polar. This could explain the relatively high level of residual activity detected (26%).

Table 4.1. *Expression[a] of MCCA and MCCB alleles.*

Gene	*Allele*	*Enzyme activity[b] (pmol/min/mg protein)*				*References*
		Experiment 1		Experiment 2		
		MCC	PCC	MCC	PCC	
MCCA	wild type	132	202	-	-	
	A291V	35	213	-	-	Dantas et al. 2005
	vector	0	287	-	-	
MCCB	wild type	168	402	115	634	
	H190Y	0	364	-	-	Dantas et al. 2005
	G352R	18.4	337	-	-	Dantas et al. 2005
	X564QLE	-	-	10	543	Not Published
	vector	0	307	0	466	

[a] Transient transfection in SV40T-transformed reference MCCα- or MCCβ-deficient cell lines.

[b] Numbers represent average of duplicates.

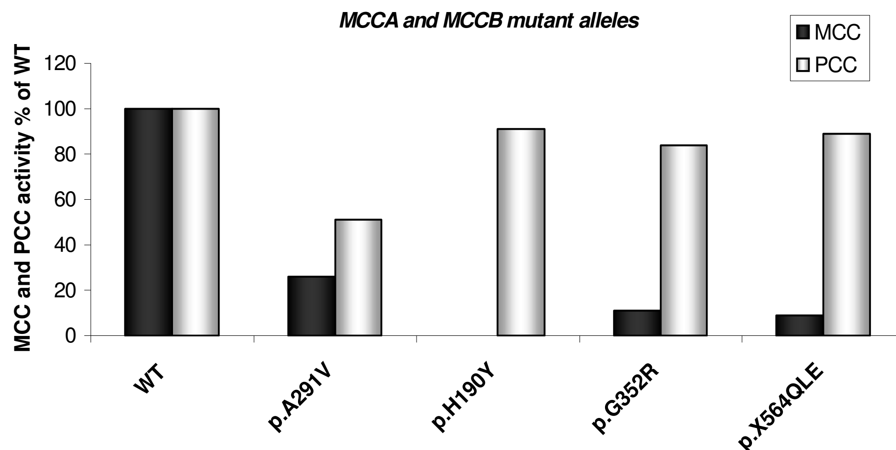


Figure 4.1. Expression of wild type (WT), MCCA-p.A291V and MCCB-p.H190Y, MCCB-p.G352R and MCCB-p.X564QLE missense alleles. 3-Methylcrotonyl-CoA carboxylase (MCC) and propionyl-CoA carboxylase (PCC) activity is expressed as % of WT.

Western Blot Analysis

To analyze the MCC polypeptides under steady-state condition in fibroblasts of *MCCA* deficient patients, we carried out Western blot analysis using rabbit anti-human MCCα antiserum. The results are shown in Figure 4.2. The MCCα protein (79 kDa) was readily detected in fibroblasts from wild type control (lane 1) and from a *MCCB*-deficient patient (compound heterozygous for *MCCB*-p.R332X and *MCCB*-p.S173FfsX25 (lane 2), and was absent in a negative control cell line homozygous for *MCCA*-Q421AfsX10 (lane 3). The MCCα protein was missing in fibroblasts of patients 036 (homozygous for p.C509X; lane 4), 043 (compound heterozygous for p.G214IfsX5 and p.E644X; lane 5), 046 (compound heterozygous for p.V697SfsX19 and p.C509SfsX38; lane 6), 050 (homozygous for p.E628X; lane 7), and 060 (homozygous for p.T693TfsX1; lane 8). All these mutations result in a truncated protein due to formation of premature termination codon (PTC) which usually leads to nonsense mediated mRNA decay (NMD).

In patients 041 (lane 9) and 064 (lane 10) only one mutant allele could be identified in the RT-PCR products (*MCCA*-p.R232W and *MCCA*-p.P632S, respectively). The second allele could not be detected indicating instability of this mutant allele and consequent degradation by the mechanisms of NMD. Usually, RNAs transcribed from mutant alleles or resulting from defective processing events in the nucleus are rapidly degraded to ensure cell growth. This is particularly important for mutations that give rise to a PTC upstream of the normal stop codon, as translation would yield a C-terminal truncated polypeptide. Such polypeptides can often act in a dominant-negative manner, leading to deleterious effects on the cell or organism. Fortunately, the eukaryotic cell has in place a surveillance mechanism, the NMD pathway, to ensure that only error-free mRNAs are accurately translated (Hentze and Kulozik 1999).

The MCCα protein was readily detected in normal amount and size in fibroblasts of five *MCCA* deficient patients, i.e. 020 (compound heterozygous for p.R385S and p.S187P; lane 11), 028 (compound heterozygous for p.R385S and exon 15 skipping due to an intragenic deletion, lane 12), 031 (compound heterozygous for p.R385S and p.E134K; lane 13), 053 (homozygous for p.R385S; lane 14), and 057 (homozygous for p.E288G; lane 15).

The missense mutation *MCCA*-p.R385S is unusual in that it is the only tested missense allele for which Western blot analysis shows normal amounts of MCCα protein (Baumgartner et al. 2001, Gallardo et al. 2001). In contrast, western blot analysis of the MCCα protein was normal in amount and size in 4 patients carrying *MCCA*-p.R385S. This is in agreement with previous studies which show that the protein product of this allele is stable (Baumgartner et al. 2001, Gallardo et al. 2001).

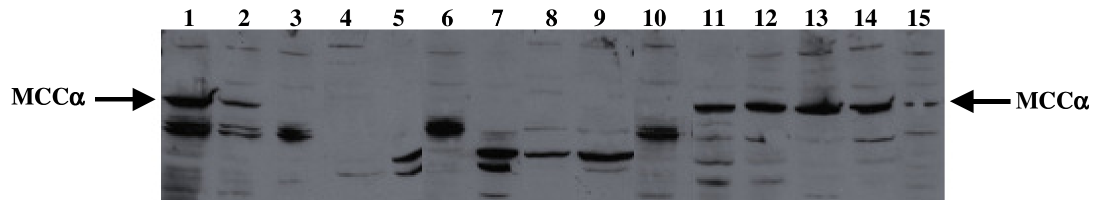


Figure 4.2. Western blot analysis of MCCα protein of wild type (lane 1), a MCCB-deficient patient (lane 2), a negative control for MCCα protein (lane 3), and 12 MCCA-deficient patients (lane 4 to 15). Equal amounts of total fibroblast protein (20 μg) from each sample were loaded on a 10% Tris-HCl precast gel and MCCα protein was detected using affinity purified MCCα antiserum as described in Methods. The identification of MCCα was determined by its mobility compared with protein standards for blotting and its absence in negative control.

5. Investigation of molecular defects with dominant-negative effect on MCC activity.

The majority of enzyme deficiencies are inherited as autosomal recessive traits. The terms dominant and recessive refer to the clinical phenotype associated with a particular allele (Beaudet et al. 2001). Although a recessive phenotype is defined as being clinically undetectable in heterozygotes, heterozygotes for many of these conditions exhibit subtle differences in phenotype when examined at the cellular or biochemical level, and these differences may be enhanced by environmental factors (Beaudet et al. 2001). Even when clinically normal, individuals who are heterozygous for recessive loss-of-function alleles demonstrate metabolic differences at the protein level. Such subtle phenotypic effects may be more common than is generally recognized and may contribute to phenotypes usually considered to be complex traits (Beaudet et al. 2001).

In 1987, Herskowitz described the term dominant-negative effect as a mutant allele in a heterozygote fashion (or, for X-linked traits, hemizygote) that interferes in one or another way with the function of the normal protein. In general, most dominant-negative mutations are considered as an altered function allele or as a particular subset of gain-of-function allele. These effects occur when proteins are stable and involved in subunit structures, or when these proteins interact with a nucleic acid or another protein in a complex. This will lead to the formation of non-functional multimers (Herskowitz 1987). Such a mutant allele has a dominant effect because its phenotype is manifested despite the presence of the wild-type allele.

Excellent examples of complex mechanisms that can underlie dominant-negative alleles can be found in the case of collagen and osteogenesis imperfecta, where many missense mutations interfere with fiber assembly and cause lethal osteogenesis imperfecta type II in heterozygotes, while null alleles cause a much milder form of the disease (Byers and Cole 2002). Heterozygosity for cystathionine β-synthase deficiency homocystinuria is also a risk factor in vascular disease (Clarke 2002), dementia in Alzheimer disease (Loscalzo 2002), and osteoporotic fractures in the elderly (Raisz 2004).

Our laboratory received two patient cell lines (047 and 048) with suspected MCC deficiency, which were shown to have partial MCC deficiency in cultured fibroblasts (20 and 21% of mean control value, respectively). Despite high residual MCC activity *in vitro*, we sequenced the entire ORF of *MCCA* and *MCCB*, and found that both patients are heterozygous for *MCCA*-p.R385S, but do not have any other coding alterations.

R385S is a missense mutation that changes an arginine to a serine at position 385 in the MCCα protein. This missense allele is of particular interest, because the mutant protein is stable and because the residue corresponding to *MCCA*-p.R385S is predicted to be part of a positively charged pocket for bicarbonate binding in the structure of the biotin carboxylase domain of *E. coli* ACC.

In the following chapter we provide evidence that *MCCA*-R385S is a dominant negative missense allele, and can lead to biochemical abnormalities and clinical symptoms in heterozygous individuals.

5.1. Isolated 3-methylcrotonyl-CoA carboxylase deficiency: evidence for an allele specific dominant negative effect and responsiveness to biotin therapy

*Baumgartner MR, **Dantas MF**, Suormala T, Almashanu S, Giunta C, Friebel D, Gebhardt B, Fowler B, Hoffmann GF, Baumgartner ER, Valle D. Am. J. Hum. Genet. 75:790-800 2004*

Abstract

Deficiency of 3-methylcrotonyl-CoA carboxylase (MCC) results in elevated excretion of 3-methylcrotonylglycine (3-MCG) and 3-hydroxyisovaleric acid (3-HIVA). MCC is a heteromeric mitochondrial enzyme comprising biotin containing α subunits and smaller β subunits, encoded by *MCCA* and *MCCB*, respectively. Mutations in these genes cause isolated MCC deficiency, an autosomal recessive disorder with a variable phenotype that ranges from severe neonatal to asymptomatic adult forms. No reported patients have responded to biotin therapy. Here, we describe two patients with a biochemical and, in one case, clinical phenotype of MCC deficiency, both of whom were responsive to biotin. The first patient presented at 3 months with seizures and progressive psychomotor retardation. Metabolic investigation at 2 years revealed elevated excretion of 3-MCG and 3-HIVA, suggesting MCC deficiency. High-dose biotin therapy was associated with a dramatic reduction in seizures, normalization of the electroencephalogram, and correction of the organic aciduria, within 4 weeks. MCC activity in fibroblasts was 25% of normal levels. The second patient, a newborn detected by tandem-mass-spectrometry newborn screening, displayed the same biochemical phenotype and remained asymptomatic with biotin up to the age of 18 months.

In both patients, sequence analysis of the complete open reading frames of *MCCA* and *MCCB* revealed heterozygosity for *MCCA*-p.R385S and for the known polymorphic variant *MCCA*-p.P464H but revealed no other coding alterations. *MCCA*-p.R385S is unusual, in that it has a normal amount of *MCCA* protein but confers no MCC activity. We show that *MCCA*-p.R385S, but not other *MCCA* missense alleles, reduces the MCC activity of cotransfected *MCCA*–wild-type allele. Our results suggest that *MCCA*-p.R385S is a dominant negative allele and is biotin responsive in vivo.

INTRODUCTION

3-methylcrotonyl-CoA carboxylase (MCC [Enzyme Commission number 6.4.1.4]) is a biotin-dependent enzyme that catalyses the fourth step in the leucine catabolic pathway. It carboxylates 3-methylcrotonyl-CoA to 3-methylglutaconyl-CoA in an ATP-requiring reaction that uses bicarbonate as the source of the carboxyl group (Sweetman and Williams 2001). MCC is a member of the family of biotin-dependent carboxylases, a group of enzymes with diverse metabolic functions but common structural features (Samols et al. 1988, Wolf 2001). In addition to MCC, there are three other biotin-dependent carboxylases in humans: propionyl-CoA carboxylase (PCC), pyruvate carboxylase, and acetyl-CoA carboxylase (Samols et al. 1988, Wolf 2001). Biotin is covalently bound to the apocarboxylases by holocarboxylase synthetase (HCS [GenBank accession number BC060787]) and, after proteolytic degradation of the active holocarboxylase into short biotinyl peptides or biocytin, it is released by biotinidase, enabling the recycling of biotin (Wolf 2001). MCC deficiency is caused either by defects of the MCC enzyme itself or by deficient activity of the enzymes involved in the metabolism of its cofactor biotin — that is, the enzymes HCS or biotinidase — deficiencies of which cause multiple carboxylase deficiency.

Isolated biotin-resistant MCC deficiency (also known as "methylcrotonylglycinuria" [MIM 210200 and 210210]) is inherited as an autosomal recessive trait (Sweetman and Williams 2001). The clinical phenotype is highly variable. Some patients present in the neonatal period with seizures and hypotonia (Bannwart et al. 1992, Lehnert et al. 1996), others are asymptomatic adult women discovered only by detection of abnormal metabolites in the neonatal-screening samples from their healthy babies (Gibson et al. 1998). Most patients are asymptomatic until an episode of acute metabolic decompensation after intercurrent illness in early childhood, which leads to their diagnosis. These patients usually respond to intravenous fluids and the cessation of protein feeding and are asymptomatic between episodes. Some children have been placed on a leucine restricted diet supplemented with oral L-carnitine, but the efficacy of this approach is unproven. In contrast to patients with multiple carboxylase deficiency due to HCS or biotinidase deficiency, no reported patient has been biotin-responsive, defined as showing a significant

clinical and/or biochemical improvement in response to oral administration of biotin at doses of 1–2 mg/kg/d or higher.

Patients with MCC deficiency have a characteristic organic aciduria with greatly increased excretion of 3-hydroxyisovaleric acid (3-HIVA) and 3-methylcrotonylglycine (3-MCG), usually in combination with severe secondary carnitine deficiency. In addition, acyl-CoA derivatives accumulate and are *trans*-esterified to acylcarnitine esters; the major abnormal metabolite, 3-hydroxyisovalerylcarnitine, is found in blood and urine (Sweetman and Williams 2001). It is surprising that MCC deficiency appears to be the most frequent organic aciduria detected in tandem mass spectrometry (TMS) newborn-screening programs in North America (Naylor and Chace 1999, Koeberl et al. 2003), Europe (Roscher et al. 2000, Schulze et al. 2003), and Australia (Wilcken et al. 2003).

Isolated MCC deficiency can be confirmed by demonstration of deficient MCC activity in the presence of normal activity of another biotin-dependent carboxylase in cultured skin fibroblasts or in isolated lymphocytes. MCC activity in cultured fibroblasts of patients is usually !2% of the mean control value. Two patients with higher residual activity, varying from 4% to 12%, in cultured fibroblasts have been reported elsewhere (Tuchman et al. 1993, Wiesmann et al. 1998). No correlation between the level of residual enzyme activity and clinical presentation has been observed.

Bovine MCC has a size of 835 kDa and appears to comprise six heterodimers: (αβ)6 (Lau et al. 1980). MCC is predominantly localized to the inner mitochondrial membrane. Like PCC, MCC is composed of larger α-subunits, which covalently bind biotin, and smaller β-subunits, encoded by the *MCCA* (*MCCC1* [GenBank accession number BC004214]) and *MCCB* (*MCCC2* [GenBank accession number BC065027]) genes, respectively, which have been cloned and characterized (Baumgartner et al. 2001, Gallardo et al. 2001, Holzinger et al. 2001). To date, 1 functionally neutral polymorphic variant, 9 functionally significant mutant *MCCA* alleles, and 13 functionally significant mutant *MCCB* alleles have been reported in probands with severe MCC deficiency (Baumgartner et al. 2001, Gallardo et al. 2001, Holzinger et al. 2001, Obata et al. 2001, Desviat et al. 2003). Several affected sibs of symptomatic patients have been clinically normal (Beemer

et al. 1982, Jurecki and Packman 1992, Mourmans et al. 1995), suggesting that the genotypes at the *MCCA* and *MCCB* loci are not the sole determinants of the clinical phenotype.

One of the identified missense alleles, *MCCA*-p.R385S, is unusual in that it is the only one of seven missense alleles detected in six MCCα-deficient patients for whom protein-blot analysis of fibroblast homogenates shows the presence of MCCα protein (i.e., shows cross reactive material [CRM]) (Baumgartner et al. 2001, Gallardo et al. 2001). Indeed, these studies suggest that the amount of MCCα protein in fibroblasts from a patient homozygous for the *MCCA*-p.R385S allele is normal or greater than normal, whereas six other *MCCA* missense alleles had little or no detectable CRM (Baumgartner et al. 2001). *MCCA*-p.R385S appears to be the most frequent allele found in MCC-deficient patients of German origin. Of seven such patients, one is homozygous and six are compound heterozygous for *MCCA*-p.R385S (M.R.B. and M.F.D, unpublished data).

Here, we describe two patients with elevated excretion of 3-MCG and 3-HIVA and with partial deficiency of MCC, one of whom has severe neurological symptoms. Both patients showed evidence of biotin responsiveness and were heterozygous for *MCCA*-p.R385S and the polymorphic variant *MCCA*-p.P464H. We provide evidence that *MCCA*-p.R385S is a dominant negative allele leading to biochemical abnormalities and clinical symptoms in heterozygous individuals and that it is responsive to pharmacologic doses of biotin in vivo.

Subjects and Methods

Case reports

Patient 1 (MCC047).—This boy was the second child of healthy, nonconsanguineous German parents. At the age of 3 wk, he developed infantile spasms. Electroencephalogram (EEG) results showed hypsarrhythmia at age 3 mo. Routine cerebrospinal-fluid and brain magnetic resonance imaging (MRI) investigations were normal. Therapeutic trials with vitamin B6 and vigabatrin did not lead to clinical improvement. At 2 years of age, he presented with severe psychomotor retardation, persistent infantile spasms, and hypsarrhythmia. MRI of the brain showed significant frontal and parietal cerebral atrophy

mainly of the white matter. Metabolic investigations revealed elevated excretion of 3-HIVA and 3-MCG without other abnormalities, suggesting MCC deficiency (table 5.1). Biotin treatment with 10 mg/d had no clinical or biochemical effect. An increase in the dose to 40 mg/d (2.7 mg/kg) was associated with dramatic reduction of seizure frequency. Within 4 wk, the EEG markedly improved, showing the disappearance of hypsarrhythmia, and the organic aciduria became virtually normal (table 5.1). On a biotin regimen (50 mg/d) and a protein-restricted diet (0.8 g/kg/d, introduced at age 2.5 years), the patient showed no further progression of symptoms, and excretion of 3-HIVA and 3-MCG completely normalized. Biotinidase activity in plasma was normal. At 7 years of age, he was seizure free on a protein-restricted diet (0.8 g/kg/d) and a regimen of vigabatrin and biotin (50 mg/d), and his EEG was unremarkable. Formal psychometrics were not obtained.

Patient 2 (MCC048).—This boy was the second child of healthy, nonconsanguineous Greek parents. His pre and postpartum history was unremarkable except for a hyperbilirubinemia requiring phototherapy. He was referred at age 5 wk because of elevated 3-hydroxyisovalerylcarnitine detected by TMS newborn screening. Urinary organic acid analysis displayed elevated 3-HIVA and trace amounts of 3-MCG with no other abnormalities (table 5.1). To test for a suspected defect in leucine catabolism, the patient received an oral leucine load (150 mg/kg). This challenge resulted in transient hyperammonemia (up to 134 μmol/L; normal < 80) and in a significant increase in urinary excretion of 3-HIVA and 3-MCG (table 5.1) that was partially suppressed by simultaneous administration of biotin (5 mg/kg) (table 5.1), suggesting in vivo biotin responsiveness similar to that of patient MCC047. Ten other infants who were referred because of elevated isovalerylcarnitine detected by TMS were similarly tested but showed normal organic acid excretion before and after leucine loading (data not shown). At 18 mo of age, patient MCC048 received biotin (5 mg/kg) and carnitine (50 mg/kg) and was developing normally. His urinary excretion of 3-HIVA and 3-MCG remained elevated (334 and 11.7 mmol/mol creatinine, respectively). Samples from the parents of patient MCC048 were not available. Informed consent for this study was obtained from the parents of both patients.

Table 5.1. *Effect of Biotin Substitution on 3-HIVA and 3-MCG concentration in Urine*

Subject and Biotin Substitution	Time after Leucine Load[a] (h)	Concentration in Urine of[b] (nmol/mol creatinine)	
		3-HIVA	3-MCG
MCC047:			
No Biotin	---	276	653
10 mg/d for 7 d	---	358	300
40 mg/d for 7 d	---	135	44
40 mg/d for 3 more wk	---	17	9
Father of MCC047:			
No biotin	---	26	ND
Mother of MCC047:			
No biotin	---	24	ND
MCC048			
No biotin	Before load	105	2.2
No biotin	0-2	183	12
No biotin	2-4	956	33
No biotin	4-6	794	35
No biotin	Before load	100	ND
5 mg/kg with leucine at 0 h	0-2	368	6.2
No biotin	2-4	471	2.5
No biotin	4-6	335	12
Controls:			
No biotin	---	<18	ND

[a]Patient MCC048 was given a leucine load of 150 mg/kg with and without a simultaneous biotin load, and urine was collected in three portions after he load.

[b]ND = not detected.

Cell cultures and carboxylase assays

Skin fibroblasts were cultured in a standard medium containing 10% fetal calf serum (FCS). The biotin concentration of this medium, supplied by the natural biotin content of FCS, was 9 nmol/L, or 5-fold the mean normal plasma biotin concentration in humans

(1.80 ± 0.84 nmol/L, range 0.65 – 4.83 nmol/L; *n*=126) (Suormala et al. 1997). We prepared a biotin-depleted medium by replacing FCS with newborn calf serum (NBCS), which resulted in a final biotin concentration of 0.1 nmol/L. To obtain physiological biotin concentration, we supplemented the low-biotin medium with 2.0 nmol/L biotin and, to obtain a high-biotin medium, with 10 µmol/L biotin (i.e., 5,000 times higher than normal plasma levels). The activities of PCC and MCC were assayed in fibroblast homogenates by measurement of the incorporation of ^{14}C-bicarbonate into acid-nonvolatile products, by methods established elsewhere (Suormala et al. 1985).

Mutation analysis by RT-PCR and genomic PCR

We extracted RNA and genomic DNA from skin fibroblast cultures between 5–15 passages, using RNA and DNA isolation kits from Qiagen. We performed RTPCR using 2–5 mg of total cellular fibroblast RNA and a cDNA cycle kit (Invitrogen) in accordance with the manufacturer's recommendations. To search for mutations, we used primer pairs in the 5_- and 3_-UTR to amplify by RT-PCR the complete *MCCA* or *MCCB* ORF, as described elsewhere (Baumgartner et al. 2001). We used the same approach to amplify the complete ORF of the *HCS* gene. The PCR products were gel purified and were sequenced directly. To confirm mutations identified in RT-PCR products, we amplified a genomic fragment containing the corresponding exon, using flanking intronic primers, and sequenced the PCR product directly (Baumgartner et al. 2001). All PCR reactions (50 ml) contained primers (100 ng of each), standard PCR buffer (Gibco-BRL), dNTPs (200 mM), and *Taq* polymerase (2.5 U) (Gibco-BRL). The sequences of all primers are available on request.

Construction of wild type and mutant MCCA expression vectors

We cloned the full-length human *MCCA* (-51 to +2275, where +1 is the A nucleotide of the initiation methionine codon) cDNA into a mammalian expression vector (pTracer-CMV2 [Invitrogen]) at the *Eco*RI sites, as described elsewhere (Baumgartner et al. 2001). To introduce R385S, A289V, L437P, and P464H into *MCCA,* we harvested an 896-bp *ACC*I fragment from RT-PCR–amplified cDNA from individuals with these variants and subcloned the fragment into the wild-type *MCCA* construct, as described elsewhere (Baumgartner et al. 2001). The pTracer-CMV2 vector contains a green fluorescent protein

(GFP) gene fused to the Zeocin-resistance gene. We sequenced all constructs in both directions to validate the sequences.

Transfections

For expression studies, we electroporated the indicated constructs into SV40T-transformed skin fibroblasts from a patient homozygous for *MCCA* Q421fs(-1), as described elsewhere (Braverman et al. 1997, Baumgartner et al. 2001). These cells have no detectable *MCCA* activity. The cells were harvested after 72 h and were assayed for MCC and PCC activities. All transfections were performed in duplicate. Transfection efficiency was assessed by scoring a subset of cells in each transfection for the presence of GFP and ranged from 10%–20% in these experiments. Transformed fibroblasts from an unaffected individual were used as control.

Allelic Variation in MCCA Expression

We used the *MCCA* SNP c.1391C→A, which results in the P464H amino acid change, to quantitate allele specific transcripts by means of single-nucleotide primer extension (SnuPE) and laser-induced fluorescence capillary electrophoresis, as described elsewhere (Mátyás et al. 2002) . This method takes advantage of distinct mobilities of SnuPE products with different nucleotides incorporated at their 3' ends and has been independently shown to yield highly quantitative results (Mátyás et al. 2002, Yan and Zhou 2003).

Results

Carboxylase activities

MCC activity in crude homogenates of fibroblasts cultured in standard medium was reduced below the normal range in patients MCC047 and MCC048, with an average of 26% and 36% of the mean control value, respectively (fig. 5.1). This residual activity is greater than that in fibroblasts from four typical CRM$^-$ MCC-deficient patients (0%–1% of mean control value) (fig. 5.1) but is less than that in fibroblasts from six heterozygotes for CRM$^-$ *MCCA* or *MCCB* alleles (72%–118% of control). The activity of another biotin-dependent carboxylase, PCC, was within the normal range in patients MCC047 and

MCC048, indicating that the deficiency of MCC is specific. Moreover, the ratio of MCC to PCC activity was consistently below the control range in both patients (MCC047: mean 0.19, range 0.18–0.19; MCC048: mean 0.24, range 0.19–0.32; 13 controls: mean 0.52, range 0.41–0.75). Fibroblast MCC activity levels from the father and the mother of patient MCC047 were both at the low end of the normal range (52% and 63% of the mean control value, respectively). However, the ratio of MCC to PCC activity was consistently below the control range in the father, who was also heterozygous for *MCCA*-R385S, whereas the ratio was normal in three of four experiments in the mother (father of MCC047: mean 0.29, range 0.24–0.36; mother of MCC047: mean 0.4, range 0.34–0.42).

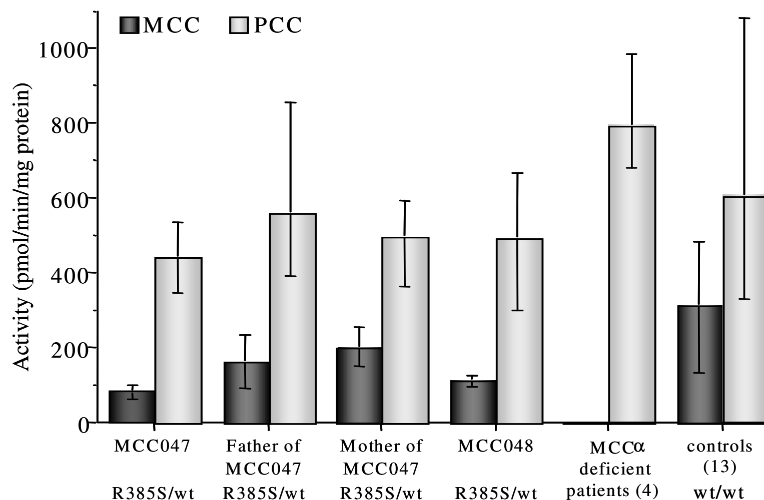


Figure 5.1. MCC and PCC activity in fibroblasts from patient MCC047 and his parents, from patient MCC048, from four typical CRM MCC-deficient patients (probands 009, 010, 012, and 013 in Baumgartner et al. 2001), and from 13 control cell lines grown in standard FCS-based medium with a biotin concentration of 9 nmol/L. The indicated activities are the mean and range (vertical lines) of four individual experiments each with duplicate determinations. Values in the CRM$^-$ MCC-deficient patients are the mean and range of activities obtained in four different patients. Control values represent the mean and range of activities obtained in 13 different cell lines.

To determine whether the extent of the MCC deficiency in our patients' fibroblasts was influenced by the biotin concentration of the medium, we cultured the cells for one passage

(17–27 d) in either biotin-depleted (0.1 nmol/L) or high-biotin (10,000 nmol/L) medium (table 5.2). After one passage in the biotin-depleted medium, MCC activities in fibroblasts from patients MCC047 and MCC048 and from the parents of MCC047 were decreased to 24%–35% of activity in the high-biotin medium. A similar decrease occurred in control cells.

Even after four passages in the low-biotin medium, MCC activity in the cells from patient MCC047 was decreased to only 27% (data not shown). Cells from patients with HCS deficiency exhibit a dramatic decrease in MCC activity when cultured in biotin-depleted medium (Suormala et al. 1997), which makes it unlikely that an abnormality of this enzyme contributes to our patients' biochemical phenotype. Furthermore, MCC and PCC activities in cells cultured in medium with a physiological concentration of biotin (2.1 nmol/L) were similar to those in cells cultured in the high-biotin medium. Increasing the biotin concentration to 100 µmol/L in the medium failed to increase the activities of MCC further (data not shown). Thus, we found no evidence for an increased biotin requirement as the cause of the low MCC activity in the patients' fibroblasts. Normal kinetics of MCC activity were obtained in crude homogenates of fibroblasts from MCC047 when they were assayed with varying concentrations of bicarbonate, 3-methylcrotonyl-CoA, ATP, magnesium, and chloride in the assay mixture (results not shown).

These results indicate that a decreased affinity of MCC for these assay components is highly unlikely to be the cause of the reduced activity in this patient. Despite these results, the ratio of MCC to PCC activity in fibroblasts from patient MCC047, his father, and patient MCC048 was reproducibly below the control range, whereas the ratio in fibroblasts from the mother of patient MCC047 was within the range of controls (table 5.2).

Furthermore, MCC and PCC activities in cells cultured in medium with a physiological concentration of biotin (2.1 nmol/L) were similar to those in cells cultured in the high-biotin medium. Increasing the biotin concentration to 100 µmol/L in the medium failed to increase the activities of MCC further (data not shown). Thus, we found no evidence for an

increased biotin requirement as the cause of the low MCC activity in the patients' fibroblasts.

Table 5.2. *Activities of 3-MCC and PCC in Fibroblasts Grown in NBCS-Based Low-Biotin Medium (0.1 nmol/L) and in the Same Medium Supplemented with Phtsiological (2.1 nmol/L) and High (10 µmol/L) Biotin Concentration.*

Subject and Biotin in Medium (nmol/L)	Carboxylase Activities[a] (pmol/min/mg protein)		
	MCC	PCC	MCC/PCC ratio
MCC047			
.1	31.5	375	.80
2.1	102	628	.16
10,000	100	638	.16
Father of MCC047:			
.1	42.0	280	.15
2.1	121	446	.27
10,000	130	498	.26
Mother of MCC047:			
.1	61.2	315	.20
2.1	176	500	.35
10,000	177	472	.38
MCC048:			
.1	30.4	283	.11
2.1	120	603	.20
10,000	128	603	.21
21 controls, mean ± SD (range):			
.1	145 ± 57 (70.4-292)	494 ± 172 (300-889)	.3 ± O.8 (.17-4.3)
10,000	340 ± 130 (140-606)	694 ± 246 (347-1,246)	.5 ± .13 (.29-.85)

[a]Values are the mean of duplicate determinations within one experiment.

Normal kinetics of MCC activity were obtained in crude homogenates of fibroblasts from MCC047 when they were assayed with varying concentrations of bicarbonate, 3-methylcrotonyl-CoA, ATP, magnesium and chloride in the assay mixture (results not shown). These results indicate that a decreased affinity of MCC for these assay components

is highly unlikely to be the cause of the reduced activity in this patient. Despite these results, the ratio of MCC to PCC activity in fibroblasts from patients MCC047, his father and patient MCC048 was reproducibly below the control range, whereas the ratio in fibroblasts from the mother of patient MCC047 was within the range of controls (Table 5.2).

Mutation analysis

Despite high residual MCC activity in vitro, the pattern of metabolic abnormalities in our patients suggested a partial defect at the level of MCC in vivo. To investigate this possibility, we sequenced the entire ORF of *MCCA* and *MCCB* in the products of RT-PCR amplification of fibroblast RNA in patients MCC047 and MCC048. We found that both patients and the father of patient MCC047 are heterozygous for an A→C transversion at position 1155, creating a new restriction site for *Nhe*I (GCTAGArGCTAGC) and substituting a serine for an arginine at position 385 (*MCCA*-R385S). Patients MCC047 and. MCC048 and the mother of MCC047 are heterozygous for the previously described neutral polymorphism 1391CrA (*MCCA*-p.P464H). The father of MCC047 does not carry this polymorphism.

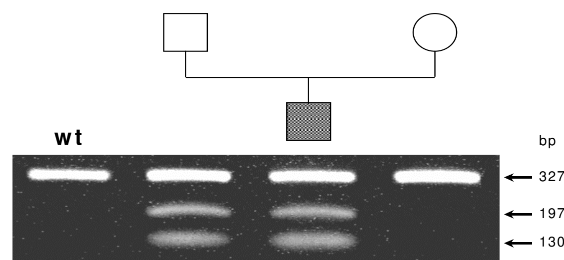


Figure 5.2. Detection of the MCCA 1155A⟶C (R385S) mutation in the MCC047 nuclear family. The 327-bp fragment amplified from genomic DNA (MCCA exon 11) was subjected to digestion with NheI. The wild-type fragment is not cut, whereas the R385S fragment is cleaved into fragments of 130 bp and 197 bp.

From this, we conclude that the *MCCA*-R385S allele encodes a proline at codon 464. We found no other sequence abnormalities over the entire length of the *MCCA* and *MCCB*

ORFs. We confirmed these results by direct sequencing of PCR-amplified genomic DNA followed by digestion with *Nhe*I (fig. 5.2). We also amplified and sequenced all *MCCA* exons and flanking intronic sequences in patient MCC047 and found no additional mutations. Because biotin responsiveness in other carboxylase-deficient patients has been shown to be due to deficiency of HCS, we also RT-PCR amplified and sequenced the entire *HCS* ORF and found no sequence abnormalities

Expression studies

In previous experiments, we showed that *MCCA*-p.R385S and several other *MCCA* missense alleles conferred no MCC activity when transfected into *MCCA* null cells (Baumgartner et al. 2001). To test if *MCCA*-p.R385S also has a dominant negative influence on the activity of *MCCA* wild type, we cotransfected both alleles into an SV40T-transformed *MCCA*-deficient reference cell line and measured MCC activity 72 h later.

In two separate experiments, cotransfection of *MCCA* wild-type allele with vector without an insert restored MCC activity to 55% of nontransfected control fibroblasts, whereas cotransfection of *MCCA* wild type with *MCCA*-p.R385S restored MCC activity to 25% — that is, to ~ 50% of that obtained with wild type coexpressed with vector without an insert. In contrast, cotransfection of *MCCA* wild type with either *MCCA*-p.A289V or *MCCA*-p.L437P restored MCC activity to the same level as that obtained with wild type coexpressed with vector without insert (fig. 5.3). Thus, the reduction of MCC activity is specific for *MCCA*-p.R385S. We repeated the experiment using the *MCCA*–wild-type construct with histidine instead of a proline at codon 464 (*MCCA*-p.P464H) with similar results. Thus, the dominant negative effect of *MCCA*-p.R385S when coexpressed with the wild-type allele is independent of the P464H polymorphism.

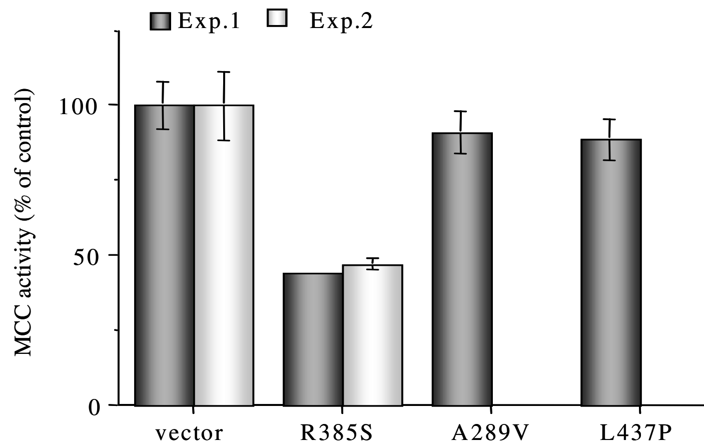


Figure 5.3. The effect of coexpression of MCCA missense alleles and wild-type MCCA. MCC-deficient cells were cotransfected with wild type MCCA plus MCCA alleles with the indicated missense mutation. Activities represent mean and range obtained in two individual experiments MCC activity of cells cotransfected with wild-type MCCA and a vector without an insert was set a 100%.

Allelic variation in expression

Recent reports have shown variation in expression of apparently wild-type alleles, for as many as 50% of all genes (Yan et al. 2002, Lo et al. 2003). This variation in expression ranges ≥ 2–4 fold, segregates with the allele, and could markedly affect the consequences of heterozygosity for a recessive loss-of-function mutation on the other allele. To determine if allele-specific variation in expression was playing a role in the manifestation of MCC deficiency in individuals heterozygous for *MCCA*-p.R385S, we used a fluorescent dideoxy-terminator–based method to distinguish the mRNA products of individual alleles in patient MCC047, his mother, and patient MCC048, by use of the *MCCA*-p.P464H polymorphism to mark the alleles. In patient MCC047 and his mother, both alleles were expressed at equal levels, whereas, in patient MCC048, the expression of the *MCCA*-S385 allele (marked by P464 in *cis*) was reduced to ~ 40% of that of the wild-type *MCCA* allele (marked by H464) (fig. 5.4). Thus, in these heterozygous individuals, we found no evidence for significant variation in expression of the *MCCA* wild-type allele.

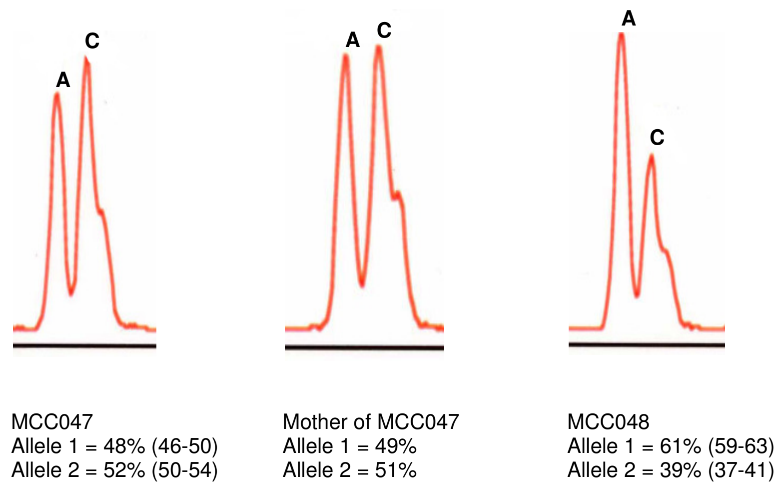


Figure 5.4. Allelic variation in MCCA expression. A sample of electrospherograms of SnuPE products from the sense strand of RT-PCR-amplified DNA from patient MCC047 and his mother and from patient MCC048. Mobility of tetramethyl-6-carboxyrhodamine (TAMRA)-labelled allele-specific SnuPE is shown for the MCCA polymorphic variant c.1391C A (MCCA-P464H). Each peack represents a specific allelic variant. Allele 1 with an A nucleotide at c.1391 encodes 464H, whereas allele 2 with a C nucleotide at c.1391 encodes 464P and the functionally significant 385S. The relative quantification of each allele represents the mean and range of two independent experiments performed on fibroblasts RNA.

Discussion

Both patients described here showed a persistent characteristic organic aciduria, with elevated excretion of 3-MCG and 3-HIVA suggesting MCC deficiency. One suffered from severe neurological symptoms starting at the age of 3 wk, whereas the other was an asymptomatic newborn detected by TMS newborn screening. In patient MCC047, excretion of the disease-specific metabolite 3-MCG was within the range found in patients with severe MCC deficiency (Sweetman and Williams 2001); in patient MCC048, urinary 3-MCG was lower but was persistently elevated. Enzymatic determination of MCC activity in cultured fibroblasts revealed a partial but specific MCC deficiency. In both patients, residual fibroblast MCC activity was not abnormally sensitive to biotin depletion, nor was it enhanced by addition of high concentrations of biotin to the medium. These results rule out HCS deficiency as the cause of reduced MCC activity and are consistent with the clinical features and the persistent organic aciduria in our patients.

Our mutation analysis of the *MCCA* and *MCCB* genes revealed that both patients are heterozygous for a previously described missense mutation, *MCCA*-p.R385S, and a known polymorphism, *MCCA*-p.P464H (Obata et al. 2001). In earlier studies, we showed that *MCCA*-p.R385S confers no MCC activity when transfected into reference MCCα-deficient cells (Baumgartner et al. 2001). We found the *MCCA*-p.R385S allele in 1 of 132 control chromosomes from white individuals (Baumgartner et al. 2001).

An arginine at position 385 (or the corresponding position) is strictly conserved in mammalian, plant, fungal, and bacterial carboxylases (Samols et al. 1988, Jitrapakdee and Wallace 1999), and the corresponding residue in the biotin carboxylase domain of *Escherichia coli* acetyl-CoA carboxylase (R338) has been shown to be part of a positively charged pocket for bicarbonate binding (Thoden et al. 2000).

To explain the biochemical abnormalities in both patients and the clinical phenotype in patient MCC047, we hypothesize a dominant negative influence of the mutant S385 *MCCA* subunit on MCC activity. A dominant negative effect of the protein product of a mutant allele usually involves homomeric or heteromeric proteins.

Typically, the protein product of a dominant negative allele is functionally inactive and has the added property of inhibiting the activity of the protein product of the normal allele (Herskowitz 1987). To achieve this, the dominant negative mutant protein product must be stable and must be able to assemble into the normal multimeric protein complex. MCCα S385 fulfills these criteria. In contrast to the protein products of all other *MCCA* missense alleles, the MCCα S385 protein is stable (Baumgartner et al. 2001, Gallardo et al. 2001). On protein-blot analysis using avidin alkaline phosphatase as a probe, MCCα S385 accumulates to levels equal to or greater than normal (Baumgartner et al. 2001). Despite this, expression studies clearly show that the MCCα S385 is catalytically inactive (Baumgartner et al. 2001, Desviat et al. 2003). Moreover, the results of our cotransfection experiments show that expression of MCCα S385 but not of two other *MCCA* missense alleles inhibits the function of coexpressed wild-type MCCα protein. Taken together, these

data strongly support our hypothesis of a dominant negative effect of MCCα S385 on the wild-type protein and thus on MCC activity.

Additional evidence comes from the recent studies of Sloane and Waldrop (2004), who used the purified homodimer of the biotin carboxylation subunit of *E. coli* acetyl CoA carboxylase to study kinetic properties of mutant proteins. They showed that *E. coli* biotin carboxylase-R338S, the missense mutant corresponding to *MCCA*-p.R385S, exhibited a dominant negative effect on the function of the wild-type active site, by negative cooperativity with respect to bicarbonate concentration (Sloane and Waldrop 2004). Furthermore, they presented evidence that the degree of negative cooperativity is decreased with increasing concentrations of biotin. These data support our hypothesis of a dominant negative effect of MCCα S385 on the wild-type protein and thus on MCC activity.

The presence of a functionally significant mutation in the second allele would also explain the reduced MCC activity in our patients. However, we searched extensively for a functional mutation on the second allele by RT-PCR of mRNA of the entire *MCCA* ORF, as well as by amplification and sequencing of all *MCCA* exons and flanking intronic splice sites from genomic DNA. By direct sequencing of the RT-PCR product, we could clearly see that both patients are heterozygous for the R385S change, which indicates that the second allele produced a stable mRNA transcript, but we found no molecular abnormalities in the *MCCA* transcript or structural gene that could explain a reduction in the function of its protein product. For all of these reasons, we concluded that MCCα S385 has a dominant negative effect on the wild-type protein.

How do we explain the lack of biochemical abnormalities in other R385S heterozygotes (e.g., the father of MCC047)? The *MCCA*-p.R385S heterozygotes with normal urinary organic acids who we have studied were all adults. Thus, one possible explanation is that the metabolic demands on the pathway are lesser in adults than in infants and children. This would also be consistent with the identification of asymptomatic adults with severe MCC deficiency (Gibson et al. 1998, Baumgartner et al. 2001). An alternative and nonexclusive explanation for the phenotypic variation in *MCCA*-p.R385S heterozygotes has to do with the amount of functional MCCα produced from the wild-type allele. Recent studies have

suggested that alleles of as many as half of all genes examined exhibit ≥2–4 fold allelic variation in expression and that this characteristic segregates with the alleles from one generation to the next (Yan et al. 2002, Lo et al. 2003). If this is the case for *MCCA,* then the expression level of the wild-type allele would be an additional variable affecting the consequences of heterozygosity for a nonfunctional allele. A heterozygote with a low-expression wild-type allele might be clinically and/or biochemically symptomatic.

Conversely, a heterozygote with a high-expression wild type allele would be normal. However, our allelic expression studies showed approximately equal expression of both alleles in patient MCC047 and his mother. In patient MCC048, we found slightly reduced expression of the *MCCA*-R385S allele (marked by P464) as compared with the wild-type allele (fig. 5.4). This result may explain the less severe biochemical abnormalities and clinical phenotype in this patient.

Additional factors that could influence the phenotypic consequences of MCC deficiency include the extent to which the pathway is stressed by dietary or other environmental factors (e.g., excessive protein breakdown associated with an intercurrent infection). In this regard, the father of patient MCC047 is described as being healthy during infancy and childhood. Finally, normal variation in genes whose protein products have the potential to modify the function of MCC or the demands on the pathway could also influence the phenotype of MCC deficiency. *MCCB* and *HCS* are obvious candidate modifier genes for *MCCA*, but we found no sequence variation in either of these genes in our patients.

It is surprising that the clinical symptoms in patient MCC047 and the biochemical phenotype in both patients appeared to respond to high doses of biotin. A biotin-responsive form of MCC deficiency has not been reported elsewhere (Sweetman and Williams 2001), even in Germany, where *MCCA*-p.R385S appears to be a common allele. In part, this may be because of failure to administer high doses of biotin on the order of 2–5 mg/kg/d. Biotin responsiveness in our patients is consistent with the observation that *MCCA*-p.R385S produces a stable protein and that other cofactor-responsive disorders are allele specific (e.g., cystathione b-synthase) (Mudd et al. 2001). It also agrees with the findings of Sloane and Waldrop (2004), who showed that biotin decreases the negative cooperativity of *E. coli*

biotin carboxylase with a missense mutation in the residue corresponding to *MCCA*-p.R385S. Alternatively, a biotin dependent increase in the level of *MCCA* mRNA also could explain biotin responsiveness. A regulatory role for biotin in the control of *HCS* and carboxylase mRNA levels through a signalling pathway that requires HCS, guanylate cyclase, and cGMP-dependent protein kinase has recently been proposed (Solorzano-Vargas et al. 2002).

To provide further evidence for biotin responsiveness in our patients, additional clinical, biochemical, and molecular studies in patients with biotin versus without biotin would be useful. This was not possible, however, because of parental unwillingness (for patient MCC047) and ethical considerations regarding the asymptomatic patient MCC048. For the future, we recommend careful documentation of the consequences of biotin administration in newly diagnosed patients, especially those carrying the *MCCA*-p.R385S allele.

6. *Genotype-Phenotype Correlation*

During our study, a number of asymptomatic but affected siblings were detected in some of the families, indicating a disease penetrance consistently lower than 100%. In general, the genotype-phenotype correlation in MCC deficiency is loose, with great variability in both genotype and clinical severity, even among family members carrying the same mutations. Thus other, still unknown, genetic or epigenetic/environmental factors must play an important role in the phenotypic expression of MCC deficiency. This can include polymorphic variants or silent mutations of the *MCCA* or *MCCB* gene itself, which may contribute to the variability in the phenotypic expression.

Combining this study with other previously reported studies, three *MCCA* and six *MCCB* mutant alleles occurred in 2 or more subjects. The majority of the MCC-deficient patients, however, carry private mutations, i.e. no prevalent mutant allele has been detected for either gene.

In the *MCCA* gene, the missense mutation *MCCA*-p.R385S, discussed in chapters 4 and 5, is the most common so far, with a total of 11 mutant alleles detected in 9 subjects (Baumgartner et al. 2001, Baumgartner et al. 2004, Dantas et al. 2005, Gallardo et al. 2001, this thesis). From these 9 subjects two were homozygous for the mutation, one with a severe phenotype and the other asymptomatic, both of German origin (Baumgartner et al. 2001, Steen et al. 1999, this thesis). One subject was compound heterozygous with mild symptoms (Gallardo et al. 2001). Two further subjects were heterozygous for the mutation, one with severe phenotype and the other asymptomatic (Baumgartner et al. 2004), and five were compound heterozygous and have remained asymptomatic (Baumgartner et al. 2001, Dantas et al. 2005). Only time will tell if these subjects will remain asymptomatic. The *MCCA*-p.Q421AfsX10 (c.1263_1264insG) mutant allele occurred in a total of 3 alleles in two subjects with mild symptoms, an American homozygote and a Swedish compound heterozygote (Baumgartner et al. 2001, Dantas et al. 2005). *MCCA*-p.T693TfsX1 (c.2079_2080delA) was identified in the homozygous state in a Turkish child with mild symptoms (not published), and in an asymptomatic compound heterozygous child of unknown origin (Holzinger et al. 2001).

With regard to the *MCCB* gene, the missense allele *MCCB*-p.E99Q has been detected in the homozygous state in a Turkish child with fatal outcome and in an adult Amish/Mennonite with mild symptoms (Bannwart et al. 1992, Baumgartner et al. 2001). However, in this study we also identified this missense allele in a further homozygous and a compound heterozygous asymptomatic subject (Dantas et al. 2005). *MCCB*-p.R155Q was identified in the homozygous state in a Turkish asymptomatic newborn, but also in a compound heterozygous Vietnamese child with mild symptoms (Baumgartner et al. 2001, Beemer et al. 1982, Dantas et al. 2005). *MCCB*-p.V339M was detected in two compound heterozygous Turkish subjects, both with severe symptoms (Baumgartner et al. 2001, Wiesmann et al. 1998). The missense allele *MCCB*-p.P310R was detected in the compound heterozygous state in a Vietnamese and in the homozygous state in a Australian child, both with mild clinical symptoms (Baumgartner et al.2001, Beemer et al.1982, Dantas et al. 2005).

At position 173 in the *MCCB* gene we identified the insertion c.517dupT (*MCCB*-p.S173FfsX75) followed by a premature stop codon thus causing a truncated β-subunit. This insertion was detected in a mildly symptomatic compound heterozygous Swiss patient (Baumgartner et al. 2001, Dantas et al. 2005, Gitzelman et al. 1987), an asymptomatic compound heterozygous American newborn (Dantas et al. 2005), and two asymptomatic homozygous Amish/Mennonite mothers (Baumgartner et al. 2001, Gallardo et al. 2001, Gibson et al. 1998). Finally, the mutant allele *MCCB*-p.F497_V562>GfsX4, a splice mutation that skips exon 16, was detected in a homozygous Arabian infant with fatal outcome and in an asymptomatic compound heterozygous Turkish newborn (Baykal et al. 2005, Dantas et al. 2005).

Based on still small numbers of subjects, this study did not reveal any obvious correlation between genotype and phenotype. No mutations were exclusively present in symptomatic or asymptomatic subjects. So far, the three *MCCA* and six *MCCB* mutant alleles that occurred in two or more subjects appear not to be predictors of a particular phenotype. Even though some patients with a severe phenotype are the product of a consanguineous union and could have an additional genetic disorder, our data strongly

support the idea that factors other than the genotype at the MCC loci have a major influence on the phenotype of MCC deficiency.

While genotypes are stable over the life of an individual, phenotypes are usually unstable and reactive (Schulze and McMahon 2004). In the Genomic Glossary web page the term phenotype is defined as "The observable structure and functional characteristics of an organism determined by its genotype and modulated by its environment" or even "The observed manifestation of a genotype, which may be expressed physically, biochemically or physiologically" (http://www.genomicglossaries.com/content/genomics_glossary.asp). The definition of a term seems to be an easier task than the definition of a phenotype itself, and the definition of a phenotype for a genetic study remains a major task for clinical research.

7. *Summary*

3-Methylcrotonyl-CoA carboxylase (MCC) deficiency is a rare disorder of leucine catabolism inherited as an autosomal recessive trait. The phenotypic expression of the disease is highly variable, ranging from neonatal onset with severe neurological involvement to asymptomatic adults. Most patients however are asymptomatic until an episode of acute metabolic decompensation following intercurrent illness leads to the diagnosis. The metabolic phenotype of MCC deficiency includes a characteristic organic aciduria with greatly increased excretion of 3-hydroxyisovaleric acid and 3-methylcrotonylglycine and elevated blood levels of 3-hydroxyisovalerylcarnitine, usually in combination with severe secondary carnitine deficiency due to urinary loss of carnitine esters. Introduction of tandem mass spectrometry (TMS) to newborn screening has revealed an unexpectedly high incidence of MCC deficiency and indicates that this disorder is the most frequent organic aciduria. The Metabolic Unit from the University Children's Hospital in Basel has a well-characterized collection of fibroblast cell lines from patients with isolated MCC deficiency, and includes 75 patients from 70 families from several different nationalities and ethnic backgrounds. This study addresses molecular characterization of MCC deficiency and functional consequences of some of the mutant alleles, and thus provides insight into the cellular and molecular biology of MCC and the molecular pathology of MCC deficiency.

7.1. Molecular characterization of MCC-deficient patients by identification of new MCCA and MCCB mutant alleles.

Following the identification of the human cDNAs encoding *MCCA* and *MCCB* by three independent groups in 2001 (Baumgartner et al. 2001, Gallardo et al. 2001, Holzinger et al. 2001), this study is focused on the molecular characterization of 70 unrelated MCC-deficient patients.

Before the introduction of TMS based newborn screening patients came to attention due to clinical symptoms associated with typical organic aciduria. Cultured fibroblasts of such patients were sent to Basel for enzymatic confirmation of suspected MCC deficiency. From

70 patients 32 presented with clinical symptoms whereas 34 patients were asymptomatic newborns detected by TMS based newborn screening, and 4 were affected but asymptomatic mothers diagnosed after detection of abnormal metabolites in the neonatal screening samples of their healthy babies.

This study shows mutation analysis in 30 patients, 10 of whom came to attention because of clinical symptoms. Another 18 patients were asymptomatic newborns detected by TMS based newborn screening, and 2 were asymptomatic but affected mothers. We identified 10 *MCCA* and 14 *MCCB* novel mutant alleles. Including earlier studies, we have characterized a total of 15 *MCCA* and 23 *MCCB* mutant alleles in our study population (Baumgartner et al. 2001, Dantas et al. 2005). None of the missense alleles were detected in a control population of at least 50 individuals indicating that each of these mutant alleles has a very low frequency in this control population (Baumgartner et al. 2001, Dantas et al. 2005). Another 3 *MCCA* and 3 *MCCB* mutations have been described by others (Desviat et al. 2003, Gallardo et al. 2001, Holzinger et al, 2001).

7.2. Consequences of MCCA and MCCB missense mutations on MCC activity and analysis of steady-state levels of mutant MCCα protein.

In order to confirm the pathogenic effect of *MCCA* and *MCCB* missense mutations we expressed 1 *MCCA* and 3 *MCCB* missense alleles using transient transfection of SV40T-transformed *MCCA* and *MCCB* deficient skin fibroblasts (Dantas et al. 2005, this thesis). The three expressed *MCCB* missense alleles showed no or very low residual activity, while the *MCCA* missense allele had 26% residual activity of wild type level, thus confirming that these changes have a deleterious effect on enzyme activity.

To analyze the MCC polypeptides under steady-state condition in fibroblasts of *MCCA* deficient patients, we carried out Western blot analysis. Results demonstrate that the MCCα protein was absent in 5 patients homozygous or compound heterozygous for nonsense or frameshift mutations that are expected to result in a truncated protein. The MCCα protein was also absent in 2 further compound heterozygous patients in whom only one mutant

allele could be identified in the RT-PCR products. The second allele could not be detected because presumably this mutant allele is unstable and degraded by the mechanisms of NMD. In contrast, western blot analysis of the MCCα protein was normal in amount and size in 4 patients carrying *MCCA*-p.R385S. This is in agreement with previous studies which show that the protein product of this allele is stable.

7.3. Investigation of molecular defects with dominant-negative effect on MCC activity.

In fibroblasts from two patients (MCC047 and MCC048) with suspected MCC deficiency, activity determination revealed only partial MCC deficiency (20 and 21% of the mean control values, respectively). In both patients, residual MCC activity in fibroblasts was not abnormally sensitive to biotin depletion nor was it enhanced by addition of high concentrations of biotin to the culture medium (Baumgartner et al. 2004). Mutation analysis of the *MCCA* and *MCCB* genes revealed that both patients are heterozygous for *MCCA*-p.R385S, and a known polymorphism *MCCA*-p.P464H (Obata et al. 2001). Despite an extensive search for a functional mutation on the second allele by RT-PCR and sequencing the entire *MCCA* and *MCCB* ORF, as well as by amplification and sequencing of all *MCCA* exons and flanking intronic sequences from genomic DNA, no other coding alterations were identified.

MCCA-p.R385S fulfills all the criteria of a dominant-negative allele. In contrast to the protein products of other *MCCA* missense alleles, the MCCα R385S protein is stable (Baumgartner et al. 2001, Gallardo et at. 2001, this study). Results of co-transfection experiments show that expression of MCCα S385 but not two other *MCCA* missense alleles reduce the activity of co-expressed wild type MCCα protein (Baumgartner et al. 2004).

Our data strongly support the hypothesis of a dominant-negative effect of MCCαS385 on the wild-type protein and thus on MCC activity. Biotin can partially reverse this dominant negative effect thus resulting in biotin responsiveness *in vivo*. This is the first example of biotin responsiveness for an isolated deficiency of any of the biotin-dependent

carboxylases, suggesting that therapeutic trials with biotin in patients carrying this mutant allele are warranted.

7.4. Genotype-Phenotype Correlation.

Molecular genetic analysis of MCC-deficient patients provided new information on the nature of mutations in the *MCCA* and *MCCB* genes and allowed the comparison of mutations in symptomatic patients and asymptomatic subjects detected by newborn screening (Baumgartner et al. 2001, Dantas et al. 2005, this thesis). Combining our present data with our previous results (Baumgartner et al 2001) we have now complete mutation analysis in 50 out of 70 probands with confirmed MCC deficiency. 22 out of 50 were detected by newborn screening. Regarding the question of whether different mutations occur in symptomatic versus asymptomatic subjects we did not identify mutations that are specific for either group. Our results provide strong evidence that factors other than the genotype at the MCC loci, such as modifying genes and environmental factors, have a major influence on the phenotypic expression of MCC deficiency.

8. *Outlook*

In conclusion, our studies provide insight into the molecular pathology of MCC deficiency. Furthermore, this work increases the understanding of the variables that influence the phenotypic consequences of MCC deficiency. This will eventually lead to guidelines for treatment which are needed for the increasing number of identified subjects due to the recent introduction of tandem mass spectrometry in newborn screening programs. Finally, this work on MCC will serve as a paradigm for the understanding of the molecular mechanism of other biotin-dependent carboxylases and of the pathophysiology in such disease states.

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EDUCATION

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FELLOWSHIP

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Mar 1996 - Nov 1996 - Member of the Board of Tutors in Histology at the Department of Histology - Campinas State University (UNICAMP)

Jan 1996 - Feb 1996 - Undergraduate Research Fellow in Phytophatology at the Department of Phytopathology - Biological Institute of Sao Paulo

SPECIAL SKILLS

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Preparation and presentation of posters and oral communications in scientific meetings
Teaching undergraduate and graduate students in the laboratory

Languages

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Fluent English
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RESEARCH ACTIVITIES

1997 to 2002 - Animals in vitro and in vivo (LTK module 1)
2002 to 2006 - Molecular biology, biochemistry and cell biology

AWARDS

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Young Investigator Award in Molecular Biology - *"Endothelium-dependent relaxation in arteries from pregnant rats involves gap junctional communication."* III Meeting for Biology Students, UNICAMP, Campinas, Brazil, 1998

PUBLICATION (FULL PAPER)

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COURSES

2003 – Protein-Nucleic Acid Interactions (Prof. Dr. T. A. Bickle)

2004 – The Molecular Basis of Human Disease (Prof. Dr. U. A. Meyer)

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