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Research paper

3-Methylcrotonyl-CoA carboxylase deficiency: Mutational spectrum derived from comprehensive newborn screening

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ABSTRACT

The deficiency of 3-methycrotonyl-CoA carboxylase (3-MCC; EC 6.4.1.4) is an autosomal recessive organic aciduria that is included in the newborn screening programs of several countries. This study reports data mainly obtained from the Portuguese newborn screening program collected over a ten-year period. Analysis of the *MCCC1* and *MCCC2* genes yielded 26 previously unreported mutations and a variant of clinically unknown significance. These mutations are discussed in the context of their likely impact on the function of the 3-MCC enzyme, with a view to exploring whether a phenotype-genotype correlation might be discerned. Further, these mutations were analysed in the context of what is known of the *MCCC1* and *MCCC2* mutational spectra, information that will be useful in both clinical and laboratory practice.

1. Introduction

The introduction of tandem mass spectrometry (MS/MS) in newborn screening (NBS) has allowed the detection of various organic acidurias, including the deficiency of 3-methycrotonyl-CoA carboxylase (3-MCC; EC 6.4.1.4). Methylcrotonylglycinuria (MCG; MIM 210200 and MIM 210210) is an autosomal recessive disorder first identified by Eldjarn et al. (1970). In MCG, the catabolism of leucine is blocked at the fourth step owing to the deficiency of the 3-MCC enzyme which catalyzes the conversion of 3-methylcrotonyl-CoA to 3-methylglutaconyl-CoA, a reversible reaction dependent upon ATP and using bicarbonate as a source of carboxyl groups. The enzymatic impairment of 3-MCC leads to the accumulation of 3-methylcrotonyl CoA inside the mitochondria where alternative pathways form 3-methylcrotonylglycine (3-MCG) and 3-hydroxyisovaleric acid (3-HIVA). The latter, after conjugation with carnitine, and having been coupled with 3-MCG, is readily excreted in the urine (de Kremer et al., 2002).

3-MCC is one of the four biotin-dependent carboxylases known in humans; the other three are acetyl-CoA carboxylase (ACC; EC: 6.4.1.2), propionyl-CoA carboxylase (PCC; EC: 6.4.1.3) and pyruvate carboxylase (PC; EC: 6.4.1.1). Of these four biotin-dependent enzymes, only ACC is cytosolic whereas the others are active in the mitochondrial matrix.

3-methylcrotonyl-CoA carboxylase deficiency is the most frequent organic aciduria detected in screening programs from European, American and Australian populations (Baumgartner et al., 2001; Frazier et al., 2006; Gibson et al., 1998; Koeberl et al., 2003; Stadler et al., 2006; Wilcken et al., 2003). Patients with 3-MCC deficiency experience normal growth and development until the emergence of an acute episode of metabolic decompensation, occurring typically between 6 months and 3 to 5 years of age (Sweetman, 2001). This episode is usually triggered by an infection or the introduction of high protein foods in the diet. The biochemical diagnosis of 3–MCC deficiency is made by means of the detection of organic acids in the urine, through gas chromatography/mass spectrometry (GC/MS), and the blood profile of acylcarnitines performed by MS/MS. The organic acid profile is characterized by a marked increase of 3-MCG and 3-HIVA acids, whilst the acylcarnitines profile reveals a highly elevated concentration of 3-hydroxyisovalerylcarnitine (C5-OH) and an increased ratio of this compound to propionylcarnitine (C3) (Holzinger et al., 2001). 3-

Abbreviations: 3-HIVA, 3-hydroxyisovaleric acid; C5-OH, 3-hydroxyisovalerylcarnitine; 3-MCC, 3-methycrotonyl-CoA carboxylase; C5:1, 3-methylcrotonylcarnitine; 3-MCG, 3-methylcrotonylglycine; ACC, acetyl-CoA carboxylase; BC, biotin carboxylase; CT, carboxyltransferase; GC/MS, gas chromatography/mass spectrometry; HMGD, Human Gene Mutation Database; MCG, methylcrotonylglycinuria; NBS, newborn screening; NMD, nonsense-mediated mRNA decay; PTC, premature termination codons; PCC, propionyl-CoA carboxylase; PC, pyruvate carboxylase; MS/MS, tandem mass spectrometry.

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Methylcrotonylcarnitine (C5:1) may or may not be present. It is common for these patients to have a secondary carnitine deficiency due to its combination with 3-HIVA for subsequent urinary excretion.

3-MCC comprises two hetero-subunits assembled into a α6β6 dodecamer. The larger α subunit contains the biotin carboxylase (BC) domain and the biotin carboxyl carrier protein domain covalently bound with a biotin prosthetic group and the binding site for the two substrates (bicarbonate and ATP). The smaller β-subunit has the carboxyltransferase (CT) domain and is essential for binding to 3-methylcrotonyl CoA which is characterized by its highly conserved functional domains (Chu and Cheng, 2007; Grunert et al., 2012; Pasquali et al., 2006; Stadler et al., 2005).

The α-subunits of 3-MCC are encoded by the *MCCC1* gene, which is located on chromosome 3q27 and spans about 70 kb of genomic DNA (Obata et al., 2001). The *MCCC1* gene encodes a protein of 725 amino acids which has a molecular weight of approximately 80 kDa (Holzinger et al., 2001). The β-subunit is a protein of 563 amino acids, with a molecular weight of approximately 61.8 kDa encoded by the *MCCC2* gene, which is located on chromosome 5q12-q13 (Baumgartner et al., 2001; Holzinger et al., 2001; Gallardo et al., 2001).

Individuals with 3-MCC deficiency harbor mutations in either *MCCC1* or *MCCC2*. Currently, the Human Gene Mutation Database (HMGD) (Stenson et al., 2014) records a total of 81 mutations associated to the *MCCC1* and 89 to the *MCCC2* gene, most of which are missense mutations although small insertions/deletions, nonsense, frameshift, and splice site mutations are also identified. The mutations identified to date are evenly distributed throughout the entire sequences of the two genes, without any evidence of mutational hot-spots (Stadler et al., 2006). Previous studies have been unable to establish a genotype-phenotype correlation since no mutations appear to be exclusively associated with symptomatic or asymptomatic cases, and no specific mutations have been associated with either milder or more severe phenotypes (Baumgartner et al., 2001; Stadler et al., 2006; Holzinger et al., 2001; Gallardo et al., 2001; Dantas et al., 2005). In this regard, it is important to note that the clinical, biochemical and genetic data appear to support the conclusion that factors other than the genotypes of the *MCCC1* and *MCCC2* loci can influence the phenotypic consequences of 3-MCC deficiency, including modifying genes and, perhaps more importantly, the extent to which the pathway is stressed by dietary or other environmental factors such as excessive protein breakdown associated with infections (Wolfe et al., 2007).

In this study, we document the molecular data covering a ten-year period of MCG identification in the Portuguese NBS program. All the mutations identified in *MCCC1* and *MCCC2* genes were analysed in the context of what is currently known of the MCC mutational spectra.

2. Materials and methods

2.1. Biochemical findings

The study presented here was based upon data obtained from extended neonatal screening, through the analysis of 903,528 newborns, corresponding to 99.8% of all births in Portugal (including Azores and Madeira) between March 2005 and December 2015. A total of 36 newborns were identified, from dried blood spots, with high values of C5OH, the primary biochemical marker of this disease. The cutoff value for a positive C5OH measure (≥1 μmol/L) was as established by the Portuguese Newborn Screening laboratory (Vilarinho et al., 2010).

For this set of 36 newborns, a second dried blood spot sample was subsequently obtained for confirmatory purposes as well as samples from the mothers. Other metabolic diseases were excluded through the analysis of the biochemical profile (urinary organic acid analysis). In 11 cases, the mothers were clinically asymptomatic and were identified by the detection of an increased C5OH acylcarnitine level identified through their infant's newborn screening. In addition to the cases identified through the Portuguese NBS program, four other cases identified through newborn screening in Spain were also studied in our Unit (two clinically asymptomatic mothers and two patients). In addition, a symptomatic 3-year old male of Spanish origin presenting delayed development and an acylcarnitine profile consistent with MCG was also included in the study.

2.2. Molecular analysis

Genomic DNA was extracted from dried blood spots (EZ1 DNA Tissue kit, QIAGEN). PCR amplification of all *MCCC1* and *MCCC2* exons and corresponding flanking intronic regions was performed using primer sequences designed using the software tool Primer3 (http://primer3.sourceforge.net/) (Table S1, Supporting information). Data from the NCBI (http://www.ncbi.nlm.nih.gov/) and Ensembl (http://www.ensembl.org/index.html) were used to obtain the reference genomic, cDNA and protein sequences for both genes. PCR-amplified fragments were purified with Exo-SAP (GE Healthcare, USA) and sequenced in an ABI PRISM™ 3130XL Genetic Analyser (Applied Biosystems, Foster City, CA) using the dye-terminator method.

2.3. Prediction of the pathological effect

In silico prediction of the impact of missense mutations was performed using the programs Polyphen2 (http://genetics.bwh.harvard.edu/pph2/) (Adzhubei et al., 2010), Mutation Taster (http://www.mutationtaster.org/) (Schwarz et al., 2014), SIFT (http://sift.jcvi.org/www/SIFT_BLink_submit.html) (Kumar et al., 2009) and I-Mutant2.0 (http://folding.biofold.org/i-mutant/i-mutant2.0.html) (Capriotti et al., 2005).

The effect of splice-site mutations was predicted by means of the Human Splicing Finder v.3.0 (http://www.umd.be/HSF/) (Desmet et al., 2009), BDGP (http://www.fruitfly.org/seq_tools/splice.html) (Reese et al., 1997) and FAS-ESS (http://genes.mit.edu/fas-ess/) (Wang et al., 2004). The nomenclature of the mutations follows the recommended format (http://www.hgvs.org/mutnomen) (den Dunnen and Antonarakis, 2000) and refers to the *MCCC1* and *MCCC2* cDNA sequence (GenBank reference sequence NM_020166.1 and NM_022132.3, respectively) in which the A of the first methionine is considered to be nucleotide +1.

2.4. Evolutionary conservation

Multiple sequence alignments were performed using 1-to-1 orthologous sequences retrieved from the Ensembl database (release 77) (Flicek et al., 2014). A total of 14 sequences were used in the comparison alignment with the human sequence (Table S2, Supporting information). Sequences were aligned using Muscule (Edgar, 2004) incorporated in Geneious v.5.5 (http://www.geneious.com).

2.5. Homology modeling

The 3D model of human 3-MCC was constructed as previously described (Azevedo et al., 2009; Lopes-Marques et al., 2012) using as template the structure of the *Pseudomonas aeruginosa* homologue (Huang et al., 2012). More specifically, we used the chain A structure (PDB 3U9S) and protein sequence (Uniprot Q96RQ3) to model human MCCC1 whereas the chain B structure (PDB 3U9S) and the protein sequence (Uniprot Q9HCC0) was used to model human MCCC2 using the MODELLER tool (Sali et al., 1995) available from the MPI Bioinformatics Toolkit (Max Planck Institute for Developmental Biology, http://toolkit.tuebingen.mpg.de; release 2.18.1) (Biegert et al., 2006). The signal peptide: residues 1 to 41 were excluded from the analysis. All structures were visualized in PyMol v.1.4.1.

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