METHYLMALONIC ACIDURIAS
mut^0/mut$^-$ and cblC Defects in Portuguese Population

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Abstract: The methylmalonic acidurias (MMAs) are metabolic disorders resulting from deficient methylmalonyl-CoA mutase (MCM) activity, a vitamin B12-dependent enzyme that uses adenosylcobalamin (Ado-Cbl) as a cofactor. Several mutant genetic classes that cause MMA are known based on biochemical, enzymatic and genetic complementation analysis. The mut^0/mut$^-$ defects result from deficiency of MCM, while the cblA, cblB and the variant 2 form of cblD complementation groups are linked to processes unique to Ado-Cbl synthesis. The cblC, cblD and cblF complementation groups are associated with defective methyl-cobalamin synthesis as well. Mutations in the genes associated with most of these defects have been described. In this study we investigate at molecular level four patients with *mut^0/mut$^-$* MMA phenotype and 19 Portuguese patients with cblC defect. We found four different mutations already described in the literature, in each MUT and MMACHC genes, respectively. Our data showed an evident difference in the prevalence of these two diseases, compared with other countries worldwide.

1 INTRODUCTION

Methylmalonic Methylmalonic acidurias (MMAs) encompass a group of genetically heterogeneous autosomal recessive disorders of methylmalonate and cobalamin metabolism caused by a defect in the conversion of methylmalonyl-CoA to succinyl-CoA. The different forms of MMAs share the biochemical marker of increased methylmalonic acid in body fluids. Methylmalonyl-CoA mutase (MCM) apoenzyme deficiency is a rare metabolic disease that may result in distinct biochemical phenotypes of MMA, namely *mut^0* and *mut$^-$*. Patients with the *mut^0* MMA phenotype exhibit the most severe, often life threatening manifestation. Treatment regimens include a protein-restricted diet, carnitine supplementation and oral antibiotic therapy. The MCM gene (*MUT*) is located in a single copy on chromosome 6 (6p21.1) and consists of 13 exons spanning over 35 kb (Jansen *et al.*, 1989). The open reading frame consists of 2.7kb, encoding 750 amino acids, the first 32 residues of which form the mitochondrial targeting sequence. It comprises different functional domains (Figure 1), the N-terminal domain (residues 1-32) followed by an extended segment (residues 32-87) involved in the dimerization of the two MCM monomers, the N-terminal $(\beta/\alpha)_8$ barrel (residues 87-416) containing the CoA binding domain and the C-terminal cobalamin-binding β/α_5 domain (residues 578-750). A linker region consisting of 160 amino acids connects the eight-stranded β/α barrel to the C-terminal β/α domain (Fuchshuber *et al.*, 2000). More than 80 mutations, including small and large scale rearrangements, truncating and missense mutations, have been described.

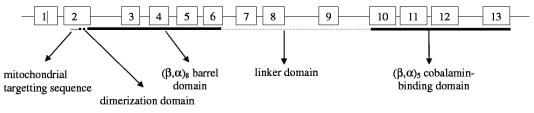

Figure 1: Functional domains of *MUT* gene (adapted from Acquaviva *et al.*, 2005).

MMA with homocystinuria is an inborn error of intracellular cobalamin metabolism resulting from impaired conversion of dietary vitamin B12 or cobalamin to its two metabolically active forms, methylcobalamin (MeCbl) and adenosylcobalamin (AdoCbl). MeCbl and AdoCbl are essential coenzymes to methionine synthase and MCM, respectively. The defect of these two cofactors causes the accumulation of methylmalonic acid and homocysteine in body fluids and a decrease of methionine. Three genetic defects of intracellular

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cobalamin metabolism, cblC (MIM 277400), cblD (MIM 277410), and cblF (MIM 277380) cause combined MMA and homocystinuria. cblC defect is the most frequent form and patients present with a heterogeneous clinical picture (Rosenblatt *et al*, 1997). Based on the age at onset, two distinct clinical forms have been recognized (early-onset and late onset form). Recently the identification of the gene responsible for cblC, MMACHC (MIM# 609831) was reported (Lerner-Ellis *et al.*, 2006). The gene is located in chromosome region 1p34.1 and has five exons. In the literature, forty-two different mutations in 204 cblC individuals were reported, including three common mutations: c.271dupA, c.394C>T, and c.331C>T. The c.271dupA and c.331C>T mutations were associated with early-onset disease while the c.394C>T mutation was associated primarily with late-onset disease.

In the present study 19 Portuguese patients with cblC defect and four patients with mut^0/mut^- MMA phenotype were investigated. We found four different mutations in *MMACHC* gene and another four in *MUT* gene. We discuss the prevalence of these diseases in our country/worldwide and the impact that mutation identification has on routine diagnostic procedures.

2 MATERIAL AND METHODS

2.1 Patients

In this cohort, the patients were selected after sharing and matching our databases. The diagnosis of mut^0/mut^- MMA and cblC defect was based on the identification of urinary and circulating metabolites and, whenever possible, confirmed with fibroblast studies. We studied at a molecular level four Portuguese patients with the mut^0 MMA phenotype and 19 with cblC defect diagnosed in our center, six of them (2/4 and 5/19, respectively) detected by extended newborn screening. The informed consent was obtained in all studied patients.

2.2 Methods

The whole coding sequence, the flanking exon–intron sequences of the *MUT* and *MMACHC* genes were PCR amplified from genomic DNA as described (Aquaviva, 2005; Lerner-Ellis, 2006). Agarose-gel purified amplicons were directly sequenced using the BigDye Terminator Cycle Sequencing Version 3.1 (Applied Biosystems, Foster City, CA), and analyzed on an ABI 3130XL DNA Analyzer. Multiple linear regression analysis was used to identify significant predictors of the genotype in the entire sample, including gender, age and clinical features. Statistical analyses were performed using a Chi-square test with Yates corrections (or, when appropriate, Fisher's exact test). Statistical significance was set at $p < 0.01$.

3 RESULTS AND DISCUSSION

In our center we diagnosed four cases of mut^0/mut^- MMA (two through newborn screening) and 19 cases of cblC defect (five from newborn screening). The number of symptomatic cases is in agreement with the prevalence found by expanded newborn screening although we know that the mild forms of cblC cannot be detected by newborn screening using the C3 (propionylcarnitine) and C3/C2 (acetylcarnitine) and C3/C16 (palmitoilcarnitine) ratios without the homocysteine determination. In most populations, mainly in Europe, the mut^0/mut^- MMA is more prevalent than the cblC defect. In the Mediterranean countries a few studies were carried out and some patients were identified although this condition is a very rare disease in middle and north of Europe (Nogueira *et al.*, 2008; Richard *et al.*, 2009).

We investigated the molecular basis of MMA in 23 unrelated patients by sequencing the entire coding region and intron-exon boundaries of the *MUT* and *MMACHC* gene using genomic DNA. The mutations were homozygous in 14 patients, and compound heterozygous in 8 patients.

All the *MUT* mutations have been previously reported; one of them is a nonsense mutation (p.R31X) and two are small deletions (p.L346del and p.G625FsX30) (Table 1).

The four different mutations found in cblC defect were: two missense (c.544T>C and c.565C>A), one nonsense (c.394C>T) and a small insertion causing frameshift (c.271dupA) (Table 2).

Our data compared with other southern-European populations, such as Italians and Spanish, have showed a less molecular heterogeneity (Nogueira *et al.*, 2008.

The recent inclusion of these conditions in the Portuguese expanded newborn screening program since 2004, resulted in a substantial improvement in the ability to identify suspected cases and allows for a more reliable determination of their incidence, considering the total number of individuals screened until now. By MS/MS 420,000 neonates were

screened and two cases of mut°/mut⁻ (1/210.000) and five cases of cblC (1/84.000) were identified.
The present study represents the first determination of the incidence of MMA in Portugal, indicating that the cblC is more frequent in our country than worldwide. All the patients identified by newborn screening revealed homozygosity for c.271dupA associated with the early phenotype. As long as more newborns will be screened, a more reliable comparison between symptomatic versus screened detected patients will be established.
Another prospect of molecular studies is to facilitate projections on the clinical type and severity of a disease. These projections may be essential to guide a proper monitoring of patients and that is why phenotype-genotype studies are needed.

Table 1: Genotype and clinical subgroup of patients with *mut⁰/mut⁻* MMA.

Patient	sex	onset	Age of diagnosis	Genotype	Phenotype
1	M	EO	3M	p.R31X / p.R31X	Severe
2	F	EO	11M	p.346delL / p.346de	Severe
3	M	EO	6D	p.G625Fsx30 / p.G625Fsx30	Severe
4	M	EO	7D	Not studied	Severe

Table 2: Genotype and clinical subgroup of patients with combined methylmalonic aciduria and homocystinuria, cblC type.

Patient	sex	onset	Age of diagnosis	Genotype	Phenotype
1	M	EO	28D	c.271dupA / c.271dupA	Severe
2	M	EO	1Y	c.271dupA / c.544T>C	Severe
3	M	EO	1Y	c.271dupA / c.271dupA	Severe
4	M	LO	8Y	c.271dupA / c.394C>T	Moderate
5	M	LO	1Y	c.271dupA / c.394C>T	Moderate
6	F	EO	25D	c.271dupA / c.271dupA	Severe
7	F	LO	15Y	c.394C>T / c.394C>T	Moderate
8	M	EO	1M	c.271dupA / c.565C>A	Severe
9	F	EO	3M	c.271dupA / c.394C>T	Severe
10	M	EO	1M	c.271dupA / c.271dupA	Severe
11	F	EO	43D	c.271dupA / c.271dupA	Severe
12	M	LO	16Y	c.394C>T / c.394C>T	Moderate
13	F	EO	4Y	c.271dupA / c.394C>T	Severe
14	F	EO	1M	c.271dupA / c.271dupA	Severe
15	M	EO	6D	c.271dupA / c.271dupA	Severe
16	F	EO	7D	c.271dupA / c.271dupA	Severe
17	F	EO	7D	c.271dupA / c.271dupA	Severe
18	M	EO	5D	c.271dupA / c.271dupA	Severe
19	M	EO	8D	c.271dupA / c.271dupA	Severe

4 CONCLUSIONS

In summary, we described the genetic background of 23 Portuguese patients with MMAs (four *mut⁰/mut⁻* MMA and 19 MMA-cblC type. Moreover, we found four different mutations already described in the literature, in each MUT and MMACHC genes, respectively. Our data showed an evident difference in the prevalence of these two diseases, compared with other countries worldwide. This study corroborate the importance of a molecular testing to confirm *mut⁰/mut⁻* MMA and MMA-cblC patients, detected by extended newborn screening programs, to offer accurate treatment and future prenatal diagnosis to couples at high risk of having affected children. The molecular data also contributes to molecular epidemiology of these diseases in our population.

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