The Molecular Landscape of Phosphomannose Mutase Deficiency in Iberian Peninsula: Identification of 15 Population-Specific Mutations

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Abstract PMM2-CDG is an autosomal recessive disorder and the most frequent form of congenital disorder of N-glycosylation, with more than 100 mutations identified to date. Sixty-six patients from 58 unrelated families were diagnosed as PMM2-CDG (CDG-Ia) based on clinical signs or because of a previous affected sibling. They all presented a type 1 serum transferrin isoform pattern, and, in most cases, the disease was confirmed by determining PMM2 activity in fibroblasts and/or lymphocytes. Residual PMM2 activity in fibroblasts ranged from not detectable to 60% of the mean controls. DNA and RNA were isolated from fresh blood or fibroblasts from patients to perform molecular studies of the *PMM2* gene, resulting in the identification of 30 different mutations, four of them newly reported here (p.Y102C, p.T118S, p.P184T, and p.D209G). From these 30 mutations, 15 have only been identified among Iberian PMM2-CDG patients. As in other Caucasian populations, p.R141H was the most frequent mutation (24 alleles, prevalence 20.6%), but less than in other European series in which this mutation represents 35–43% of the disease alleles. The next frequent mutations were p.D65Y (12 alleles, prevalence 10.3%) and p.T237M (9 alleles, prevalence 7.6%), while p.F119L and p.E139K, the most frequent changes in Scandinavian and French populations, respectively, were not found in our patients. The most common genotype was [p.R141H]+[p.T237M], and four homozygous patients for p.Y64C, p.D65Y, p.P113L, and p.T237M were detected. The broad mutational spectrum and the diversity of phenotypes found in the Iberian populations hamper genotype–phenotype correlation.

Keywords CDG · CDG-Ia · PMM2-CDG · Congenital defects of glycosylation · Mutations

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Introduction

PMM2-CDG (CDG-Ia; MIM 212065) is the most frequent congenital disorder of the N-glycosylation pathways. It is caused by a deficiency in the cytosolic enzyme phosphomannomutase (PMM 2; EC 5.4.2.8) that converts mannose-6-phosphate to mannose-1-phosphate, by means of transient phosphorylation of the catalytic site of the enzyme. Mannose-1-phosphate is precursor in the formation of guanosine diphosphate-mannose (GDP-Man), the main mannose donor for *N*-, *O*-, and *C*-mannosylglycans and glycosylphosphatidylinositol (GPI)-anchor biosynthesis (Jaeken et al. 1997). The *PMM2* gene is located on chromosome 16q13 and has 8 exons and a reading frame of 738 base pairs encoding for a protein of 246 amino acids. Up to 109 mutations (HGMD Professional®) have been identified along the *PMM2*

gene (MIM 601785), yielding a broad range of enzyme deficiencies (Matthijs et al. 1997; Haeuptle and Hennet 2009). There are mutational hot spots in exons 5 and 8. The majority of mutations (81%) are missense changes affecting different amino acid residues; 10% are nonsense or single or multiple base pair deletions which lead to early stop codons or frameshifts and thus to truncated proteins. Ten splicing defects have also been described due either to disruption of conserved sequences at the exon–intron junctions or at the polypyrimidine tract or to an intronic change that activates a pseudoexon sequence or to a deletion mediated by an Alu retrotransposition (Schollen et al. 2007a; Vega et al. 2009). The most common mutations are p.R141H and p.F119L, representing nearly 88% of mutant alleles described to date, both are probably founder mutations in Northern European countries (Bjursell et al. 1998). Most PMM2-CDG patients are compound heterozygotes for two different mutations, and homozygosity for p.R141H or other severely inactivating mutations has never been reported (Matthijs et al. 1998; Kjaergaard et al. 1998). Functional studies of several mutations have been performed to investigate their effect on activity and stability of the protein leading to the conclusion that a genotype retaining some residual PMM2 catalytic activity is required for survival (Pirard et al. 1999; Kjaergaard et al. 1999). It is worth noting that none of the amino acids constituting the active catalytic center by extrapolation in the crystal structure of the PMM1 isoenzyme has been found to be mutated (Silvaggi et al. 2006). The clinical course of patients with PMM2-CDG usually progress through four stages (Grunewald 2009): the infantile multisystemic or "visceral" stage, the childhood ataxia and mental retardation stage, the teenage leg atrophy stage, and the adult hypogonadal stage. Any organ system can be affected and severity of symptoms can widely vary. The most common presentation in infancy is multisystemic with central nervous system involvement. Mortality is as high as 20% of patients with the most severe form of the disease.

In a previous paper (Briones et al. 2002), we reported the biochemical and molecular characteristics of 26 Spanish PMM2-CDG patients. In this work, we update and report the predominant clinical signs and the spectrum of *PMM2* mutations in 66 PMM2-CDG patients from Portuguese and Spanish families.

Patients and Methods

Patients

The 58 families included were unrelated and originated from different regions of Portugal and Spain. In two families, one of the parents was from England and another from Russia, respectively. All cases were suspected due to clinical symptoms or because of a previous affected sibling, and they were referred for study by pediatricians from different hospitals since 1995.

Methods

Analysis of serum transferrin was performed as previously described by %CDT, isoelectric focusing (IEF) (Pérez-Cerdá et al. 2008; Colome et al. 2000) or by high-pressure liquid chromatography (HPLC) or capillary zone electrophoresis (Quintana et al. 2009). PMM2 activity in lymphocytes and fibroblasts was determined essentially as described in Van Schaftingen and Jaeken 1995. Fibroblasts were cultured according to standard procedures in minimal essential medium (MEM) supplemented with 1% glutamine, 10% bovine calf serum, and antibiotics in humidified atmosphere containing 5% of CO_2.

Genetic analysis was performed using whole blood or fibroblast cell lines from patients as source of mRNA and gDNA. Total mRNA was isolated by Tripure Isolation Reagent (Roche Applied Sciences, Indianapolis) adhering to the manufacturer's protocol, followed by two-step RT-PCR using oligodT as primer and using the SuperScript III First-Strand enzyme (Invitrogen, Carlsbad, CA).Genomic DNA was isolated using MagnaPure system, following the manufacturer's protocol (Roche Applied Science). The primers used for cDNA and gDNA amplifications were designed using the ENSEMBL database (http://www.ensembl.org/index.html; ENSG00000140650) and GenBank accession number NM_000303.2 as described in Vega et al. 2009. Amplifications of exons and flanking intronic sequences were performed using the FastStart kit (Roche Applied Sciences, Indianapolis). The PCR products were sequenced with the same primers used for amplification, using the BigDye Terminator v.3.1 mix (Applied Biosystems, Foster City, CA), following the manufacturers protocol. The sequenced products were then purified in Performa V3 96-Well Short Plates (EdgeBio, Gaithersburg, MD) and analyzed by capillary electrophoresis in an ABI Prism 3700 Genetic Analyzer (Applied Biosystems). DNA mutations numbering is based on cDNA reference sequence (GenBank accession number NM_000303.2) considering nucleotide +1 as the A of the ATG translation initiation codon and DNA reference sequence NT_010393.

Parental DNA was analyzed, when available, in heterozygous patients for allele assignment and in homozygous patients to rule out the presence of a genomic deletion.

Results

Sixty-six patients from 58 families were diagnosed as PMM2-CDG based on clinical signs, altered transferrin pattern, and deficient PMM2 activity in cells. All patients were of Caucasian origin. Collected clinical data of patients demonstrated that most of them presented with the "typical" clinical signs of a defect on protein glycosylation: cerebellar hypoplasia (82%), some degree of psychomotor retardation (76%), eye disturbances (54%), hypotonia (51%), ataxia (50%), liver dysfunction (36%), inverted nipples (36%), failure to thrive (36%), abnormal clotting factors (36%), dysmorphic features (29%), lipodystrophy (27%), and skeletal abnormalities (17%). Fifteen patients died, most of them in the first years of life, except for one in whom death occurred at age 13. Biochemically, they all presented an altered serum transferrin isoform pattern type 1, with increased amounts of asialo- and disialo-transferrin isoforms, compatible with a CDG type I diagnosis. PMM2-CDG was confirmed in most cases by determining PMM2 activity in fibroblasts and/or lymphocytes. Residual PMM2 activity in fibroblasts ranged from not detectable to as high as 60% of the mean of control values. In some patients in whom a high PMM2 activity value in fibroblasts was found, a lower enzyme activity in leukocytes ranging from 6% to 33% of the control values was demonstrated, confirming that the assay in leukocytes is more reliable for PMM2-CDG diagnosis. Molecular studies of the *PMM2* gene in RNA or DNA from 58 unrelated patients resulted in the identification of 30 mutations, four of them newly reported in this work (p.Y102C, p.T118S, p.P184T, and p.D209G). The results are shown in Table 1. As in other Caucasian populations, p.R141H is the most frequent mutation in the Spanish and Portuguese PMM2-CDG patients, accounting for 19.7% (17/86) and 21.8% (7/32) of the alleles, respectively. The second most frequent mutation in both populations was p.D65Y, accounting for 10% (12/118) of the disease alleles; but among the Spanish group of patients, the second most frequent was p.T237M that was found in 10.4% (9/86) of the disease alleles. Of the 30 identified variations, 13 have been reported only in our group of patients (p.M1V; p.Y64C; p.Y76C; IVS3-1G>C; p.E93A; p.Y102C; p.T118S; p.R123X; p.P184T; p.F207S; p.D209G; IVS7-9T>G; Del 28KB+exon8 Alu mediated). Forty-four different genotypes were detected; the most common one found in five Spanish families was [p.R141H]+[p.T237M]. Four homozygous patients for p.Y64C, p.D65Y, p.P113L, and p.T237M mutations were identified.

Discussion

PMM2-CDG is an autosomal recessive inherited disorder with an estimated incidence of 1:20,000 (Matthijs et al. 2000; Schollen et al. 2000). Due to the broad spectrum of clinical signs, including the very mild phenotypes recently described (Perez-Duenas et al. 2009; Grunewald 2009), the disease is probably still underdiagnosed. Of the patients of our series, 75% are currently alive and near a third of them are adults enjoying a good quality of life. This fact may be reflecting a lower prevalence of severe mutations in our population. Moreover, in contrast to other reported series where a low PMM2 activity (<15%) was found in patients' fibroblasts (Kjaergaard et al. 1998; Imtiaz et al. 2000), most of our patients did also show an enzyme activity below 25–30% of the control mean, but 15 patients presented a high activity up to 60% of control value. Some of these patients were functional hemyzygous for a mutation that retains in vitro a residual activity as high as 60% for p.C241S, 43% for p.P113L, and 48% for p.T237M or that affect the folding and/or stability of the enzyme (p.D65Y, p.V44A, and p.L32R) (Silvaggi et al. 2006; Vega et al. 2011), maybe contributing to the high residual activity found in vivo.

A large variety of mutations have been identified in our Iberian cohort of unrelated PMM2-CDG patients: 25 (83%) are missense mutations (Briones et al. 2002; Quelhas et al. 2007), four of them newly reported here (p.Y102C, p.T118S, p.P184T, and p.D209G); one nonsense (p.R123X) (Briones et al. 2002), three affecting the splicing of mRNA (IVS3+2T>C; IVS3-1G>C and IVS7-9T>C) (Briones et al. 2002; Vega et al. 2009), and a deletion mediated by an Alu retrotransposition displaying the complete loss of exon 8 (Schollen et al. 2007a). Of these 30 identified mutations, 13 have only been reported in our series of patients. The p.D65Y mutation was reported in a French study in a Portuguese patient, and a haplotypic association study confirmed its Iberian origin (Quelhas et al. 2007), and the p.V44A mutation was reported in an Ecuatorian patient probably of Spanish ancestors, adding these two mutations to our population-specific group of mutations. It is to note that mutations p.N216I and p.T226S were also previously reported in patients of Mediterranean origin (France, Italy, and Greece) (Matthijs et al. 2000). As expected, p.R141H was the most frequent mutation detected in our group of patients, but we have identified neither the severe mutation p.F119L, the second more frequent mutation among Scandinavian populations (Bjursell et al. 2000), nor p.E139K, the most prevalent change among French patients (Le Bizec

Table 1 Mutations in the *PMM2* gene in Iberian (Spanish and Portuguese) PMM2-CDG-Ia patients

Exon/intron	Base change	Amino acid change	N° alleles, Spain (prevalence %)	N° alleles, Portugal (prevalence %)	Geographical origin[a]	Reference
1	c.1 A>G	p.M1V	1 (1.16)		ES	Perez-Duenas et al. (2009)
2	c.95TA>GC	p.L32R	1 (1.16)		AUS, CAN, ES, F, I	Matthijs (2000), Vega et al. (2009)
2	c.131 T>C	p.V44A	6 (6.97)		EC, ES	Matthijs et al. (1999), Briones et al. (2002)
3	c.191A>G	p.Y64C	3 (3.48)		ES	Briones et al. (2002)
3	c.193 G>T	p.D65Y	6 (6.97)	6 (20.0)	ES, F (Portuguese), PT	Matthijs et al. (1999), Briones et al. (2002), Quelhas et al. (2007)
3	c.227A>G	p.Y76C	1 (1.16)		ES	Matthijs et al. (2000), Briones et al. (2002)
In 3	c.255+2 T>C (IVS3+2 T>C)	Splice variant	1 (1.16)		D, ES	Matthijs et al. (1999), Briones et al. (2002)
In 3	c.256-1 G>C (IVS3-1 G>C)	p.Val60GlyfsX11	1 (1.16)		ES	Vega et al. (2009)
4	c.278A>C	p.E93A	1 (1.16)		ES	Briones et al. (2002)
4	**c. 305A>G**	**p.Y102C**	**1 (1.16)**		**ES**	**In this study**
4	c.338 C>T	p.P113L	6 (6.97)	1 (3.33)	AU, B, D, ES, F, NL, PL, PT, S, USA, JP	Matthijs et al. (1997), Briones et al. (2002)
5	**c.353 C>T**	**p.T118S**	**1(1.16)**		**ES**	**In this study**
5	c.367 C>T	p.R123X	1 (1.16)	1 (3.33)	ES, PT	Matthijs et al. (2000), Briones et al. (2002), Quelhas et al. (2007)
5	c.368 G>A	p.R123Q	5 (5.81)	3 (10.0)	AU, ES, F, I, NL, PT, S, USA	Matthijs et al. (1999), Briones et al. (2002), Quelhas et al. (2007)
5	c.385 G>A	p.V129M	3 (3.48)		CAN, ES, F, I, TK, USA	Matthijs et al. (1997), Briones et al. (2002)
5	c.422 G>A	p.R141H	17 (19.76)	7 (23.33)	AR, AU, AUS, B, CAN, CH, Czech, D, DK, EC, ES, F, I, IR, N, NL, PE, PT, S, UK, USA	Matthijs et al. (1997), Briones et al. (2002), Quelhas et al. (2007)
6	c.458 T>C	p.I153T	1 (1.16)	1 (3.33)	ES, F, PT, USA	Matthijs et al. (2000), Briones et al. (2002)
6	c.470 T>C	p.F157S	4 (4.65)	2 (6.66)	D, ES, F, I, PL, PT, USA	Matthijs et al. (1999), Briones et al. (2002), Quelhas et al. (2007)
6	c.484 C>T	p.R162W	2 (2.32)	3 (10.0)	B, ES, F, NL, PT, UK	Matthijs et al. (1997), Briones et al. (2002), Quelhas et al. (2007)
7	c.548 T>C	p.F183S	1 (1.16)		D, ES, S, UK	Matthijs et al. (2000), Briones et al. (2002)
7	**c.550 C>A**	**p.P184T**	**1 (1.16)**		**ES**	**In this study**
7	c.620 T>C	p.F207S	3 (3.48)		ES	Briones et al. (2002)
7	**c.626 A>G**	**p.D209G**	**1 (1.16)**		**ES**	**In this study**
In 7	c.640-9 T>G (IVS7-9 T>G)	p.Pro213_Gly214ins 23	2 (2.38)		ES	Vega et al. (2009)

8	c.647A>T	p.N216I	1 (1.16)		ES, Gr, I	Matthijs et al. (1997), Briones et al. (2002)
8	c.677 C>G	p.T226S	2 (2.32)	1 (3.33)	ES, F, PT	Matthijs et al. (2000), Briones et al. (2002), Quelhas et al. (2007)
8	c.691 G>A	p.V231M		3 (10.0)	AR, AU, B, CAN, D, F, I, PE, PT, S, UK, USA	Matthijs et al. (1997), Quelhas et al. (2007)
8	c.710 C>T	p.T237M	9 (10.465)		CAN, ES, F, I, IR, UK, USA	Matthijs et al. (1997), Briones et al. (2002)
8	c.722 G>C	p.C241S	4 (4.65)	1 (3.33)	B, ES, F, I, NL, PT, USA	Matthijs et al. (1999), Briones et al. (2002), Quelhas et al. (2007)
8	Alu retrotransposition-mediated deletion of 28 kb	Loss of exon 8	1 (1.16)		ES	Schollen et al. (2007b)

Mutations in bold are reported for the first time in this article. Changes p.T118S and p.P184T are located on the same allele

86 Spanish alleles (43 patients) and 30 Portuguese alleles (15 patients) were studied. All Spanish mutant alleles were identified and in one Portuguese allele (3.33%) the mutation remains unidentified

Aus Australia, *AU* Austria, *AR* Arab origin, *B* Belgium, *CAN* Canada, *CH* Switzerland, *Czech* Czech republic, *D* Germany, *DK* Denmark, *EC* Ecuador, *ES* Spain, *F* France, *GR* Greece, *I* Italy, *IR* Ireland, *JP* Japan, *N* Norway, *NL* the Netherlands, *PE* Peru, *PL* Poland, *PT* Portugal, *S* Sweden, *TK* Turkey, *UK* United kingdom, *USA* United States of America

[a]Information on geographical origin of mutations was obtained from database of Euroglycanet web: http://www.euroglycanet.org

et al. 2005). In summary, the different mutational spectrum with half of the mutations not reported till now in other populations, and the absence of the frequent mutations p. F119L and p.E139K, probably reflect the impact of Mediterranean migrations, as occurs for other metabolic inherited diseases such as phenylketonuria (Desviat et al. 1999; Mallolas et al. 1999) or galactosemia (Gort et al. 2006, 2009). It is noticeable that subtle differences were found between Spanish and Portuguese patients; so the third most frequent mutation (p.V231M) among European patients was only present in three Portuguese disease alleles, while p.T237M was only identified in Spaniards. It is difficult to know whether this observation is related to different population influences in Spain and Portugal or, more probably, to the short number of mutant alleles studied due to the rarity of the disease.

Most patients were functionally hemyzygous with one severe mutation and other that retains considerable residual activity, and no patients with two null mutations were detected, as it has ever been reported (Freeze and Westphal 2001; Le Bizec et al. 2005; Pirard et al. 1999), supporting again the hypotheses that the combination of two inactivating mutations is lethal (Schollen et al. 2000). Four homozygous patients were found, with mutations that probably affect the folding of the PMM2 monomer (Silvaggi et al. 2006). Those homozygosities result in mild phenotypes. Overall, 83% of the mutant chromosomes identified in our population are missense changes, most of them affecting the folding and stability of the PMM2 homodimer (misfolding or conformational disease) (Silvaggi et al. 2006; Pirard et al. 1999; Kjaergaard et al. 1999; Le Bizec et al. 2005; Vega et al. 2011), and thus probably contributing to the milder phenotypes found in comparison to those reported in other series (Erlandson et al. 2001; Imtiaz et al. 2000). Nevertheless, the highly variable phenotype described in PMM2-CDG, as in other monogenic diseases, is probably determined not only by the *PMM2* mutant alleles but also by the other genes modulating the effect on the final functional enzyme (Dipple and McCabe 2000).

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