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Absence of homozygosity for predominant mutations in *PMM2* in Danish patients with carbohydrate-deficient glycoprotein syndrome type 1

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Carbohydrate-deficient glycoprotein syndrome type 1 (CDG1; McKusick No. 212065) is an autosomal recessively inherited disease characterised clinically by central nervous system dysfunction and biochemically by hypoglycosylation of many serum proteins. Most patients with CDG1 have deficient activity of phosphomannomutase. The locus for this enzyme has been mapped to 16p13, and a gene, *PMM2*, encoding phosphomannomutase has been isolated. We identified 34 mutations on 36 disease chromosomes in 18 unrelated Danish patients with CDG1. All patients have less than 15% residual activity of phosphomannomutase. Two mutations account for 88% of all mutations: F119L and R141H were each found in 16 out of 36 CDG1 alleles. These two mutations were found to be in linkage disequilibrium with two different alleles of the marker D16S3020, suggesting that there is one ancestral origin for each mutation. Two new mutations, G117R and D223E, were identified also. Surprisingly, no patient was homozygous for either of the two common mutations, suggesting that homozygosity for these mutations is either lethal or so benign that such patients are not detected.

Keywords: CDG1; *PMM2*; mutation; phosphomannomutase; linkage disequilibrium

Introduction

Carbohydrate-deficient glycoprotein syndrome type 1 (CDG1) is an autosomal recessively inherited disorder with multisystemic abnormalities including psychomotor retardation, hepatic dysfunction and dysmorphic features.[1,2] Biochemical diagnosis relies on isoelectric focusing of serum glycoproteins which shows a cathodal shift of isoforms due to undersialylation.[3] The majority of CDG1 patients have a deficiency of phosphomannomutase which converts mannose-6-P to mannose-1-P.[4–6]

A locus for CDG1 has been mapped to 16p13,[7–9] and overrepresentation of a certain haplotype in CDG1 families from southern Scandinavia suggests the presence of a specific mutation with a founder effect in this

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area.[9] Recently, two genes, both encoding a phosphomannomutase, have been cloned on the basis of their homology to yeast phosphomannomutase SEC53.[10–12] The first gene, *PMM*1, is localised on chromosome 22q13 and can therefore be excluded as a CDG1 candidate gene.[10,11] The second gene, *PMM*2, is located on 16p13, and a number of different mutations in *PMM*2 have been identified in patients with CDG1,[12] strongly suggesting that deficiency of the phosphomannomutase encoded by *PMM*2 is responsible for the phenotype in these patients, although expression studies have not yet been published. Furthermore, the role of the phosphomannomutase encoded by *PMM*1 is not clear. We now report our investigation of 18 Danish CDG1 patients, including the determination of their specific activity of phosphomannomutase, a comparison of their CDG1 chromosome DNA haplotypes, and a search for their mutations in *PMM*2.

Materials and Methods

Patients

Twenty-two patients and their parents from 18 unrelated families were included in this study. The clinical diagnosis of CDG1 had previously been substantiated by isoelectric focusing of transferrin and determination of carbohydrate-deficient transferrin in serum according to Stibler *et al*.[3]

Phosphomannomutase Assay

The specific activity of phosphomannomutase was determined in cultured fibroblasts by the method described by Van Schaftingen and Jaeken.[4]

Microsatellite Analysis

All family members were typed for the microsatellite markers GATA-P6084, AFMa284wd5, D16S406 and D16S3020.[13] Primer sequences were obtained from Genome Data Base. One primer in each set was 5'-labelled with FITC (fluorescein-isothiocyanate). The PCR products were analysed on an A.L.F. DNA sequencer using the Fragment Manager software (Pharmacia Biotech, Uppsala, Sweden).

Screening for Mutations by Single Strand Conformation Analysis (SSCA)

PCR All eight exons and flanking introns were amplified using the primers described by Matthijs *et al*.[14]

SSCA PCR products were denatured by mixing 3 μl with an equal volume of loading mixture (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol), followed by boiling for 5 min and cooling on ice. Of each denatured sample 2 μl were loaded on a 20% homogeneous PHAST-gel with native buffer strips (Pharmacia Biotech, Uppsala, Sweden). The separation conditions consisted of a prerun for 10 avh (average volt hours) at 5 mA, 1 W, and 400 V; a sample application for 2 avh at 5 mA, 1 W, and 25 V; and a separation for 310 avh at 5 mA, 2 W, and 450 V. Single-strand products were visualised by silver staining according to the PHASTsystem recommendations. PCR products with altered electrophoretic mobility were reamplified and sequenced.

Direct DNA Sequencing

5'-biotinylated reverse primers with sequences identical to those used for SSCA for the amplification of exons 5 and 8 were employed. The PCR products were purified using the QIAquick PCR purification kit (Qiagen, Düsseldorf, Germany). Single-strand DNA was isolated by the use of Dynabeads M-250 Streptavidin (Dynal, Oslo, Norway) and a Dynal magnetic separator and subsequently sequenced with the Sequenase kit version 2.0 (USB, Cleveland, Ohio), using the forward primers in the PCR amplification as sequencing primers.

Confirmation of Mutations by Restriction Analysis

Simultaneous detection of F119L and R141H: these mutations are located in exon 5 of *PMM*2. Using the primers R141HmisR and intU2 (Table 2) a PCR product of 250 bp spanning both mutations was obtained. The primer R141HmisR introduces a restriction site for *Mlu*I in the PCR product of the normal allele, but not in that of the allele harbouring R141H. The mutation F119L creates a restriction site for *Mse*I. After amplification two 10 μl aliquots of the amplification mixture were incubated with the appropriate restriction enzyme/buffer in a total volume of 20 μl and subsequently analysed by agarose gel electrophoresis.

The presence of G117R was detected using the primers G117Rmis and 2BF. The primer G117Rmis will introduce a restriction site for *Ava*I, except in the presence of G117R. D223E introduces a restriction site for *Bsp*1286I, using the primers int7BB and 8Rnew (Figure 1).

Results

Cultured skin fibroblasts were available from 16 unrelated patients with CDG1. The specific activity of phosphomannomutase in extracts of these cells ranged

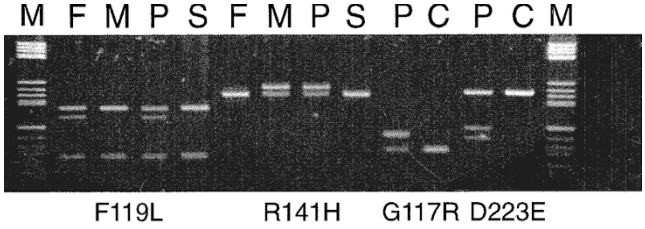

Figure 1 *Restriction analyses for detection of the mutations F119L, R141H, G117R and D223E. M (molecular size markers). The next eight lanes show the restriction analyses of family 2. The patient (P) is positive for both F119L (*Mse*I) and R141H (*Mlu*I). The father (F) is heterozygous for F119L, the mother (M) is heterozygous for R141H, whereas a healthy sib (S) does not harbour any of the mutations. The next four lanes show the restriction analyses for patient 1 G117R (*Ava*I) and for patient 8 with the mutation D223E (*Bsp*1286I).*

Table 1 *PMM* activities and mutations identified in 18 CDG1 patients of Danish origin

Fam nr.	*nmol/min/ mg protein*[a]	*Mutation 1*	*Amino acid change*	*Mutation 2*	*Amino acid change*
1	<0.2	669C→G	D223E	?	?
2	0.4	357C→A	F119L	425G→A	R141H
3	0.5	357C→A	F119L	425G→A	R141H
4	<0.2	357C→A	F119L	425G→A	R141H
5	0.3	357C→A	F119L	425G→A	R141H
6	<0.2	357C→A	F119L	425G→A	R141H
7	0.3	357C→A	F119L	425G→A	R141H
8	0.4	357C→A	F119L	349G→C	G117R
9	<0.2	?	?	425G→A	R141H
10	0.5	357C→A	F119L	425G→A	R141H
11	<0.2	357C→A	F119L	425G→A	R141H
12	<0.2	357C→A	F119L	425G→A	R141H
13	<0.2	357C→A	F119L	425G→A	R141H
14	<0.2	357C→A	F119L	425G→A	R141H
15	n.d.	357C→A	F119L	425G→A	R141H
16	n.d.	357C→A	F119L	425G→A	R141H
17	<0.2	357C→A	F119L	425G→A	R141H
18	0.4	357C→A	F119L	425G→A	R141H

[a]Control: 3.1±1.0 (n=9). n.d.=not determined.

from undetectable to 15% of normal activity (Table 1).

In order to identify the disease-causing mutations in *PMM2* all eight exons and flanking introns were initially screened by means of SSCA, using genomic DNA from 18 unrelated patients with CDG1. Exon 5 was analysed in two fragments, 5A and 5B. Several different aberrant SSCA patterns of 5A, 5B and exon 8 were detected. Direct sequencing of reamplified 5A and 5B products revealed the mutations 357C→A (F119L) and 425G→A(R141H), respectively (Table 1). Using a primer pair flanking both mutations (Table 2), it was possible to verify the presence or absence of both of the above mutations by restriction analysis of a single PCR product. Compound heterozygosity for R141H and F119L was found in 15 of the 18 patients, and their parents were heterozygous for the mutations in question.

Furthermore, we identified two novel missense mutations, 349G→C (G117R) in exon 5 of *PMM2* in patient 8 and 669C→G (D223E) in exon 8 in patient 1. In addition to D223E we identified a 3 bp deletion, 348-58delATG, on the paternal chromosome of patient 1. No mutation was identified on this patient's maternal chromosome. The presence of G117R and D223E was confirmed by restriction analysis of appropriate PCR products (Figure 1). No patient was homozygous for either of the common mutations.

It has previously been reported that a marker haplotype, A, defined by the microsatellites GATA-P6084 (or D16S768), AFMa284wd5, and D16S406 is markedly overrepresented on CDG1 chromosomes in patients from southern Scandinavia.[9] In order to determine which of the two common mutations was associated with this haplotype we determined the haplotypes in all available family members using the

Table 2 Primers used for confirmation of the mutations by restriction analysis

Mutation	*Name*		*Ann temp (°C)*
F119L/ R141H	intU2	GCACAGAGCTGAGAAACATT	60
	R141HmisR[a]	GTACTTTATCGAGTTCGTAGAACTCAA*C*G	
G117R	2BF	TGCGTTCTTCTTGGCTGCAGC	55
	G117Rmis[a]	AGAAACTCTGTCACCCTTTCATTCCC*C*GG	
D223E	int7FBB	TCCAGGGTCACATCAGCAATGG	52
	8Rnew	CCTATGCCTGCTTGTCAGCCG	

[a]Mismatch primers. The mismatch bases are in underlined italic.

Table 3 Expected and observed numbers of CDG1 patients with different genotypes. Frequencies, by counting, of F119L and R141H are 16/36 = 0.44 and 16/36 = 0.44, respectively

Genotypes	Expected frequency	Expected number	Observed number
F119L/F119L	$(0.44)^2$	3.5	0
F119L/R141H	$(2 \cdot 0.44 \cdot 0.44)$	7.0	15
R141H/R141H	$(0.44)^2$	3.5	0
Other combinations[a]	0.26	4.0	3

[a]F119L/other, R141H/other, and other/other.
$P = 0.0009$; $\chi^2 = 16.4$; df = 3.

above markers and the additional marker D16S3020[9,13] (Table 4). The mutation F119L was found with one specific haplotype in nine of 16 CDG1 chromosomes. Since some of our patients were included in the investigation of Bjursell *et al*,[9] we conclude that the A haplotype is associated with F119L. No specific haplotype was associated with the R141H mutation when all four markers were included. When only D16S406 and D16S3020 were considered, seven of 16 R141H chromosomes shared the same haplotype. We observed one recombination event between GATA-P6084, AFMa284wd5, D16S406 and (F119L/D16S3020) in one family. Two different D16S3020 alleles were found to be in linkage disequilibrium with F119L (allele 11) and R141H (allele 16), respectively.

Discussion

Most patients with CDG1 have a deficiency of phosphomannomutase, and intermediate activity of this enzyme is present in their parents.[4,6] The locus for CDG1 has been mapped to 16p13,[7,9] but the observation of families without evidence of this linkage suggests genetic heterogeneity.[8] Two genes both encoding phosphomannomutase have been isolated, *PMM*1 and *PMM*2.[10,11,13] *PMM*1 maps to chromosome 22 and can therefore be excluded as a candidate gene for CDG1. *PMM*2 maps to 16p13, and mutations in this gene have been identified, strongly suggesting that *PMM*2 is the gene responsible for CDG1.[12,13]

We investigated 18 Danish families with a total of 22 children with CDG1 and identified 34 mutations on 36 disease chromosomes. Two mutations were found to account for 88% of the mutations. Sixteen of 36 CDG1 alleles (44%) harboured the F119L mutation and 16 of 36 CDG1 alleles (44%) the R141H mutation. The activity of phosphomannomutase in 16 patients was low to undetectable. The very low activity of phosphomannomutase in extracts of normal cultured fibroblasts made it difficult to compare the residual activities of mutant fibroblast extracts.[4] Fifteen of 18 patients were compound heterozygous for F119L/R141H, but only seven of these had detectable residual activity. It is not clear whether this is due to residual activity of the mutant enzyme or a contribution from the phosphomannomutase encoded by *PMM*1.[16]

Linkage analysis has previously suggested that a significant proportion of Scandinavian patients with CDG1 have an ancestral mutation which segregates with a common haplotype A.[9] We conclude from our results that this haplotype is associated with F119L. The linkage disequilibrium between both of the common mutations, F119L and R141H, and two different alleles of D16S3020 strongly suggests the presence of two ancestral mutations. R141H was associated with a haplotype defined by only two markers D16S406 and D16S3020 (Table 2), suggesting that this mutation is older than F119L. The predominance of these mutations in Danish CDG1 patients emphasises the genetic homogeneity of the Danish population known from previous studies of cystic fibrosis[15] and galactosaemia (M. Salamon, personal communication 1997).

Our other findings include two new *PMM*2 mutations, G117R and D223E. The conservation of the involved amino acids in the highly homologous phosphomannomutases of man (*PMM*1 and *PMM*2), yeast (SEC53), and *Candida albicans*[10] suggests that these amino acids are crucial to the catalytic activity of the enzyme and that G117R and D223E are of pathogenic importance. In two of the patients, 1 and 9, only one mutation was found. The haplotypes defined by the markers AFMa284wd5, D16S406 and D16S3020 on the two chromosomes with an unknown mutation are identical and not found on any normal chromosome. We therefore expect these chromosomes to harbour identical mutations in *PMM*2.

In view of the predominance of only two mutations we were surprised to find that no patient was homozygous for either one. The expected frequencies of homozygotes for both F119L and R141H were 0.20. This lack of homozygosity is highly significant ($P = 0.0009$) (Table 3). So far no patient has been reported to be homozygous for any mutation in *PMM*2.[12] However, two patients are known to be homozygous for the A haplotype,[9] strongly suggesting homozygosity for F119L. The most likely explanation is that the R141H homozygous genotypes are lethal. If, on the other hand, homozygozity for F119L results in a

Table 4 Haplotypes found on 36 CDG1 chromosomes and 36 normal chromosomes

	CDG1 chromosomes					*Normal chromosomes*			
Mutation	GATA-P6084	AFMa284 wd5	D16S 406	D16S 3020	*fam. no*	GATA-P6084	AFMa284 wd5	D16S 406	D16S 3020
F119L	1	2	3	11	8	3	2	2	1
	2	7	3	11	6	3	5	5	1
	4	**4**	**4**	**11**	2	1	2	0	4
	4	**4**	**4**	**11**	4	1	2	0	4
	4	**4**	**4**	**11**	10	3	2	1	4
	4	**4**	**4**	**11**	11	2	1	5	4
	4	**4**	**4**	**11**	12	3	5	1	10
	4	**4**	**4**	**11**	13	3	3	1	11
	4	**4**	**4**	**11**	14	2	6	1	11
	4	**4**	**4**	**11**	16	3	2	2	11
	4	**4**	**4**	**11**	18	3	2	2	11
	2	2	5	11	3	2	2	4	11
	2	2	6	11	7	2	2	5	11
	2	2	6	11	15	2	5	5	11
	3	2	6	11	5	3	2	5	11
	n.i.	n.i.	n.i.	11	17	4	2	5	11
R141H	3	2	1	16	9	1	2	7	11
	1	2	**3**	**16**	16	16	2	7	11
	2	2	**3**	**16**	4	4	2	9	11
	2	2	**3**	**16**	6	6	2	5	12
	2	2	**3**	**16**	11	11	3	4	13
	2	1	**3**	**16**	13	13	2	5	13
	3	2	**3**	**16**	3	3	5	1	14
	4	0	**3**	**16**	14	14	2	4	14
	2	2	4	16	10	10	1	5	14
	2	6	4	16	15	15	2	5	14
	1	2	5	16	2	2	2	2	15
	1	2	5	16	5	5	2	2	15
	2	2	5	16	18	18	2	5	15
	3	2	6	16	7	7	1	5	15
	3	2	6	16	12	12	5	1	16
	n.i.	n.i.	n.i.	16	17	17	2	5	16
G117R	2	2	6	11	8	9	2	5	16
D223E	2	3	4	15	1	1	2	6	16
Unknown	2	2	4	12	1	4	2	8	16
	3	2	4	12	9	3	2	6	17

GATA-P6084: allele 1 = 109 bp; AFMa284wd5: allele 1 = 238 bp; D16S406: allele 1 = 180 bp; 16S3020: allele 1 = 62 bp.
n.i.: parents not informative.
Numbers in bold represent the alleles in linkage disequilibrium with F119L and R141H, respectively.

very mild phenotype, such patients could be underdiagnosed. This would also explain why compound heterozygosity would convey a phenotype compatible with survival, albeit with substantial morbidity.

The phosphomannomutase encoded for by yeast SEC53 is a dimeric protein.[16,17] If the product of *PMM*2 in man has a similar structure, interallelic complementation could rescue a genotype with one lethal and one mild mutation.

The role of the *PMM*1 enzyme in mannose metabolism is still puzzling. The two *PMM* genes are highly homologous, catalyse the same reaction and are apparently expressed in the same tissues.[10,12] All *PMM*2 patients express no or very low phosphomannomutase in leucocytes, although *PMM*1 mRNA is found in normal levels in these cells.[10] A possible explanation could be that the activity of *PMM*1 is dependent on *PMM*2 phosphomannomutase. Future *in vitro* expression studies may clarify the phenotypic consequences of mutations in *PMM*2.

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