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Two novel mutations and coexistence of the 991C>T and the 1339C>T mutation on a single allele in the coproporphyrinogen oxidase gene in Swedish patients with hereditary coproporphyria

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Abstract Hereditary coproporphyria (HCP) is an autosomal dominant disorder, resulting from a partial deficiency of the enzyme coproporphyrinogen oxidase (CPO). This enzyme catalyzes the sixth step of the heme biosynthetic pathway, and mutations in the *CPO* gene have been coupled to HCP. The present study was undertaken to identify disease-producing mutations in the *CPO* gene in nine Swedish families with HCP. Exon 1 of the *CPO* gene of the nine probands was analyzed directly by sequencing, and exons 2–7 were screened by denaturing gradient gel electrophoresis, followed by sequencing of exons showing abnormal band pattern. Mutations were detected in five of the nine families. In two of these families, the novel mutations 623C>T (S208F, exon 2) and 982C>T (R328C, exon 5) were identified, respectively. In the affected members of the other three families, the previously reported mutations 991C>T (R331W, exon 5) and 1339C>T (R447C, exon 7) were shown to coexist on one allele. The present study contributes 2 novel mutations to the 34 that have been previously reported to cause HCP. In addition, this is the first report on patients carrying two HCP-coupled mutations on one allele.

Key words Coproporphyrinogen oxidase · Hereditary coproporphyria · DNA mutational analysis · Heme biosynthesis *CPO* mutations

Introduction

Hereditary coproporphyria (HCP) is an autosomal dominant disorder, resulting from a partial deficiency of the enzyme coproporphyrinogen oxidase (CPO) (EC 1.3.3.3.) (Anderson et al. 2001). This mitochondrial enzyme (Grandchamp et al. 1978) operates as a homodimer (Kohn et al. 1993) and catalyzes the conversion of coproporphyrinogen III to protoporphyrinogen IX in the sixth step of heme biosynthesis (Dailey 1997).

Clinical manifestations of HCP are similar to those seen in acute intermittent porphyria and variegate porphyria (VP), including neurovisceral symptoms (such as abdominal pain, vomiting, and constipation), motor and sensory neuropathy, and psychiatric symptoms. HCP and VP are also cutaneous porphyrias because patients may develop skin fragility and bullous eruptions on sun-exposed areas of the body. These skin lesions, in the absence of engagement of the nervous system, cannot be clinically differentiated from the skin lesions seen in porphyria cutanea tarda (PCT), which is a nonacute and nonneurologic form of cutaneous porphyria (Anderson et al. 2001). The clinical penetrance of HCP may be limited because it has been reported (Martasek 1998) that many of the genetically predisposed individuals never experience an acute attack. Biochemical findings in HCP include increased urinary excretion of 5-aminolevulinic acid (ALA), porphobilinogen (PBG) and porphyrins, and increased fecal excretion of coproporphyrins (Anderson et al. 2001). In feces, the ratio of coproporphyrin III: coproporphyrin I is likely to be increased also in latent cases of HCP (Blake et al. 1992). Early detection of latent cases is important so that precipitating factors such as certain drugs, alcohol, hormones, and fasting can be avoided (Anderson et al. 2001). Carriers of HCP traits can be identified by genetic analysis of the *CPO* gene or by measuring the activity of CPO in lymphocytes or fibroblasts, which is reduced to about 50% of normal in heterozygous HCP (Elder et al. 1976; Grandchamp and Nordmann 1977).

The gene encoding human CPO has been cloned, sequenced (Martasek et al. 1994a; Taketani et al. 1994),

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Table 1. Biochemical data (at time of diagnosis) of affected HCP family members

	Urine			Feces			Blood	
	ALA (mmol/ mol crea)	PBG (mmol/ mol crea)	Porphyrins (μ mol/ mol crea)	Total porphyrins (nmol/g dry weight)	Coproporphyrins (% of total porphyrins)	Coproporphyrin III/I ratio	Plasma porphyrins (fluorescence at 620 nm)	Erythrocyte porphyrins (μ mol/l)
Family A								
Proband	1.3	0.3	11	98	61	7.0	ND	2.2
Family B								
Proband	2.1	0.6	23	480	93	24.0	D	—
Sister	1.8	0.2	28	380	76	16.0	ND	0.8
Son	1.5	0.3	14	160	77	11.5	ND	1.0
Nephew	1.0	0.2	7	253	76	7.3	ND	0.8
Family C								
Proband	1.2	0.2	1	36	28	0.8	ND	6.9
Son	2.0	0.3	82	1624	81	19.0	ND	3.4
Son	1.5	0.3	19	23	19	0.8	ND	2.0
Niece	2.4	0.6	15	681	89	24.0	ND	2.4
Granddaughter	1.1	0.3	13	211	62	9.0	ND	1.1
Granddaughter	2.2	0.3	11	343	49	16.0	ND	1.4
Granddaughter	1.4	0.1	70	874	75	32.0	ND	—
Family D								
Proband ^a	1.0	0	27	117	76	10.0	—	—
Brother	1.4	0.5	18	114	77	9.0	D	5.6
Niece	3.8	1.1	81	1061	92	19.0	D	8.7
Family E								
Proband	1.8	0.4	25	249	84	16.0	ND	2.7
Father ^a	1.4	0.5	15	—	—	—	—	0.7
Aunt ^a	1.0	0.2	4	138	88	1.2	ND	0.9
Reference value	<3.1	<1.2	<25	<200	$\approx$ 20	$\approx$ 0.4	ND	<1.2

All patients in which erythrocyte porphyrin was measured had normal B-hemoglobin values

All investigated patients had decreased lymphocyte coproporphyrinogen oxidase (CPO) activity, about 50% of a normal control included in each assay

^aCPO activity not investigated

HCP, Hereditary coproporphyrria; ALA, 5-aminolevulinic acid; PBG, porphobilinogen; Crea, creatinine; D, detectable; ND, not detectable

and mapped to chromosome 3q12 (Cacheux et al. 1994). The gene contains seven exons, encoding a 454-amino acid peptide, of which the 110 N-terminal amino acid residues constitute a putative mitochondrial target sequence (Delfau-Larue et al. 1994).

Several HCP-coupled mutations have been identified in the *CPO* gene (Doss et al. 1999; Lamoril et al. 2001; Petersen et al. 2000; Sassa et al. 1997, and Human Gene Mutation Database). The mutations identified so far are, with a few exceptions, restricted to single families, making HCP a genetically heterogeneous disease.

HCP has previously been reported in only 1 Swedish family (Haeger-Aronsen et al. 1968). At the Swedish Porphyria Centre there are now 11 registered HCP families. This study was undertaken to identify HCP-coupled mutations in the *CPO* gene in 9 of these families.

Subjects and methods

Subjects

The probands and their affected and unaffected relatives had been referred to the Swedish Porphyria Centre during the last two decades. The HCP diagnosis in affected indi-

viduals had been based on clinical observations and was confirmed by biochemical analysis including urinary ALA and PBG, plasma porphyrin fluorescence, urinary and fecal total porphyrins, and the coproporphyrin III:I ratio in feces. In most of the patients, the activity of lymphocyte CPO had been determined as well. The biochemical data of affected members of those families where mutations were identified are presented in Table 1.

Fifty unrelated unaffected individuals of Caucasian origin were used as controls. The study was approved by the Ethics Committee of the Karolinska Institute (395/00).

Case reports

Family A. The proband experienced her first acute porphyric crisis at the age of 43 years. She was severely affected by abdominal pain, which had started after parenteral administration of barbiturate. After several investigations, the diagnosis acute porphyria was established and she was informed about which drugs to avoid. Since then she had only presented mild symptoms such as paresthesia (tingling) and periods of depression. She had never experienced skin symptoms.

During reinvestigation 30 years later, she had normal concentration of urinary and fecal porphyrin metabolites, but in the feces the porphyrin pattern was characteristic for HCP and the CPO activity was decreased.

Family B. The proband had her first symptoms at the age of 29 years. She suffered from skin fragility and blisters on sun-exposed areas. She was supposed to have PCT and was treated with a series of phlebotomies, which resulted in a complete remission of the skin lesions.

Several years later, she experienced periods of abdominal pain and paresthesias and the PCT diagnosis was reconsidered. The results of biochemical screening were consistent with HCP. Her son had been affected by severe abdominal pain at the age of 17 years, after a festive period. At the age of 30 years, his fecal porphyrin profile showed a pattern characteristic for HCP and he also showed decreased lymphocyte CPO activity. A sister of the proband and her son had also been affected by both acute symptoms and skin symptoms.

Family C. The proband had suffered from abdominal pain and skin fragility during the third decade of life. The diagnosis of HCP was established many years later when his children were investigated for porphyria. At diagnosis he had no clinical symptoms and the biochemical picture was nearly normal with the exception of a surprisingly high concentration of erythrocyte porphyrins. The diagnosis of HCP was confirmed by the decreased CPO activity. Two of his children were also diagnosed with HCP. They complained of skin fragility and one of them had periods of depression. The disease had been confirmed in several other family members as well.

Family D. The proband was clinically suspected to suffer from acute porphyria. Biochemically, she showed only slightly increased concentration of fecal porphyrins with a subfraction pattern consistent with the diagnosis of HCP.

Her brother showed a similar excretion pattern in feces and a high concentration of erythrocyte porphyrins. In his early youth, he had a period of 4 to 5 years of abdominal pain and myalgias, and these symptoms later reappeared after alcohol intake. His daughter had been affected by abdominal pain, arthralgia, anxiety, asthenia, and lability since puberty. At the time of diagnosis, she had a very high concentration of fecal porphyrins and also a high concentration of erythrocyte porphyrins.

Family E. The proband was referred for investigation of porphyria at the age of 46 years. She was affected by sensory axonal polyneuropathy, and the diagnosis of acute porphyria was suggested as a possible cause. For several years she had recurrently been affected by periods of abdominal pain and chronic constipation. The biochemical pattern was consistent with HCP. The proband was not aware of the fact that her father had once been diagnosed with acute porphyria. He is now deceased and cannot be reinvestigated. The paternal aunt showed almost normal porphyric biochemical

screening but a slightly increased coproporphyrin III:I ratio.

DNA extraction

DNA was extracted from whole blood using QIAamp DNA Mini Kit (Qiagen, Chatswell, CA, USA).

Denaturing gradient gel electrophoresis (DGGE) analysis

Exons 2–7 of the *CPO* gene were screened for sequence variations by DGGE, using primers described previously (Petersen et al. 2000). The polymerase chain reaction (PCR) reaction mixture contained approximately 2 ng/μl genomic DNA, 2 mM MgCl₂, 0.15 mM deoxyribonucleoside triphosphates (dNTPs), 2 μM of each primer, and 0.025 U/μl Ampli Taq Gold DNA Polymerase (Applied Biosystems, Foster City, CA, USA) in Ampli Taq Gold Buffer (Applied Biosystems). The PCR program was 10 min at 95°C, followed by 40 cycles of 1 min at 95°C, 1 min at 58°C (exon 5 and 7) or 60°C (exons 2–4 and 6), and 1 min at 72°C. This was followed by an additional extension for 10 min at 72°C. Samples were then heated to 99°C for 5 min and thereafter incubated for 60 min at 60°C, followed by 60 min at 37°C to allow formation of heteroduplexes. The reaction was carried out in a Mastercycler Gradient thermal cycler (Eppendorf, Hamburg, Germany).

PCR products, mixed 1:1 with loading dye (Bio-Rad, Hercules, CA, USA) were loaded onto an 8% polyacrylamide gel (acrylamide: bisacrylamide 37.5:1) with a linear gradient of denaturant (100% denaturant: 7 M urea and 40% (v/v) formamide). A gradient of 20%–60% was used for analysis of all exons, as described previously (Petersen et al. 2000). In addition, exons 2, 3, and 4 were analyzed on 15%–40% gels and exon 6 on a 10%–40% gel. Electrophoresis was performed at 150 V at 60°C for 5 h, using equipment included in the DCode Universal Mutation Detection System for DGGE (Bio-Rad). For prediction of an appropriate gradient of denaturing agents in the gels, computer analysis of the PCR fragments was performed with Mac Melt Software 1.0 (Bio-Rad).

Sequencing analysis

Sequencing of exons 1, 2, 4, 5, and 7 was performed using primers described previously (Petersen et al. 2000). For exons 3 and 6, the primers 3S, 5'-GAGTGAATTTGTA TCAAGGC-3', 3AS, 5'-ATGTTATGCCCTCTTATCTG-3' and 6S, 5'-GTTATGGGTTAGTCTTAAG-3', 6AS, 5'-TTGTGGGTGTTTGGGAATTGG-3' were used, respectively. The PCR reaction mixture contained approximately 2 ng/μl genomic DNA, 2 mM MgCl₂, 0.15 mM dNTPs, 2 μM of each primer, 0.025 U/μl Ampli Taq Gold DNA Polymerase (Applied Biosystems) in Ampli Taq Gold Buffer (Applied Biosystems). The PCR program was 10 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at

55°C (exons 2–7) or 65°C (exon 1), and 30s at 72°C. At the end of the program, there was a final extension for 5 min at 72°C. The PCR products were purified using QiaQuick PCR Purification Kit (Qiagen) and sequencing was then performed using either the forward or reverse primer together with ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems). The sequencing reaction conditions used were 25 cycles of 30s at 96°C, 15s at 50°C, and 4min at 60°C. The samples were then analyzed (Cybergene AB, Huddinge, Sweden) on an ABI PRISM 377 DNA Sequencer (Applied Biosystems).

Protein sequence alignment

Alignment of protein sequences was performed using Fasta3 version 3.3t09, obtained from The European Bioinformatics Institute (May 17, 2001; <http://www.ebi.ac.uk/fasta33>).

Numbering system

Nucleotides were numbered from the first nucleotide of the translation initiation codon according to the cDNA sequence derived from the *CPO* genomic sequence (Delfau-Larue et al. 1994).

Databases

Human Gene Mutation Database (July 25, 2001; <http://www.hgmd.org>) reported for mutations in the *CPO* gene and European Molecular Biology Laboratory Nucleotide Sequence Database (May 17, 2001; <http://www.ebi.ac.uk/embl>) reported for sequences used in the protein sequence alignment.

Results

Screening for mutations in exons 2–7 of the *CPO* gene by DGGE, followed by sequencing to identify the sequence variation and direct sequencing of exon 1 revealed mutations in five of the nine probands. In the remaining four probands, no mutation was identified, not even after sequencing of all exons.

In the probands of families A and B, the novel mutations 623C>T (S208F, exon 2) and 982C>T (R328C, exon 5) were identified, respectively. DGGE analysis of symptomatic and asymptomatic members of family B showed that the 982C>T trait segregates with the HCP diagnosis in this family (Fig. 1). In family A, no relatives were available for investigation. In the probands of families C, D, and E, the two previously reported mutations 991C>T (R331W, exon 5) (Lamoril et al. 2001; Martasek et al. 1994b) and 1339C>T (R447C, exon 7) (Lamoril et al. 2001) were shown to coexist. Investigation of the members of these three families revealed that both these mutations are located on one

allele, and that they segregate with the HCP diagnosis (Fig. 1). None of the four mutations was identified among the 50 controls.

Three previously reported polymorphisms, 814A/C (exon 4), 880G/A (exon 4), and 990G/A (exon 5) (Fujita et al. 1994; Lamoril et al. 2001; Martasek et al. 1994b; Petersen et al. 2000; Sassa et al. 1997) were identified among the investigated individuals. The frequency of these alleles in the control individuals were 0.87A/0.13C (814), 0.94G/0.06A (880), and 0.72G/0.28A (990). The 991C>T and 1339C>T mutations in families C, D, and E were located on an 814A:880G:990G allele (data not shown).

The sequence variations in exons 2 and 4 were detectable on both 20%–60% and 15%–40% DGGE gels, but were more prominent on the 15%–40% gel.

Discussion

Until now, a total of 34 different mutations had been identified in the *CPO* gene of patients suffering from HCP in heterozygous or homozygous form (Doss et al. 1999; Lamoril et al. 2001; Petersen et al. 2000; Sassa et al. 1997; Human Gene Mutation Database). In the present study, DGGE analysis and sequencing identified two novel and two previously reported mutations in five of nine investigated Swedish HCP families. The four identified mutations are all missense mutations, corresponding to the amino acid substitutions S208F, R328C, R331W, and R447C, respectively.

The novel mutations 623C>T (S208F) and 982C>T (R328C) were detected in families A and B, respectively. Alignment of the human CPO amino acid sequence with corresponding sequences from other species shows that S208 and R328 are totally conserved among the listed species (Table 2). In addition, these traits were not detected in any of the 50 normal controls, and the 982C>T (R328C) trait was shown to segregate with the HCP diagnosis in family B (Fig. 1). Thus, these variations seem to be the cause of HCP in these families.

According to the protein sequence alignment, the R331 and R447 residues (affected by the previously reported mutations 991C>T and 1339C>T) are poorly conserved among the listed species (Table 2). These residues, however, seem to be important for the full activity of the enzyme because a previous report has shown that expression of *CPO* carrying the 991C>T (R331W) or the 1339C>T (R447C) mutation decreases the CPO activity to 32% and 14% of normal, respectively (Lamoril et al. 2001). In addition, the 991C>T mutation has been shown to result in a less stable protein that binds an excessive amount of porphyrins (Martasek et al. 1994b; Martasek et al. 1997).

The 991C>T mutation has previously been identified in a true homozygous case of HCP, with the patient exhibiting a CPO activity of 2% of normal, whereas the heterozygous parents with 50% activity were latent carriers (Grandchamp et al. 1977; Martasek et al. 1994b). This mu-

Fig. 1. Pedigrees of the Swedish hereditary coproporphyria families (A–E), showing the inheritance of 623C>T in family A, 982C>T in family B, and 991C>T and 1339C>T in families C, D, and E. The mutations were identified by denaturing gradient gel electrophoresis

(DGGE) analysis, followed by sequencing of the *CPO* gene of each proband. The mutation status of the family members was determined by DGGE analysis

Table 2. Alignment of CPO amino acid sequences from nine different species

Species ^a	Overall identity (%) ^b	Amino acid position			
		S208	R328	R331	R447
1. <i>Homo sapiens</i> (Human)	—	S	R	R	R
2. <i>Mus musculus</i> (Mouse)	86.4	S	R	R	R
3. <i>Drosophila melanogaster</i> (Fruit fly)	61.5	S	R	S	K
4. <i>Glycine max</i> (Soybean)	52.1	S	R	R	I
5. <i>Saccharomyces cerevisiae</i> (Baker's yeast)	51.9	S	R	T	T
6. <i>Nicotiana tabacum</i> (Common tobacco)	50.4	S	R	R	I
7. <i>Salmonella typhimurium</i>	47.9	S	R	R	K
8. <i>Escherichia coli</i>	47.6	S	R	Q	K
9. <i>Pseudomonas aeruginosa</i>	44.5	S	R	P	L

Fasta3, version 3.3t09 used for alignment

^a Accession numbers at European Molecular Biology Laboratory Nucleotide Sequence Database: (1) Q14060; (2) P36552; (3) Q9V3D2; (4) P35055; (5) P11353; (6) Q42946; (7) P33771; (8) P36553; (9) P43898

^b Compared with the normal human sequence

tation was later reported to cause HCP in heterozygous form as well (Lamoril et al. 2001). In the present study, the patients diagnosed with HCP in families C, D, and E carry both the 991C>T and the 1339C>T mutation on one allele. This genetic constellation probably affects the enzyme more severely than does each mutation alone. In fact, the clinical penetrance of the disease in these families seems to be high because no asymptomatic carriers could be identified (Fig. 1). In family E, the aunt of the proband is a carrier of only the 1339C>T mutation. She recently went through a biochemical investigation, and the porphyrin profiles in

urine and blood were normal, but the fecal porphyrin pattern showed a slightly increased coproporphyrin isomer III:I ratio (Table 1).

Because the 991C>T and the 1339C>T mutation were shown to be located on an 814A:880G:990G allele in all three families, a common ancestry for this allele cannot be excluded. Although there are no previous reports on intragenic recombination points in the *CPO* gene, a meiotic crossing-over point somewhere between exon 5 and exon 7 might be the explanation for the loss of the 991C>T mutation in the aunt of the proband in family E.

The allele carrying the two mutations is most likely a result of a normal allele being mutated twice. Another, but probably less likely explanation, is that a meiotic crossing-over event in a compound heterozygous individual resulted in the creation of an allele carrying both mutations. This theory is, however, based on the assumption that compound heterozygosity for these two mutations is compatible with life.

In the four families in which no mutation was identified, the gene defects might be of a kind that could not be detected by the methods used in this study, such as gross deletions or mutations located in the parts of the introns that were not covered by the primers used for the PCR amplification.

In summary, the likely disease-causing mutations have been identified in five of nine Swedish families with HCP. Two novel mutations were identified and can be added to the 34 that have been previously reported to cause HCP. In addition, this is the first report on patients carrying two HCP-coupled mutations on one allele.

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