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FOUR NOVEL MUTATIONS OF THE COPROPORPHYRINOGEN III OXIDASE GENE

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Abstract – Here we report the characterization of four novel mutations and a previously described one of the coproporphyrinogen III oxidase (CPO) gene in five Italian patients affected by Hereditary Coproporphyria (HCP). Three of the novel genetic variants are missense mutations (p.Gly242Cys; p.Leu398Pro; p.Ser245Phe) and one is a frameshift mutation (p.Gly188TrpfsX45).

Key words: Hereditary Coproporphyria, coproporphyrins, coproporphyrinogen III oxidase

INTRODUCTION

Hereditary Coproporphyria (HCP, OMIM 121300) is an autosomal dominant disease classified as acute hepatic porphyria. Rarely, a homozygous variant may occur, associated with more severe clinical and biochemical manifestations (6, 13). Clinical penetrance is incomplete and depends on environmental and acquired factors such as hormones, drugs, nutritional status, alcohol, infectious diseases. The disorder is due to the reduced activity of coproporphyrinogen III oxidase (CPOX, EC 1.3.3.3), the mitochondrial enzyme in the heme biosynthetic pathway that catalyzes the conversion of coproporphyrinogen III to protoporphyrinogen IX (4). CPOX is encoded by the coproporphyrinogen III oxidase gene (CPO), that is 14 kb long, contains 7 exons and is located on chromosome 3q11.2 (1, 3).

Clinical symptoms are very rare before puberty, and are neurological and less commonly cutaneous. Biochemically, overproduction and excretion of coproporphyrin III in urine and feces is the hallmark of the disease. Increased levels of delta aminolevulinic acid (ALA) and porphobilinogen (PBG) are generally observed during the acute crisis.

Abbreviations: ALA, δ-Aminolevulinic Acid; **PBG,** Porphobilinogen; **TP,** Total Porphyrins; **CP,** Coproporphyrins; **PP,** Protoporphyrins; **N.P,** not performed ; **HCP,** Hereditary Coproporphyria ; **CPO ,** coproporphyrinogen III oxidase; **d.w,** dry weight

Latent HCP patients or HCP gene carriers are clinically and sometimes biochemically normal although being at risk of life-threatening acute crises. Therefore their detection is extremely important in the management of the disease. To this aim the usual screening method used was based on the determination of the CPOX activity, which is reduced in asymptomatic as well as in symptomatic patients (7). This method has been shown to be poorly reliable for diagnostic purposes (9), and the identification of the gene defect at molecular level has become a useful diagnostic method, as it plays a key role in preventing behaviors that could be triggering factors for the clinically overt disease.

To date over 45 mutations in the CPO gene have been identified (Human Gene Mutation Database, http://www.hgmd.cf.ac.uk/ac/index.php). Here we report the characterization of four novel mutations and a previously described one of the CPO gene in five coproporphyiria Italian patients from different families.

MATERIALS AND METHODS

Patients and Biochemical findings

A total of 17 individuals, representing five unrelated Italian coproporphyria patients and some of their relatives, were studied. The diagnosis of Hereditary Coproporphyria was based on the observation of typical clinical manifestations and increased excretion of urinary and fecal porphyirins with a predominance of copro III isomer.

The concentration of porphyrin precursors in urine was measured spectrophotometrically after ion-exchange chromatography (δ-

ALA/PBG column test; Bio-Rad GmbH, München, Germany). Total porphyrins in urine and feces were determined by spectrophotometer methods (2) (12). Urine and fecal porphyrin profiles were analyzed by reverse-phase high-pressure liquid chromatography (11).

PCR amplification of CPO gene

Genomic DNA was extracted from blood using commercially available kits (QIAGEN). The entire coding sequence and exon/intron boundaries of CPO gene were amplified in 8 different PCR fragments with the following primers: CPOX1p1FOR (5'-AGCGGCTCTTCTCGAAAGGT-3') and CPOX1p1REV (5'-GCCAGCTCATCCTCCTCCT-3') for exon 1 (partial); CPOX1p2FOR (5'-ATGTGCAGCGGGCGGAGAT-3') and CPOX1p2REV (5'-TGACAGCTGATCCGGGTCCAT-3') for exon 1 (partial); CPOX2FOR (5'-GATTTGGGAAACGGGAAAATA-3') and CPOX2REV (5'-TTGTGGGCAAAATAAGGTTTG-3') for exon 2; CPOX3FOR (5'-AAAAAGCCATTCGGGTTCATA-3') and CPOX3REV (5'-ATTGCCTTTACATTGCCTCCT-3') for exon 3; CPOX4FOR (5'-CTGCCTAGGCCTTACTGGTCT-3') and CPOX4REV (5'-CCATTTTCATAAGCAGAAGAGG-3') for exon 4; CPOX5FOR (5'-GGACAGCAAGTAAGGACACCA-3') and CPOX5REV (5'-GACAACACAACACCCGCTATT-3') for exon 5; CPOX6FOR (5'-CTGTAGGCTGGTGTCCTCTGT-3') and CPOX6REV (5'-CACCATGTCACCGCTTAAAAT-3') for exon 6; CPOX7p1FOR (5'-TTTAAGTAAAGAGGTATGTGTGTCA-3') and CPOX7p1REV (5'-AACAAGATGAGAGATTTCCTGATA-3') for exon 7 (partial). All PCR reactions were carried out in a 50 µl reaction volume containing genomic DNA, 2.5 Unit Taq DNA Polymerase (Invitrogen), 50 pmol of each primer, 0.2 mM dNTPs (Invitrogen) and 1.5 mM $MgCl_2$ in the buffer supplied by the manufacturer. The amplification was performed using an initial denaturation step at 94 °C for 3 min, followed by 35 cycles of 94 °C for 45 s, annealing at the annealing temperature of the specific primer pair for 30 s, extension at 72 °C for 1 min followed by a 10 min final step at 72 °C. All PCR products were checked for purity on agarose gel. PCR products intended for DNA sequencing were cleaned up with NucleoSpin kit (Macherey Nagel), checked on agarose gel for yield and subjected to direct sequencing for mutation screening. Mutations not previously described are validate by screening of 50 unrelated healthy controls.

DNA sequencing and mutation detection

DNA sequencing was performed by Eurofins MWG GmbH, Martinsried Germany, using the same primers as in PCR. Sequences data were analyzed using the software Chromas Pro Version 1.33. Once identified, all mutations have been confirmed by sequencing on a second amplified fragment from each patient.

All mutations are described as recommended by HGVS (Human Genome Variation Society, http://www.hgvs.org/). Nucleotide numbers are referred to coding DNA sequence (cDNA) derived from genomic CPO sequence (GenBank accession NC_000003.10, GI 89161205).

RESULTS

Direct sequencing of the seven exons and exon-intron boundary regions of the CPO gene from the five unrelated patients revealed four novel mutations (p.Gly242Cys; p.Leu398Pro; p.Ser245Phe; p.Gly188TrpfsX45) and a previously described one (p.Ala203Thr). All were in the heterozygous state. Where appropriate, DNA samples from 50 unrelated healthy controls were analyzed to exclude the possibility these were common polymorphisms.

Biochemical features, clinical symptoms and CPO gene mutations from HCP patients and their relatives are reported in table 1.

The mutation detected in the index HCP 1 is a novel, missense mutation: a single nucleotide substitution, at position 724 of the coding sequence (c.724G>T) in exon 3, causes the replacement of the Glycine at the amino acid position 242 with a Cysteine (p.Gly242Cys). Sequencing of exon 3 of CPO gene in family members of the patient revealed that this mutation was inherited from the maternal grandmother and was present in the mother and sister of index 1. Mother and grandmother had biochemical alterations but only the first one showed poor clinical symptoms (she could remember of recurrent, mild abdominal pain) The sister showed normal values of total urinary and fecal porphyirins with only an inversion of copro III:I ratio in fecal porphyrins (8). She has never suffered from any of the clinical symptoms of HCP. The mutation was absent in 50 control subjects.

In patient HCP 2 a novel missense mutation was found. This is a T>C substitution at position 1193 of the coding sequence (c.1193T>C) in exon 6 which leads to an aminoacid change from Leucine to Proline at position 398 of the amino acid sequence. This genetic variant was presumptively inherited from the father, who showed a typical biochemical pattern of HCP. He had recurrent abdominal pain and neurological problems such as paresthesias of limbs and emotional instability. He admitted his severe clinical symptoms and made contact with a doctor only after he saw the same pathological condition in his son. It was not possible to perform mutation analysis prior to his death. The mutation was absent in 50 control subjects.

In patient HCP 3 a duplication of a T in position 561 of the coding sequence (c.561dupT) in exon 2 was found. This is a novel mutation and resulted in a frameshift that gives rise to a stop codon 45 residues downstream Glycine at position 188 (p.Gly188TrpfsX45). The biochemical analyses of urine and feces were in accordance with a diagnosis of HCP.

The mutation identified in patient HCP 4 was previously described (9). The patient was found to have a G>A substitution in position 607 of the coding sequence (c.607 G>A) of exon 2, which is a missense mutation that results in an Alanine to Threonine change at position 203 of the corresponding protein (p.Ala203Thr). Sequencing of exon 2 of sister and brother of the index revealed that both had the mutation and that their biochemical pattern was characteristic of HCP. Both of them showed poor clinical symptoms: exhaustion and mild abdominal pain.

The fourth novel mutation was found in patient HCP 5, who showed a C>T substitution in position 734 of the coding sequence (c.734 C>T) of exon 3. This missense mutation results in a Serine to Phenylalanine substitution at position 245 of the aminoacid sequence (p.Ser245Phe). The mutation was absent in 50 control subjects. The biochemical analyses of urine and feces revealed patterns characteristic of HCP.

TABLE 1. Biochemical features and CPO gene mutations from HCP patients and their relatives

PATIENTS	AGE / SEX	URINE				FECES			MUTATION	CLINICAL SYMPTOMS
		ALA	PBG	TP	CP	TP	CP	PP		
HCP 1 index	9/M	15.3	17.3	8.18	6.02	7512	7512	0	c.724G>T (p.Gly242Cys)	present
father	42/M	0.69	1.0	0.132	0.093	27	8	11	Wild type	absent
mother	37/F	2.0	0.91	0.240	0.206	1418	1418	0	c.724G>T (p.Gly242Cys)	present (poor)
sister	4/F	0.80	0.16	0.022	0.019	20	14	6	c.724G>T (p.Gly242Cys)	absent
maternal grandmother	57/F	1.9	0.23	0.280	0.193	1449	1449	0	c.724G>T (p.Gly242Cys)	absent
maternal grandfather	62/M	1.3	0.48	0.050	0.038	35	15	20	Wild type	absent
maternal uncle	36/M	0.38	0.47	0.020	0.014	66	32	34	Wild type	absent
maternal aunt	24/F	1.5	0.53	0.012	0.011	17	11	6	Wild type	absent
maternal grandmother's nephew	59/M	1.8	1.9	0.100	0.087	56	14	36	Wild type	absent
maternal grandmother's niece	59/F	0.88	0.68	0.002	0.001	57	15	35	Wild type	absent
HCP 2 index	21/M	3.3	1.9	0.170	0.156	833	806	27	c.1193T>C (p.Leu398Pro)	present
father	68/M	5.8	5.7	5.80	4.64	4395	3736	659	N.P.	present
HCP 3 index	50/F	3.4	2.1	0.280	0.224	2549	2506	43	c.561dupT (p.Gly188TrpfsX45)	present
HCP 4 index	35/F	3.4	1.5	0.320	0.244	1271	1254	17	c.607G>A (p.Ala203Thr)	present
brother	43/M	2.9	1.4	0.200	0.160	1181	945	236	c.607G>A (p.Ala203Thr)	present (poor)
sister	41/F	7.0	3.5	0.530	0.463	4101	4025	50	c.607G>A (p.Ala203Thr)	present (poor)
HCP 5 index	50/F	5.7	3.1	1.06	0.919	1719	1664	55	c.734C>T (p.Ser245Phe)	present
NORMAL VALUES		*≤7.5 mg/24h*	*≤2.5 mg/24h*	*≤ 0.150 mg/24h*	*≤0.120 mg/24h*	*≤200 nmol/g d.w.*	*≤50 nmol/g d.w.*	*≤150 nmol/g d.w.*		

ALA=δ-Aminolevulinic Acid; PBG=Porphobilinogen; TP=Total Porphyrins; CP=Coproporphyrins; PP=Protoporphyrins; N.P.= not performed

DISCUSSION

The genetic characterization of five unrelated Italian coproporphyria patients led us to the identification of four novel mutations of the CPO gene. Three of them are missense mutations and one is an insertion of a single nucleotide. Expression studies were not performed but considering that the described genetic alterations were not found in any of the subjects of our control group and that no other mutation was identified in our HCP cases, it may be concluded that they are involved in causing the disease.

Moreover it has to be considered that, according to the assignment of residues at the active site of CPOX protein (10), subjects HCP1 and HCP5 have mutations (p.Gly242Cys, p.Ser245Phe) at positions very close to Ser244, an aminoacid directly involved in the catalytic mechanism. The missense mutation p.Gly242Cys of HCP 1 replaces a highly conserved aminoacid located in the β4 strand of the CPOX structure (10). It is reported that the high content of Glycine residues of the β-strands (β2, β3, β4, β6, β7) is an important feature to maintain the flatness of the CPOX sheet.

The functional consequence of the HCP 2 mutation (p.Leu398Pro), located in α9 helix (10), is not easy to predict, even though it involves the substitution of a large, hydrophobic aminoacid with a small, polar one. But it is known that the deletion of the region comprising residues 392-418 results in a protein unable to dimerize, and that this novel missense mutation is located between two regions of conserved aminoacids in the primary sequence of the protein.

The mutation identified in HCP 3 represents a significant change in the protein because it leads to a premature stop codon and results in a truncated protein of 233 amino acids. The 221 aminoacids encoded by the deleted fragment include residues from codon 234 to 454 of the wild type protein. Such a large deletion would clearly have major consequences for the catalytic activity of the enzyme.

All the index cases and their relatives with identified mutations had a profile of both fecal and urinary porphyrins characteristic for HCP, with the exception of the HCP 1 young sister, who at age 4 years old did not have biochemical expression of this autosomal dominant acute hepatic porphyria (5).

The variety of the mutations we found furtherunderlines the heterogeneity of the molecular defects of HCP.

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