Hum Genet (1997) 99 : 199–201

ORIGINAL INVESTIGATION

Makoto Daimon · Eishirou Gojyou
Makoto Sugawara · Keiichi Yamatani
Makoto Tominaga · Hideo Sasaki

A novel missense mutation in exon 4 of the human coproporphyrinogen oxidase gene in two patients with hereditary coproporphyria

Received: 23 April 1996 / Revised: 15 July 1996

Abstract Hereditary coproporphyria (HCP) is an autosomal dominant disease characterized by a deficiency of coproporphyrinogen oxidase. To date, four mutations of the gene have been reported. We report here another mutation in two Japanese families with HCP, which was revealed by analysis of polymerase chain reaction (PCR)-amplified DNA fragments of the gene by a direct-sequencing method. A point mutation, G to A, was found in exon 4 of the gene at position 538 of the cDNA from the reported putative translation initiation codon ATG. This mutation results in a glycine to arginine substitution at amino acid 180. Two carriers in the family were successfully diagnosed by detecting the mutation using restriction analysis of the PCR products.

Introduction

Hereditary coproporphyria (HCP) is inherited as an autosomal dominant trait, and is characterized by attacks of abdominal pain, neurological disturbances, and psychiatric symptoms (Kappas et al. 1989). HCP results from a partial deficiency of the sixth enzyme in heme biosynthesis, coproporphyrinogen IX oxidase (CPX) [EC 1.3.3.3.] (Grandchamp and Nordmann 1977; Kappas et al. 1989). The symptoms are generally manifested with rapid onset, and can be precipitated by drugs, alcohol, caloric deprivation, infection, endocrine factors or stress (Kappas et al. 1989). Therefore, early detection of carriers of the defective gene is important in the prevention of attacks, since carriers can be advised to avoid the precipitating factors. The condition of most carriers of the defective gene is clinically latent, and accurate diagnosis of carrier status is not always possible by conventional biochemical methods (Kappas et al. 1989). The CPX enzyme activity in lymphocytes has been used for the clinical diagnosis of the disease, but there is some overlap between the affected and the normal values (Kappas et al. 1989). Hence, identification of the gene abnormality itself may be suitable for a more reliable diagnosis.

Identification of the gene abnormality in HCP families is now feasible since the cDNA and gene of human CPX have been isolated and sequenced (Delfau-Larue et al. 1994; Martasek et al. 1994a; Taketani et al. 1994). To date, four different mutations have been reported (Delfau-Larue et al. 1994; Fujita et al. 1994; Martasek et al. 1994b; Lamoril et al. 1995). Here, we report another mutation of the gene found in two Japanese patients.

Materials and methods

Two Japanese women with HCP (patients 1 and 2), three children of patient 1, and the husband of patient 2 were studied. Patient 1 was 45 years old and patient 2 was 35 years old at the time of diagnosis. Diagnosis of the patients was established on the basis of the clinical symptoms of the disease, increased excretion of δ-aminolevulinic acid and porphobilinogen in urine and coproporphyrinogen III in feces and urine. Other than the patients, no individual in their families has ever shown clinical symptoms corresponding to the disease. These two families are not related, but they live in the same area, Akita prefecture, Japan. One hundred non-porphyria individuals were also examined as normal controls.

Genomic DNA was extracted from peripheral blood leukocytes as described by Sambrook et al. (1989). Single-strand conformation polymorphism (SSCP) analysis was performed on all seven exons of the CPX gene. The nucleotide sequences of the primers used are described together with the sizes of each polymerase chain reaction (PCR)-amplified product (Table 1). Primers E1F and E7R were made to match the reported CPX cDNA sequence in the 5′- and 3′-noncoding regions, respectively. These two primers match exons 1 and 7, respectively. The other primers were made to match the reported CPX genomic sequence in the intron regions (Delfau-Larue et al. 1994). Therefore, this analysis covered all genomic DNA regions corresponding to the coding region of the cDNA. The 5′- and 3′-most positions analyzed in this study were 9

M. Daimon (✉) · K. Yamatani · M. Tominaga · H. Sasaki
The Third Department of Internal Medicine,
Yamagata University School of Medicine, 2-2-2 Iida-Nishi,
Yamagata 990–23, Japan
Tel.: +81-236-28-5316; Fax: +81-236-28-5318

E. Gojyou
Gojyou Clinic, 7-4-17 Ushijima-Higashi, Akita, Japan

M. Sugawara
Sugawara Clinic, 6–60 Higashidouri-Kannonnmae, Akita, Japan

bp upstream from the reported putative translation initiation codon and 104 bp downstream from the termination codon of the cDNA, respectively (Martasek et al. 1994a; Taketani et al. 1994).

The nucleotide sequence of the DNA fragment containing exon 4 of the CPX gene was determined by direct sequencing using a Cycle Sequencing Kit from Pharmacia LKB Biotechnology (Tokyo). The PCR primers were also used as the sequencing primers.

The new mutation abolishes the *Fok*I restriction site. Hence, the PCR product containing the mutation site was amplified with the primers E4F and E4R, and then used for restriction analysis with *Fok*I (Takara, Tokyo, Japan) as reported previously (Daimon et al. 1994). Genomic DNAs from four other individuals of the patients' families as well as from the patients were used for this analysis. Genomic DNAs from 100 control individuals were also analyzed.

The amino acid sequence around the newly discovered mutation site was compared with those of other species, such as mouse (Kohno et al. 1993), soybean (Madsen et al. 1993), yeast (*Saccharomyces cerevisiae*) (Zagorec et al. 1988) and *Escherichia coli* (Troup et al. 1994). The FASTA program was used to maximize similarity. The secondary structures of the proteins were predicted by Chou-Fasman (1978) analysis using Genetyx-Mac (Software Development Co., Tokyo, Japan).

Results and discussion

We used SSCP analysis to screen all seven exons of the CPX gene for mutations. This analysis covered all coding region of the CPX cDNA as described in Materials and methods. The sizes of the PCR-amplified products ranged from 220 to 364 bp in length (Table 1). We analyzed two HCP patients as well as several control individuals. For each PCR product, all samples were run side-by-side to facilitate comparison of the banding patterns. SSCP analysis of the PCR product containing exon 4 of the two patients showed the same banding patterns, which were different from those observed in normal controls (data not shown). As shown in Fig. 1, direct sequencing of this PCR product from patient 1 revealed a single base substitution, G to A, in exon 4 of the CPX gene at position 548 of the cDNA from the reported putative translation initiation codon ATG (Martasek et al. 1994a; Taketani et al. 1994). The sequencing ladder of the sense strand around the mutation in exon 4 showed double bands at the mutation site, indicating that the patient is heterozygous for the normal allele and the mutant allele (Fig. 1a). Direct sequencing of the antisense strand of the PCR product also revealed the mutation. This mutation resulted in a Gly180 to Arg substitution in the abnormal protein (Fig. 1b).

Only four different mutations of the CPX gene responsible for HCP have been reported so far (Delfau-Larue et al. 1994; Fujita et al. 1994; Martasek et al. 1994b; Lamoril et al. 1995). All are single base substitutions, and found in exons 2 (Fujita et al. 1994), 5 (Martasek et al. 1994b) and 6 (Delfau-Larue et al. 1994; Lamoril et al. 1995). Three other point mutations of the gene have also been reported as common genetic polymorphisms. Two of them, A514C and G580A, were found in exon 4, and resulted in the amino acid substitutions Asn172 to His and Val194 to Ile, respectively (Delfau-Larue et al. 1994). The other polymorphism, G690A, in exon 5 was silent. Comparison of the amino acid sequence of human CPX with those of other species reveals that Gly180 is completely conserved among human, mouse, soybean, yeast and *E. coli*, while

Table 1 Primers used for amplifying the coproporphyrinogen oxidase *(CPX)* gene. The first and second primers of each set are presented in the forward and reverse orientations relative to CPX gene transcription, respectively

Amplified region	Primer	Sequence (5′……3′)	PCR product (bp)
Exon 1	E1F	CGGGCGGAGATGTTGCCTAAGAC	364
	E1R	CCCCATCCCATATTGTGAACTAT	
Exon 2	E2F	AAACGGGAAAATAACCATCTTCA	298
	E2R	AAAACTTGTGGGCAAAATAAGGT	
Exon 3	E3F	TGTATCAAGGCATTCAAGGTAAC	263
	E3R	TATTCTTTCTCGCTTTTAGATGT	
Exon 4	E4F	ATAGCCAGTAATGCTGAATCTCA	248
	E4R	ACAGAATGTAATTTTGGGGTCAT	
Exon 5	E5F	TAACCTGAAAGGCTCACATTGAT	352
	E5R	GCTGCTCCACCTCCCCCACTTAG	
Exon 6	E6F	CCCCTTTTAAGTATTTGTTATGG	232
	E6R	GGAATTGGGAGTGTAGGGATAAC	
Exon 7	E7F	TTTTTGCTTTTGTTTTGGACA	220
	E7R	AAGACTAAGGCACGGGTAACT	

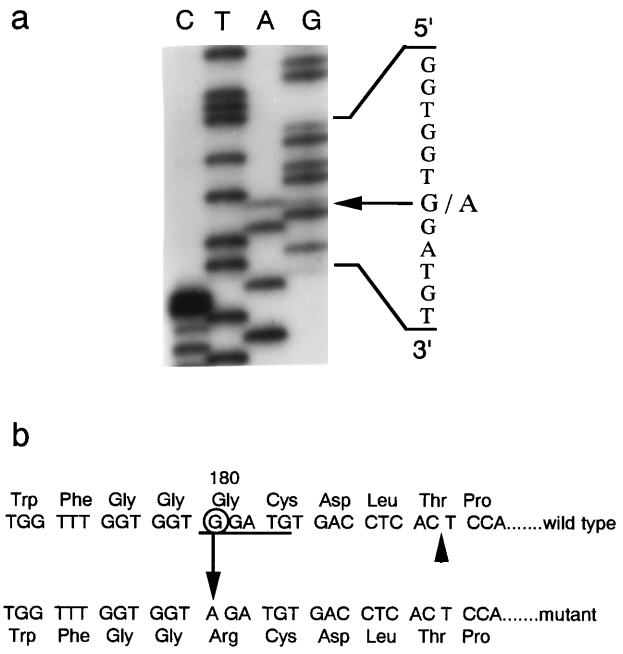

Fig. 1a, b Direct-sequencing analysis of the amplified DNA fragment from an hereditary coproporphyria patient. **a** Nucleotide sequence ladder around the mutation site. The position of the mutation is revealed by double bands on the autoradiogram and indicated by an *arrow* on the right. **b** Deduced amino acid sequence of the wild-type CPX and the predicted result of the amino acid 180 missense mutation. The G to A transition leading to the glycine to arginine substitution is indicated with an *arrow*. The *Fok*I recognition site abolished by the mutation is *underlined*, and the cleavage site of the enzyme is indicated by the *arrowhead*

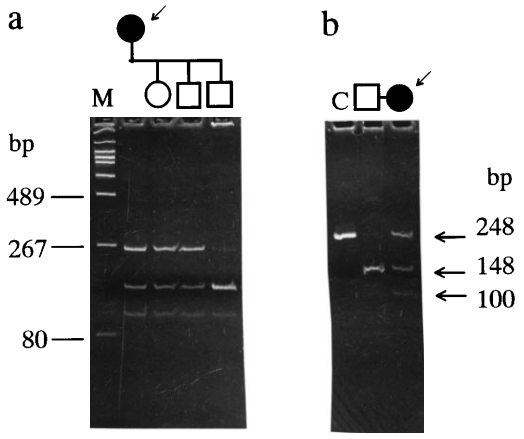

Fig. 2a, b Restriction analysis of polymerase chain reaction (PCR) products. The PCR products containing exon 4 were analyzed as described in Materials and methods. The members of the patients' families examined are indicated at the top of each panel. Patients 1 and 2 are indicated by *closed circles* and *arrows* in panels **a** and **b**, respectively. *Lane C* undigested PCR product of patient 2. *Lane M* DNA molecular weight standard pHY size marker (Toyobo, Tokyo, Japan). The *numbers on the left and right* indicate the sizes of several DNA size markers and fragments observed in this analysis, respectively

those amino acids previously reported as polymorphisms are within nonconserved regions (data not shown). Furthermore, Chou-Fasman (1978) analysis for the secondary structure of the protein predicts that the Gly180 substitution changes the structure at this position from turn to β-sheet (data not shown). Thus, these analyses together suggest that the newly discovered mutation impairs the function of the protein and hence is responsible for the disease.

*Fok*I digestion of the PCR products containing the new mutation site of the two patients and two out of three children of patient 1 produced the 248-bp abnormal fragment, as well as the 148- and 100-bp normal fragments, whereas those of one child of patient 1 and the husband of patient 2 produced normal fragments only (Fig. 2). This analysis clearly indicates that patients 1 and 2 are both heterozygous for the normal allele and the mutant allele, and that the mutation is transmitted from patient 1 to her children. The finding that two unrelated patients from the same area had the same mutation suggests that this mutation may be common, at least in the Akita prefecture, Japan, although more cases have to be examined before making this conclusion.

Genomic regions corresponding to the whole coding region of the cDNA were examined by SSCP analysis, and the PCR product containing exon 4 was the only product showing unique banding patterns. Furthermore, none of 100 normal control individuals (200 alleles) examined had the mutation (data not shown), which indicates that this mutation is not a polymorphism but is unique for the HCP patients studied. Thus, all the observations mentioned above together strongly indicate that the mutation found is the causative abnormality in the patients.

References

Chou PY, Fasman GD (1978) Prediction of the secondary structure of proteins from their amino acid sequence. Adv Enzymol 47: 45–148

Daimon M, Yamatani K, Igarashi M, Fukase N, Morita Y, Ogawa A, Tominaga M, Sasaki H (1994) Acute intermittent porphyria caused by a single base insertion of C in exon 15 of the porphobilinogen deaminase gene that results in a frame shift and premature stopping of translation. Hum Genet 93:533–537

Delfau-Larue MH, Martasek P, Grandchamp B (1994) Coproporphyrinogen oxidase: gene organization and description of a mutation leading to exon 6 skipping. Hum Mol Genet 3:1325–1330

Fujita H, Kondo M, Taketani S, Nomura N, Furuyama K, Akagi R, Nagai T, Terajima M, Galbraith RA, Sassa S (1994) Characterization and expression of cDNA encoding coproporphyrinogen oxidase from a patient with hereditary coproporphyria. Hum Mol Genet 3:1807–1810

Grandchamp B, Nordmann Y (1977) Decreased lymphocyte copro-porphyrinogen III oxidase activity in hereditary coproporphyria. Biochem Biophys Res Commun 74:1089–1095

Kappas A, Sassa S, Galbraith RA, Nordmann Y (1989) The porphyrias. In: Stanbury JB, Wyngaarden JB, Fredrickson DS, Goldstein JL, Brown MS (eds) The metabolic basis of inherited disease, 6th edn. McGraw-Hill, New York, pp 1305-1365

Kohno H, Furukawa T, Yoshinaga T, Tokunaga R, Taketani S (1993) Coproporphyrinogen oxidase: purification, molecular cloning, and induction of mRNA during erythroid differentiation. J Biol Chem 268:21359–21363

Lamoril J, Martasek P, Deybach JC, Da Silva V, Grandchamp B, Nordmann Y (1995) A molecular defect in coproporphyrinogen oxidase gene causing harderoporphyria, a variant form of hereditary coproporphyria. Hum Mol Genet 4:275–278

Madsen O, Sandal L, Sandal NN, Marcker KA (1993) A soybean coproporphyrinogen oxidase gene is highly expressed in root nodules. Plant Mol Biol 23:35–43

Martasek P, Camadro JM, Delfau-Larue MH, Dumas JB, Montagne JJ, de Verneuil H, Labbe P, Grandchamp B (1994a) Molecular cloning, sequencing, and functional expression of a cDNA encoding human coproporphyrinogen oxidase. Proc Natl Acad Sci USA 91:3024–3028

Martasek P, Nordmann Y, Grandchamp B (1994b) Homozygous hereditary coproporphyria caused by an arginine to tryptophan substitution in coproporphyrinogen oxidase and common intragenic polymorphisms. Hum Mol Genet 3:477–480

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning – a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Taketani S, Kohno H, Furukawa T, Yoshinaga T, Tokunaga R (1994) Molecular cloning, sequencing, and expression of cDNA encoding human coproporphyrinogen oxidase. Biochim Biophys Acta 1183:547–549

Troup B, Jahn M, Hungerer C, Jahn D (1994) Isolation of the hemF operon containing the gene for the Escherichia coli aerobic copro-porphyrinogen III oxidase by in vivo complementation of a yeast HEM13 mutant. J Bacteriol 176:673–680

Zagorec M, Buhler JM, Treich I, Keng T, Guarente L, Labbe-Bois R (1988) Isolation, sequence, and regulation by oxygen of the yeast HEM13 gene coding for coproporphyrinogen oxidase. J Biol Chem 263:9718–9724