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A Systematic Analysis of the Mutations of the Uroporphyrinogen III Synthase Gene in Congenital Erythropoietic Porphyria

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Porphyria
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Abstract

Congenital erythropoietic porphyria (CEP) or Günther's disease is an inborn error of heme biosynthesis, transmitted as an autosomal recessive trait and characterized by a profound deficiency of uroporphyrinogen III synthase activity (UROIIIS). The molecular defects observed in CEP are mainly heterogeneous, except for one missense mutation, C73R (Cys to Arg substitution at codon 73) which represents nearly 40% of the disease alleles. A convenient strategy was designed to establish a rapid diagnosis at the genetic level in samples from patients with CEP. As a first step, the most frequent mutation is searched for by restriction analysis from genomic DNA amplified by PCR. Next, the nine coding exons and intron-exon boundaries are sequenced from genomic DNA. As an alternative, the mutation can be determined by sequencing the UROIIIS cDNA of the patient, using the RT-PCR technique on RNAs when a lymphoblastoid cell line can be established. Finally, for each new mutation in UROIIIS coding sequence, the corresponding mutant protein is expressed in *Escherichia coli,* in order to demonstrate the pathological significance of the mutation. This work describes the analysis of UROIIIS gene mutations in 10 new families with CEP and summarizes the data from 20 unrelated families studied in our laboratory. Three new missense mutations of UROIIIS coding sequence (H173Y, Q187P and P248Q) have been observed together with 8 known mutations. The significance of three intronic base changes (476 –31 T→C; 562 –4 A→T; 562 –23 A→G) is discussed. In 6 alleles out of 40 (15%), the mutation remains undetermined.

Introduction

Porphyrias are a group of inherited disorders caused by specific defects along the heme biosynthetic pathway. Congenital erythropoietic porphyria (CEP) or Günther's disease is a rare disease that is inherited as an autosomal recessive trait. CEP is characterized by severe cutaneous photosensitivity, chronic hemolysis and massive porphyrinuria resulting from the accumulation in the bone marrow, peripheral blood and other organs, of large amounts

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of predominantly type I porphyrins, which are not substrates for heme synthesis [1]. A characteristic abnormality of the disease is an 80–98% decrease in the erythrocytic activity of uroporphyrinogen III synthase (UROIIIS), the fourth enzyme of the heme biosynthetic pathway: hydroxymethylbilane hydrolase [cyclizing] (EC.4.2.1.75) [2, 3]. Human UROIIIS enzyme has been purified to homogeneity from erythrocytes as a monomeric protein, with an apparent molecular mass (M_r) of 29,500 [4]. The determination of the nucleotide sequence of the cDNA encoding UROIIIS [5] and the characterization of the UROIIIS gene structure [6] has made it possible to study the molecular lesions responsible for the disease. The gene encoding UROIIIS protein is unique, contains 10 exons and has been assigned to the chromosomal region 10q25.3→q25.6. The size of the coding sequence and the gene are 795 bp and 60 kb, respectively [6, 7].

The mutations causing CEP are mainly heterogeneous [8–16]. Eleven missense mutations are associated with different levels of residual UROIIIS activity: L4F, Y19C, P53L, T62A, A66V, C73R, V99A, A104V, S212P, G225S and T228M. Two point mutations are responsible for a truncated protein: the nonsense Q249X and the frameshift 633insA mutations. Four putative splicing defects have been observed: the deletion of exon 2, 4, or 9, corresponding to the changes (63 +1 G→A), (148 del 98) and (660 +4 del A), respectively, and an 80-bp insertion between the last two exons of UROIIIS gene (660ins80). The mutations are designated according to the nomenclature recommended by Beaudet and Tsui [17]. The missense mutation C73R is the most common, representing at least one third of the mutated alleles studied, while the other mutations are rarely observed in more than one family [10, 14].

In this work, we describe the analysis of UROIIIS gene mutations in 10 new families and summarize the analysis in twenty-one CEP patients, using cDNA and genomic sequencing together with cDNA-directed expression in a prokaryotic system of the corresponding mutant protein.

Materials and Methods

In vitro Amplification of Genomic DNA

High-quality genomic DNA was prepared from whole blood by extraction with phenol and chloroform after overnight digestion with proteinase K. Two hundred to 500 ng of genomic DNA were amplified on a Hybaid Omnigene thermal cycler. The reaction mix contained 50 m*M* KCl, 10 m*M* Tris-HCl pH 9, 0.1% Triton X-100, 0.15 m*M* $MgCl_2$, 10 pmol of each primer, 200 n*M* of the four dNTPs and 1 U *Taq* polymerase (Promega, France) in a final volume of 50 µl. After a first denaturation step at 94 °C during 3 min, 30 cycles consisted in denaturation at 92 °C for 10 s, annealing at 45 °C to 56 °C for 30 s, depending on the Tm of the primers, and elongation at 72 °C for 1 min, followed by a last elongation at 72 °C for 5 min. The sequences of the primers are detailed in table 1. Each sense primer is biotinylated at the 5′ end to allow the purification of single-stranded DNA using its affinity for streptavidin for the subsequent sequencing step.

Restriction Analysis of the C73R Mutation

The fourth coding exon of the UROIIIS gene was amplified from genomic DNA using the primer set 4 (4S, 4AS1). One third of the PCR product was digested with the restriction enzyme *Mae*II, at 65 °C for 3 h and analysed on a 3% agarose gel. The enzyme recognizes a new site when the mutation is present. A single 140-bp undigested fragment was detected in normal samples, a 100-bp fragment in homoallelic samples and both fragments in heteroallelic samples.

Direct Sequencing Using Paramagnetic Beads

A solid-phase sequencing protocol, using single stranded PCR products immobilized on paramagnetic beads, was chosen to obtain unambiguous sequencing results. The biotinylated strand of each PCR product was purified by affinity with streptavidin-coated paramagnetic beads by means of a magnetic separator (Dynabeads M-280 Streptavidin, Biosys, France).

40 µl of the PCR reaction were incubated with the beads, the strands were separated in 0.1 *M* NaOH and the nonbiotinylated strand was removed as recommended by the supplier. The biotinylated strand was used as template for dideoxy chain termination sequencing using a T_7 sequencing kit (Pharmacia, France). The antisense primer was used for both PCR and sequencing with consistent results. An unusually high level of secondary structure was observed when sequencing exon 9 with primer set (9S1, 9AS) and the sequence was determined from the nonbiotinylated strand using a sense primer (9S2). Exon 10 was sequenced with an internal antisense primer (10AS2) in order to read directly the coding sequence of this last exon of the UROIIIS gene. The results of the sequence analysis of the mutant P248Q were ambiguous because of the present band compression. The mutation was confirmed by hydridization of the PCR product with an allele-specific mutated oligonucleotide as described [10].

In vitro Amplification of UROIIIS cDNA

Lymphoblastoid cell lines were successfully established in some patients by Epstein-Barr virus (EBV) transformation of peripheral B lymphocytes at the Généthon laboratory (Evry, France). Total RNAs were prepared by phenol-chloroform-isothiocyanate extraction [18] and reverse-transcribed from oligo(dT) primers, using Moloney murine leukemia virus reverse-transcriptase (RT), according to the manufacturer's instructions (Gibco BRL). A first PCR was performed with the primers US2 and 10AS2, followed by a nested PCR with a biotinylated sense primer, US12B, and the antisense primer US4. PCR conditions were the same as those described for genomic DNA. The resulting PCR product was sequenced directly as a single-stranded product using M280-streptavidin beads and a T_7 sequencing kit for manual sequencing.

Table 1. Oligonucleotide sequences

A. Genomic primers used for amplification and sequencing UROIIIS exons

Coding exon primer set	UROIIIS cDNA sequence amplified		Oligonucleotide primer
2	[-26, 63]	S:	5'-BTTGCTTAGGAAGAGTCT-3'
		AS:	5'-CTGTGGGATAAGGAGTC-3'
3	[64, 147]	S:	5'–BGGTTTTGCAAAACCTCAGA-3'
		AS:	5'-GTCCCTCTCTGGCTTCA-3'
4	[148, 244]	S:	5'-BGTCTTTATTGCTTTTTGG-3'
		AS1:	5'-GGGAGTTTAGGAGATAG-3'
		AS2:	5'-GACCCACCCTCACCTTC-3'
5	[245, 319]	S:	5'-BATAGTTTGCTTTGCTCACA-3'
		AS:	5'-CTGCATTCTTATCAGTAGT-3'
6	[320, 394:	S:	5'-BATACCTGTGTATTTGCACG-3'
		AS:	5'-CAAGAATGCACTGAGGAAA-3'
7	[395, 475]	S:	5'-BCTGAGTCCTAGAAGCAGAG-3'
		AS:	5'-CACCCACTTCTATCACTGC-3
8	[476, 561]	S:	5'-BAGGAGGGTGGTGCTGTATT-3'
		AS:	5'-GGGACAGTGAAACCACATA-3'
9	[562, 660]	S1:	5'-BTTGGTGGCCTGTGGCTTG-3'
		S2:	5'-GGCTTGAGGTCTTGATGCG-3'
		AS:	5'-TAAGGCACCTGCTAGGCCA-3'
10	[661, 1083]	S:	5'-BCGTCACATGAGCAGTAACG-3'
		AS1:	5'-ATACCTGTCTCCTCCCTG-3'
		AS2:	5'-CAGAGCCAGCCCAGCCCA-3'

B. Primers used for cDNA analysis

Primer	Position on UROIIIS cDNA		Oligonucleotide primer
US2	[191, 211]	S:	5'-CCAGAGCAGTGGAAGCAGCAG-3'
US4	[796, 819]	AS:	5'-CAGCGCTAGGTGGCTGACTCA-3'
US12B	[280, 298]	S:	5'-BCAAGTCAGTGTATGTGGTTG-3'
US13E	[474, 492]	S:	5'-AGGGATTGCCATGGAAAGC-3'
248MS	[735, 749]	S:	5'-GCCCCACGCaACAAGC-3'
248MAS	[737, 751]	AS:	5'-GGGCTTGTtGCGTGG-3'

Genomic primers were chosen according to previously published sequences [11, 14]. In 248MS and 248MAS primers used for mutagenesis, the mutated base is indicated by small lettering. B indicates a biotine, S = sense, AS = antisense.

Prokaryotic Expression of UROIIIS Mutant Proteins

The normal and mutant UROIIIS alleles were expressed in *E. coli* using the pKK 223.3 vector (Pharmacia, France) as previously described [10, 13]. The mutations H173Y, Q187P and P248Q are included in a 300-bp *Nco*I fragment of UROIIIS cDNA. Each mutated *Nco*I fragment was cloned as a mutated cassette in the normal pKK UROIIIS plasmid. The mutated cassettes for H173Y and Q187P were obtained from total RNAs by RT-PCR and a nested PCR using the primers US13E and US4. The mutated cassette for P248Q was constructed by mutagenesis using sequential PCR [19]. First, two overlapping mutated PCR fragments were obtained from the normal cDNA using the primer pairs US12B-US248MAS and US248MS-US4 and gel purified with the Wizard PCR purification system (Promega, France). The corresponding PCR products were self annealed and amplified with the external primers US12B and US4. The resulting PCR product was digested with *Nco*I, gel purified and cloned in the normal pKK UROIIIS plasmid. In the three constructs, the sequence of the mutated cassette was checked in both

Table 2. Mutations determined in 20 unrelated families

Family	Sex	Age	Origin	Severity	Genotype	Reference
I	F	17 y	French	severe	C73R/P53L	8
II	F	6 y	Tunisian	severe[1]	C73R/C73R	8
III	M	67 y	French	mild	C73R/	10
IV	F	5 y	Italian	moderate	148del198/L04F	10
V	M		French	mild	C73R/T228M	10
VI	M	26 y	Pakistani	mild	148del198/	10
VII	F	61 y	English	mild	660ins80/	10
VIII	F	22 y	Algerian	moderate	H173Y/H173Y	this study
IX	F	40 y	French	mild	C73R/T228M	this study
X	M	1 d	Belgian	severe	C73R/C73R	12
XI	M	2 y	English	severe	C73R/	this study
XII	M	21 y	French	moderate	C73R/562-23A→G	this study
XIII	M	14 y	African Black	moderate	V99A/633insA	13
XIV	M	50 y	Spanish	moderate	C73R/	this study
XV	F	2 y	French	severe	C73R/Q187P	this study
XVI	M	5 y	Italian	severe	C73R/C73R	this study
XVII	F	1 m	French	severe	C73R/C73R	16
XVIII	M	10 y	Spanish	severe	C73R/P248Q	this study
XIX	F	stillborn	French	severe	C73R/	this study
XX	M	2 m	Spanish	severe	C73R/P248Q	this study
	M	2 y	Spanish	severe	C73R/P248Q	this study

Probands IV, X, XIII have been studied in both our and Dr. Desnick's laboratories [11–14]. Two patients are described for family XX. Age is given at the time of the genetic analysis (y = year, m = month, d = day).

[1] Coexistence of hereditary coproporphyria.

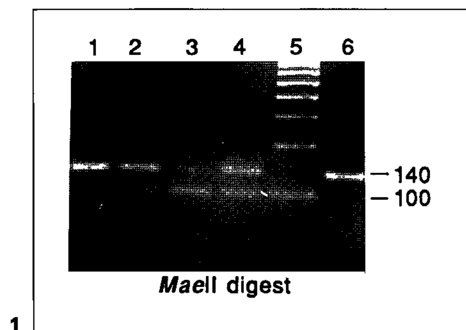
directions to confirm the engineered sequence and the absence of PCR errors. Bacterial growth, IPTG induction, and UROIIIS enzyme assay were performed as described [10, 13].

Results

Mutations in the UROIIIS gene were systematically searched for by cDNA and/or genomic sequencing in 11 new patients with CEP from 10 families (VIII, IX, XI, XII, XIV, XV, XVI, XVIII, XIX, and XX) described in table 2. A brief description of the patients is given in table 2, concerning sex, age at the time of the genetic analysis, geographic origin and disease severity. In mild and moderate forms, the symptoms were mainly cutaneous and often allowed a normal life in adulthood. In severe forms, transfusion dependency was observed due to recurrent anemia. Two infants (X and XVII) died from acute hemolysis at the age of 1 day and 2 months, respectively; a 3rd one was stillborn (XIX). Two patients were born of consanguineous parents: patient II (the parents are first cousins) and patient VIII (multiple consanguinity).

The most common mutation in CEP, C73R, was observed in 9 patients: 8 of them were heteroallelic (patients IX, XI, XII, XIV, XV, XVIII, XIX, and XX) and 1 was homoallelic for the mutation (patient XVI). The restriction patterns of normal (lane 1), homoallelic (lane 3) and heteroallelic (lanes 2, 4) samples are shown in figure 1. One previously reported mutation, T228M, was observed in 1 of the 9 new patients presented in table 2: patient IX was shown to be a compound heterozygote C73R/T228M. The analysis of the patients I-VII, X, XIII, and XVII have been published previously [8, 10, 12, 13, 16].

Three new exonic mutations were identified in four different patients: patient VIII was homoallelic for the mutation H173Y, patients XV was heteroallelic for the mutation Q187P, and patients XVIII and XX were both heteroallelic for the mutation P248Q. The missense mutation H173Y was identified by cDNA sequencing (fig. 2) and confirmed by sequencing the genomic fragment which contains exon 8. The mutation abolished a *Nsi*I restriction site. The homozygosity was not surprising since multiple consanguinity was observed in the family.



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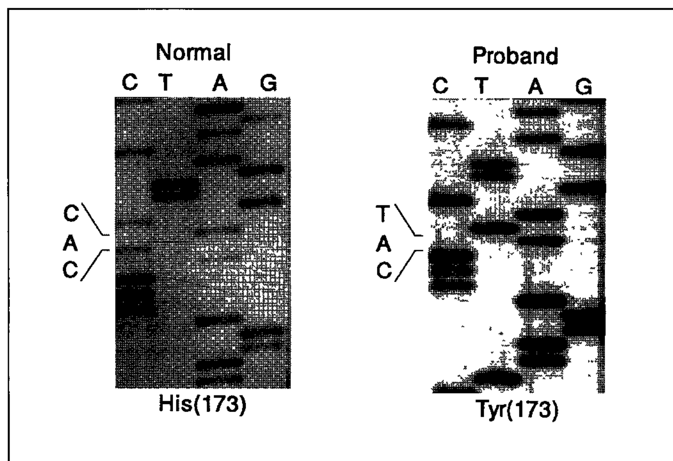

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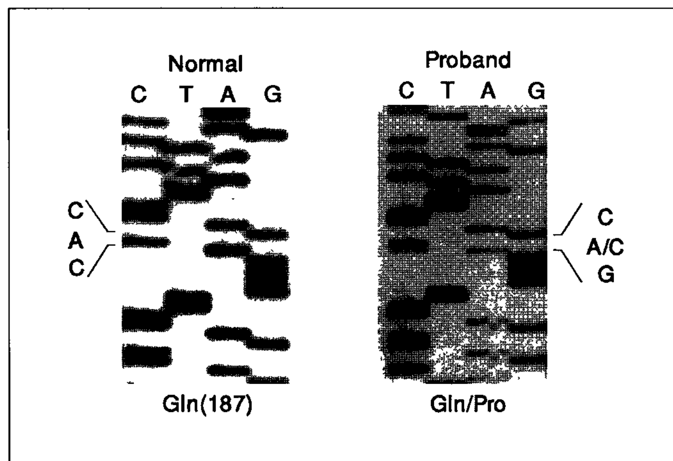

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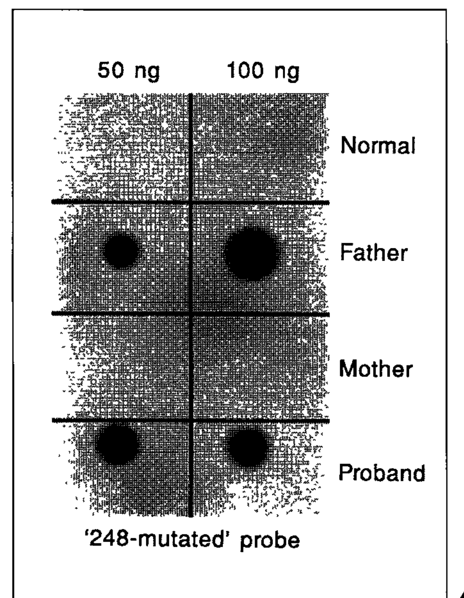

4

Fig. 1. Identification of the C73R mutation by restriction analysis. A 140-bp PCR product containing exon 4 is amplified from genomic DNA and digested with *Mae*II enzyme which recognizes a new site when the mutation is present. A single 140-bp fragment is seen in normal samples (lane 1), a 100-bp fragment in homoallelic samples (lane 3) and both fragments in heteroallelic samples (lanes 2 and 4). A 100-bp ladder and a 140-bp undigested PCR product are loaded in lanes 5 and 6, respectively.

Fig. 2. Identification of the H173Y mutation by direct sequencing. Direct sequencing was performed on RT-PCR products obtained from lymphoblastoid RNAs. The substitution CAC→TAC at codon 173 (H173Y) is present at the homoallelic state in patient VIII.

Fig. 3. Identification of the Q187P mutation by direct sequencing. Direct sequencing was performed on RT-PCR products obtained from lymphoblastoid RNAs. The substitution CAG→CCG (Q187P) is present at the heteroallelic state in patient XV.

Fig. 4. Confirmation of sequencing results by allele specific hybridization. The mutation P248Q was identified by direct sequencing of the last exon of UROIIIS gene, amplified from genomic DNA. Sequencing data were confirmed by hybridization of the PCR product with a 19-mer oligonucleotide containing the substitution. The samples from the proband and his father were both recognized by the mutated oligonucleotide allele while the sample from the mother was not.

The missense mutation Q187P was also identified by cDNA sequencing (fig. 3) and confirmed by sequencing an exon 8 genomic fragment. The C73R mutant allele was transmitted by the mother and the Q187P mutant allele by the father of the proband. In patient XVIII, the mutation P248Q was identified by sequencing the genomic fragment containing exon 10. However, the sequence was ambiguous because of band compressions in the region of the nucleotide change C→A at nucleotide 743. RNAs were not available to analyze the cDNA in this patient. Therefore, the mutation was confirmed by allele specific oligonucleotide hybridization using the genomic fragment containing exon 10 (fig. 4). The same mutation was detected by automatic sequencing in a second family (XX) in two brothers aged 2 months and 2 years, respectively. The C73R mutant allele was transmitted by the mother,

Table 3. Expression of mutated UROIIIS proteins

Specific activity (U/mg of protein)				
Plasmid	Mean	SD	Assays	Residual activity
pKK	0.35	±0.1	5	–
pKK-UROIIIS	143	±35	4	100
pKK-H173Y	1.03	±0.6	5	<1
pKK-Q187R	0.48	±0.2	5	<1
pKK-P248Q	0.58	±0.1	4	<1

Values for specific activity (SA) are the mean of 4–5 experiments. Residual activity was determined by dividing 100 × [SA – SA (pKK)] by [SA (pKKUROIIIS) – SA (pKK)].

the P248Q mutant allele by the father of the proband in both pedigrees (XVIII and XX).

The three new exonic mutations were introduced in the expression vector pKK 223.3 containing the normal UROIIIS cDNA sequence either by cloning the mutated cDNA obtained from RT-PCR products or by site-directed mutagenesis. None of the three mutated constructs expressed any residual UROIIIS activity in *E. coli* lysates (table 3).

In 4 patients (XI, XII, XIV and XIX), heterozygous for the common mutation C73R and in 3 previously described patients (III, VI, VII) a systematic analysis of the 9 exonic fragments of UROIIIS gene was performed. The genomic sequencing showed three intronic nucleotide changes in three of these heterozygous patients (III, VII, XII). In patient III, an A→T substitution is observed at position –4 from the 3′ acceptor site of intron 8: CTCAG is read instead of CACAG (542 –4 A→T). In patients VII and XII, the substitution is located in the region of the branch points of introns 7 and 8, 476 –31 T→C and 562 –23 A→G, respectively. The pathological significance of these intronic base changes is discussed in the next section.

Discussion

Different techniques were combined to analyze the mutations in amplified segments of the UROIIIS gene in CEP patients: restriction analysis of genomic exonic fragments, direct sequencing on genomic DNA or cDNA, allele-specific hybridization. Finally, the exonic mutations were expressed in a prokaryotic system in order to

Table 4. Frequency of the different mutations in CEP

Mutation	Alleles		References
Nonsense and missense mutations			
C73R	30*	(38.5)	8–12, 14, 16
L04F	4	(5.1)	10, 11, 14
T228M	4	(5.1)	10, 11, 14
G225S	3	(3.8)	14
S212P	3**	(3.8)	15
H173Y	2**	(2.6)	this study
P248Q	2	(2.6)	this study
Q249X	2	(2.6)	14, 15
Y19C, P53L, T62A, A66V, V99A, A104V	1	(1.3)	8, 10, 11, 13, 14
Q187P	1	(1.3)	this study
Insertions			
633insA	1	(1.3)	13, 14
660ins80	1	(1.3)	10
Splicing defects			
63 +1 G→A (IVS2^{+1})	2	(2.6)	14
148del98 (V82F)	2	(2.6)	10, 14
660 +4 delA (IVS9$^{\Delta A+4}$)	2**	(2.6)	14
562 –23 A→G	1	(1.3)	this study
Unknown alleles	12	(15.4)	8–16
Total	78	(100)	

* 6 homoallelic patients; ** 1 homoallelic patient. Values in parentheses are percentages.

measure the UROIIIS residual activity of the corresponding mutated proteins.

The mutations determined in 20 unrelated CEP families are presented in table 2 and the relative frequency estimated in table 4. The high frequency of the C73R mutation is obvious with 19 mutant alleles out of 40 (47.5%) in 15 independent families out of 20. As a consequence, in each new CEP patient, the C73R mutation is investigated first using a simple restriction analysis, and the systematic sequencing of the exons of the UROIIIS gene is undertaken afterwards. A lower frequency (26%) of the C73R mutation was described by Xu et al. [14] in patients of various ancestry, as opposed to the Caucasian origin of the patients described in this work. No clear racial or sexual predominance has been observed in CEP. However, the disease is too rare (200 cases reported before 1992 [1] to allow a valuable analysis of the allelic frequency as a function of ethnic origins.

The C73R mutation is usually associated with a severe phenotype at the homoallelic state and the family may ask

		Branch point								3' Acceptor site				
Base location	5'-			-26			-23			-4	-3	-2	-1	- 3'
Consensus sequences		Py	N	Py	Py	Pu	A	Py	$(Py)_{12}$	N	C	A	G	Exon
Nucleotide frequency (%)		80		80	87	75	99	95			65	100	100	
UROIIIS gene sequence	TCTCATGGCAAG	G	A	C	T	G	[A]	T	CTTGTTTTCTGTTCCCC	A	C	A	G	Exon 9
Base change in patient XII							G							
Base change in patient III										T				
Common polymorphism				T										
Base location					-31		-29			-4	-3	-2	-1	
UROIIIS gene sequence	GTGTGCCCAGTG	C	G	G	T	A	[A]	A	GTCTTTCCTGCTTTTGATTGTGC	C	C	A	G	Exon 8
Base change in patient VII					C									

Fig. 5. Consensus sequences for the branch point and the 3′ acceptor sites and location of base changes in UROIIIs introns 7 and 8. The consensus sequences for the branch point and the 3′ acceptor sites are indicated together with the nucleotide frequency of each position. The consensus sequences are aligned with the normal sequences observed in UROIIIS introns 7 and 8. The best guess position for the A of the branch point is −23 in intron 8 and −29 in intron 7. Three individual base changes and a common polymorphism are shown.

for a prenatal diagnosis. In family XVII, a first child was diagnosed as homoallelic for the mutation C73R. On the next pregnancy the amniotic cells and the amniotic fluid were analysed for porphyrin accumulation and UROIIIS genotype at 16 weeks of gestation. The fetus was also homoallelic for the mutation and a large increase in uroporphyrin I was noted. The diagnosis led to the interruption of the pregnancy [16].

The strategy allowed a rapid determination of the sequence of the entire coding region and all intron/exon boundaries of the UROIIIS gene, a 60-kb gene encoding 10 exons [6]. A biotinylated primer was used in each amplification of either genomic DNA or cDNA to separate single strands for direct solid-phase sequencing [20]. This approach has proved to be more efficient than mutation detection methods such as the single strand conformation polymorphism technique, [21] heteroduplex analysis, [22] or the use of denaturing gradient gel electrophoresis [23] since each of them requires one round of mutation detection followed by sequencing. In the future, the use of an automatic sequencer should shorten the analysis and facilitate the comparison of the sequencing patterns of different patients. In the last patients studied (family XX), the mutations were identified by automatic sequencing using the cycle sequencing strategy and dye terminator labeling (ABI 377, Perkin Elmer).

Three new missense mutations were identified, H173Y, Q187P and P248Q, which markedly altered the nature of the encoded amino acid. The resulting mutant protein had no residual activity in the expression system. The H173Y mutation was observed at the homoallelic state in a consanguineous family and was associated with a severe phenotype. The Q187P and P248Q mutations were associated with the C73R mutant allele and resulted also in severe forms with mutilating cutaneous lesions and transfusion dependency. Unexpectedly, the P248Q mutation was detected in two unrelated Spanish families (XVIII and XX) who lived in distant regions, Madrid and Seville, respectively.

Recently, three point mutations responsible for a splicing defect in UROIIIS gene have been described: 63 +1 G→A, 148 del 98 (244 G→T) and 660 +4 del A, resulting in the deletion of exon 2, 4 and 9, respectively [14]. Special attention was given to intron-exon boundaries and three nucleotide changes were observed in three patients heteroallelic for the most common mutation C73R. In patient III, the A→T substitution at nucleotide −4 of the 3′ acceptor site of intron 8 should not influence the splicing since any nucleotide can be present at this position in the consensus sequence (fig. 5) [24]. In patient VII the T→C substitution at nucleotide −31 is located in the best-guess region for the branch point consensus sequence of intron 7 [24]. However, both nucleotides C or T can be

seen at this position, as shown in figure 5. Therefore, these nucleotide changes were considered as rare polymorphisms rather than mutations linked to the disease. Three other polymorphisms were observed in the patients described in this report: one in intron 5, at position +64, and two in intron 8, at positions −26, and +19, respectively. For the 18 alleles tested, in intron 5, the allelic frequency was 0.89 and 0.11 for G and C, respectively. In the same sample, the allelic frequency was 0.72 and 0.28 for C and T, respectively, at both positions in intron 8. In patient XII the A→G substitution at position −23 in intron 8 is the best guess location for the A of the branch point consensus [25], as shown in figure 5. In a number of β-globin thalassemia variants, mutations of the pre-mRNAs generate new splice sites or activate cryptic ones, leading to aberrant splicing patterns [26] and providing a convenient model to study exon and intron sequences required for splicing [27]. Recently, in intron 7 of the IL-3 receptor gene (α-subunit) a 5-bp deletion at the branch point has been demonstrated to cause an aberrant splicing. This nucleotide change was considered as responsible for the mutant phenotype observed in the A/J mouse [28]. However, in patient XII, the immortalization of lymphoblastoid cells failed, blood samples were not suited for RNA preparation and the presence of abnormality spliced transcripts could not be documented.

The mutation was considered not determined in 6 of the 40 alleles described in table 2, from the sequencing of all 9 coding exons of the UROIIIS gene. The overall frequency of unknown alleles in CEP patients is 15% as shown in table 4. The undetected mutations may involve gene deletions, promoter mutations or other splicing defects. Unfortunately, the large size of UROIIIS gene precludes a complete analysis of gene defects as a routine analysis in patients with CEP.

Finally, the mutations of the UROIIIS gene are mainly heterogeneous since most defects appear only in individual families. Only four families were known to be consanguineous among 39 families mentioned in table 4 and 4 different mutations were observed: C73R [8], 660 +4 del A [14], S212P [15], and H173Y (this work). Of note, a second mutant allele (Q249X) was observed in the consanguineous family showing the S212P allele. In six homoallelic patients for the C73R mutation, only 1 was born of consanguineous parents (first cousins). Indeed, the prevalence of the disease is too low (less than 10^{-6}) to analyze the genetic heterogeneity of the disease from the estimation of consanguineous marriages, as previously described [29].

As a conclusion, the identification of UROIIIS gene mutations in CEP allows a precise characterization of the disease, the detection of heterozygotes for accurate genetic counselling and improved prenatal diagnosis in severe cases.

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