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Demystification of Chester Porphyria: A Nonsense Mutation in the *Porphobilinogen Deaminase* Gene

*P. POBLETE-GUTIÉRREZ[1], *T. WIEDERHOLT[2, 3], A. MARTINEZ-MIR[4], H. F. MERK[2], J. M. CONNOR[5], A. M. CHRISTIANO[4, 6], J. FRANK[1,2,3]

[1]Department of Dermatology, University Hospital Maastricht, The Netherlands, [2]Department of Dermatology and Allergology and [3]Porphyria Center, University Hospital of the RWTH Aachen, Germany, [4]Department of Dermatology, Columbia University, New York, NY, USA, [5]Institute of Medical Genetics, Yorkhill Hospitals, Glasgow, Scotland, [6]Department of Genetics and Development, Columbia University, New York, NY, USA

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Summary

The porphyrias arise from predominantly inherited catalytic deficiencies of specific enzymes in heme biosynthesis. All genes encoding these enzymes have been cloned and several mutations underlying the different types of porphyrias have been reported. Traditionally, the diagnosis of porphyria is made on the basis of clinical symptoms, characteristic biochemical findings, and specific enzyme assays. In some cases however, these diagnostic tools reveal overlapping findings, indicating the existence of dual porphyrias with two enzymes of heme biosynthesis being deficient simultaneously. Recently, it was reported that the so-called Chester porphyria shows features of both variegate porphyria and acute intermittent porphyria. Linkage analysis revealed a novel chromosomal locus on chromosome 11 for the underlying genetic defect in this disease, suggesting that a gene that does not encode one of the enzymes of heme biosynthesis might be involved in the pathogenesis of the porphyrias. After excluding candidate genes within the linkage interval, we identified a nonsense mutation in the *porphobilinogen deaminase* gene on chromosome 11q23.3, which harbors the mutations causing acute intermittent porphyria, as the underlying genetic defect in Chester porphyria. However, we could not detect a mutation in the coding or the promotor region of the *protoporphyrinogen oxidase* gene that is mutated in variegate porphyria. Our results indicate that Chester porphyria is neither a dual porphyria, nor a separate type of porphyria, but rather a variant of acute intermittent porphyria. Further, our findings largely exclude the possibility that a hitherto unknown gene is involved in the pathogenesis of the porphyrias.

Key words
Porphyria • Chester porphyria • Porphobilinogen deaminase • Protoporphyrinogen oxidase

Introduction

The porphyrias are a group of rare metabolic diseases which arise from an either inherited or acquired dysfunction of one of the eight enzymes involved in heme biosynthesis. Disturbance of any of these enzymes leads

* Both authors contributed equally to this manuscript.

to an accumulation of the preceding metabolites with a measurable increase of porphyrins and/or porphyrin precursors (Kauppinen 2005).

According to the major site of expression of the particular enzymatic deficiency, the porphyrias can be classified into erythropoietic and hepatic forms. From a clinical point of view however, it seems more suitable to classify the porphyrias in acute and non-acute forms, thereby primarily considering whether the patient does or does not experience acute neurovisceral attacks (Lip *et al.* 1993). These acute porphyric attacks are life-threatening events and can present with such heterogeneous symptoms as colicky abdominal pain, vomiting, electrolyte dysregulation (hyponatremia), constipation, tachycardia, hypertension, muscle pain and weakness, seizures, paresis of the upper and lower extremities, paralysis, and a variety of often confusing neurological and psychiatric symptoms (Crimlisk 1997).

The acute porphyrias are comprised of acute intermittent porphyria (AIP), variegate porphyria (VP), hereditary coproporphyria (HCP), and δ-aminolevulinic acid dehydratase (ALA-D) deficiency porphyria (Frank and Christiano 1998). Whereas AIP is the most frequent type of porphyria worldwide, in some countries other acute forms might be predominant, as is the case for VP in South Africa and in Chile (Groenewald *et al.* 1998, Frank *et al.* 2001). Beside the aforementioned neurovisceral attacks, individuals suffering from VP or HCP can present with cutaneous symptoms, including increased photosensitivity, abnormal skin fragility, blisters on sun-exposed areas, erosions, chronic scarring, and post-imflammatory hyperpigmentation. By contrast, however, AIP and ALA-D deficiency porphyria do not present with cutaneous symptoms (Kauppinen 2005).

Diagnose in an acute porphyria traditionally involves three major steps. First, the medical history including the frequency of acute attacks, the family history, and a thorough physical examination. Second, biochemical measurement of porphyrins and porphyrin precursors in urine and feces. Third, determination of specific enzymatic activities in lymphocytes or fibroblasts which is usually only possible in specialized laboratories. But even when using all these diagnostic tools, the results might still be confusing (Da Silva *et al.* 1995, Grandchamp *et al.* 1996). This is particularly true for the so-called dual porphyrias.

In families with dual porphyrias, simultaneous defeciency of two enzymes along the heme biosynthetic pathway are detected. Single cases have been described but, overall, seem to be extremely rare (Watson *et al.* 1975, Levine *et al.* 1978, Day *et al.* 1982, Doss 1988, Doss 1989a, Doss 1989b, Sturrock *et al.* 1989, Nordmann *et al.* 1990, Gregor *et al.* 1994, Sieg *et al.* 1995, Freesemann *et al.* 1997, Doss *et al.* 2002).

In 1985, a new form of porphyria with autosomal dominant inheritance, designated Chester porphyria (CP), was described in a large kindred from the United Kingdom. Affected family members presented with acute porphyric attacks and revealed biochemical characteristics of both VP and AIP, with some patients showing intermediate patterns. Measurement of enzymatic acitivities demonstrated reduced activity of porphobilinogen deaminase (PBGD) and protoporphyrinogen oxidase (PPOX) in affected individuals (McColl *et al.* 1985). In 1992, the results of a genome wide linkage analysis performed in the CP family suggested that a novel gene residing on chromosome 11q might be involved in the pathogenesis of this subtype of dual porphyria (Norton *et al.* 1993). Interestingly, this locus did not contain any of the genes encoding enzymes catalyzing major steps in heme biosynthesis.

In an effort to define the molecular basis of this novel porphyria we cloned and screened two candidate genes, *SDHD* and *FDX1*, on chromosome 11q, which we considered good candidates for CP. However, direct sequencing analysis of these genes did not reveal any mutation underlying CP.

Although the original linkage report from Norton and coworkers excluded the *PBGD* and *PPOX* gene as candidates (Norton *et al.* 1993), we nevertheless sequenced these genes because the biochemical and enzymatic data indicated deficiencies of the encoded enzymes. Sequencing analysis of the coding regions of the *PPOX* gene and its promoter region revealed no mutations. In exon 9 of the *PBGD* gene, however, we detected a nonsense mutation, designated R149X, in all affected individuals studied.

Although proposed by previous biochemical and enzymatic studies, we herein excluded on the basis of molecular genetic analyses the existence of a novel type of autosomal dominant porphyria resulting in a dual enzyme deficiency.

Methods

Samples and DNA extraction

A detailed pedigree of the extensive CP family was previously published elsewhere (Norton *et al.* 1993).

Here, we studied DNA samples of ten individuals from this family. Five of them had been classified as affected and the other five as unaffected, based on the broad clinical, biochemical, and enzymatic studies previously performed (McColl *et al.* 1985, Qadiri *et al.* 1986). Additionally, 100 healthy, unrelated Caucasian control individuals were included in this study. All individuals provided informed consent for inclusion in the study according to guidelines set forth by the local institutional review board. Genomic DNA was isolated according to standard techniques (Sambrook et al. 1991).

PCR amplification and mutation screening

A mutation detection strategy for the *PBGD* and *PPOX* gene was developed based on PCR amplification of all *PBGD* and *PPOX* exons and their adjacent splice sites using PCR primers and conditions that were either published recently as is the case for *PPOX* [6] or specifically designed for this study using the primer design program Oligo 4 (Molecular Biology Insights, Inc., Cascade, CO 80809-1333) as is the case for *PBGD*. For amplification of exon 9 of the *PBGD* gene in this study, the following primers were used:

PBGD exon 9F: 5'-GGAAAGACAGACTCAGGCAG-3'
PBGD exon 9R: 5'-GAAAGGAGATGCAGATGAGC-3'.

PCR was carried out according to the following program: initial denaturation at 95 °C for 5 minutes, followed by 35 cycles of denaturation at 95 °C for 45 seconds, annealing at primer specific temperatures as outlined in Table 1 for 1 minute, and extension at 72 °C for 1 minute and 15 seconds, followed by a final extension at 72 °C for 10 minutes, in a Biometra® TGradient thermal cycler (Whatman Biometra®, Göttingen, Germany). Each amplification reaction contained ~100 ng of genomic DNA, 50 ng/µl of each forward and reverse primer and 35 µl of Platinum Taq® PCR Super Mix (Invitrogen® Life Technologies, Karlsruhe, Germany), in a total volume of 38 µl.

PCR products were purified in a first step, using the High Pure PCR product purification kit (Roche, Basel, Switzerland). In a second step, PCR fragments were purified on Edge Centriflex columns (Edge BioSystems, Gaithersburg, MD) and sequenced directly with POP-6 polymer using an ABI Prism 310 Genetic Analyzer from Applied Biosystems Inc. (Applied Biosystems, Foster City, CA).

Screening of the SDHD and FDX1 gene as candidates

Upon screening the OMIM genome database, we detected several genes in the vicinity of the Chester porphyria locus on chromosome 11q, two of which, *SDHD* and *FDX1*, we considered as good candidates. From both genes human cDNA sequences were available (accession numbers NM_003002 and XM_016467, respectively) and, subsequently, we screened available genomic databases, cloned these genes *in silico* and established their exon-intron-boundaries using methods previously described in detail (Frank et al. 2001). Primer pairs to amplify coding regions and adjacent splice sites of both genes were generated using the primer design program Oligo 4 (Molecular Biology Insights, Inc., Cascade, CO 80809-1333). DNA from affected and unaffected individuals of the CP family was PCR amplified and screened for mutations as aforementioned for the *PBGD* and *PPOX* gene.

Results

Exclusion of the SDHD and FDX1 gene as candidates

Both the *SDHD* and *FDX1* gene consist of 4 exons, respectively. Automated sequencing analysis of all exons and adjacent splice sites in both genes did not reveal any mutation in the ten individuals from the CP family (data not shown).

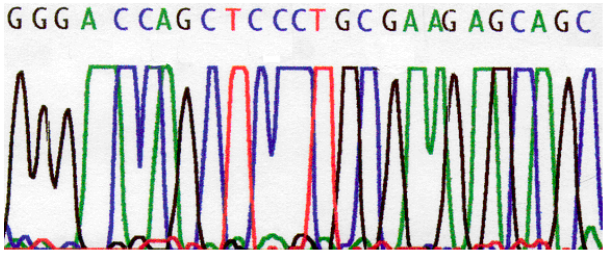
Mutation screening in the PPOX gene

In all members of the CP family, automated sequencing of the entire coding regions, adjacent splice sites and the promoter region of the *PPOX* gene did not reveal any sequence varaiation suggestive of a mutation (data not shown).

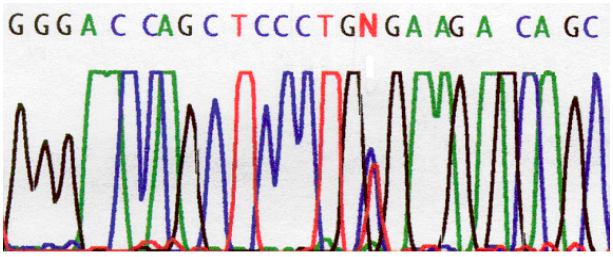
Mutation detection and verification in the PBGD gene

Amplification of exon 9 of the *PBGD* gene resulted in a PCR product 209 bp in size, containing 63 bp of intron 8, 76 bp of exon 9, and 70 bp of intron 9. Automated sequence analysis of this PCR product revealed a sequence variation in four of the five CP family members previously classified as being affected. The mutation consisted of a C-to-T transition at nucleotide position 445 of the *PBGD* cDNA, numbered according to GenBank accession number NM_000190, counting the first base of the initiating methionine as number 1 (Figure 1). This base substitution leads to a nonsense mutation, consisting of an amino acid conversion from arginine to a premature termination codon at position 149 in the deduced amino acid sequence, designated R149X.

Since the mutation did not introduce a restriction site for a restriction endonuclease we used automated sequencing of newly synthesized PCR products to verify the sequence variation detected in exon 9 of the *PBGD* gene. Hereby, we confirmed the absence of this nucleotide deviation in 200 chromosomes from unaffected, unrelated Caucasian control individuals and excluded it as a common polymorphism (data not shown).



exon 9
wild-type allele



exon 9
R149X mutant allele

Fig. 1. Results of mutation analysis in the Chester porphyria family. Nonsense mutation R149X in exon 9 of the PBGD gene, consisting of a heterozygous C-to-T transition (lower panel), indicated by an arrow.

Discussion

In this study we sought to define the molecular basis of CP by screening two putative candidate genes, *SDHD* and *FDX1*, as well as the *PPOX* and *PBGD* genes for mutations underlying this unique disorder.

The first report of the CP family was published in 1985. McColl and coworkers described a large family residing in Chester, England, that revealed typical acute porphyric attacks and the biochemical features of both VP and AIP, with some family members even demonstrating overlapping values of porphyrins and porphyrin precursors in the urine and feces. Interestingly, confirmation of these biochemical data by enzyme assays performed on peripheral blood mononuclear cells showed decreased activities of PBGD in some affected individuals while other family members also revealed decreased activities of PPOX. Thus, the authors concluded that affected family members were suffering from a novel type of one of the so called dual porphyrias in which the simultaneous dysfunction of two enzymes of heme biosynthesis is proposed (McColl *et al.* 1985).

In general, the occurrence of dual porphyrias seems to be extremely rare and only occasionally single cases with no obvious Mendelian transmission in affected individuals and families have been reported to date (Watson *et al.* 1975, Levine *et al.* 1978, Day *et al.* 1982, Doss 1988, Doss 1989a, Doss 1989b, Sturrock *et al.* 1989, Nordmann *et al.* 1990, Gregor *et al.* 1994, Sieg *et al.* 1995, Freesemann *et al.* 1997, Doss *et al.* 2002). The majority of theses cases comprise a combination of porphyria cutanea tarda (PCT) with one of the dominantly inherited acute porphyrias (AIP, VP, or HCP) [Watson *et al.* 1975, Levine *et al.* 1978, Day *et al.* 1982, Doss 1988, Doss 1989a, Doss 1989b, Sturrock *et al.* 1989, Sieg *et al.* 1995, Freesemann *et al.* 1997, Doss *et al.* 2002). PCT is the most frequent type of porphyria worldwide and, further, represents the only porphyria variant that is not exclusively hereditary since a sporadic occurrence (type I or acquired PCT) as well as an autosomal dominant transmission of the disease (type II or inherited PCT) have been reported, with some authors even proposing the existence of a third subtype of PCT (type III PCT) (Elder 1998). In two case reports, a simultaneous occurrence of the acute porphyria HCP with either AIP or the autosomal recessive disorder congenital erythropoietic porphyria (CEP) have been described (Nordmann *et al.* 1990, Gregor *et al.* 1994).

In all aforementioned reports, the diagnosis of a dual porphyria was established on the basis of clinical symptoms, biochemical measurement of porphyrins and/or porphyrin precursors, and enzymatic assays (Watson *et al.* 1975, Levine *et al.* 1978, Day *et al.* 1982, Doss 1988, Doss 1989a, Doss 1989b, Sturrock *et al.* 1989, Nordmann *et al.* 1990, Gregor *et al.* 1994, Sieg *et al.* 1995, Freesemann *et al.* 1997, Doss *et al.* 2002). Surprisingly, however, in none of these patients and families the diagnosis of a double porphyria could be confirmed on the molecular genetic level by demonstrating the simultaneous occurrence of disease causing mutations in those individuals obviously affected by a combined deficiency of two enzymes of heme biosynthesis. In the majority of these cases this might possibly be due to the fact that the frequency of type I PCT is much higher than that of type II PCT. Taking this into consideration, it is understand-

able that there has been no report about a patient with mutations in two genes encoding enzymes of heme biosynthesis thus far.

Since the co-existence of both VP and AIP had not been previously reported it was tempting for McColl and colleagues to propose a new type of porphyria at that time (McColl *et al.* 1985). Consistent with that notion, Norton and coworkers performed a genome wide linkage analysis in search of a candidate locus for a putative gene harboring mutations contributing to the unique biochemical and enzymatic expression pattern observed in affected individuals from the CP family, in which the disease segregated in an autosomal dominant fashion. The results of their approach indicated linkage of CP to a locus on chromosome 11q23.1 within an interval of approximately 1 Mb, thereby excluding both the *PPOX* and the *PBGD* gene as candidates since they reside on chromosomes 1q22-23 and 11q23.3, respectively. Although the *PBGD* gene on 11q23.3 was in close proximity to the candidate locus, the authors excluded this gene as a candidate on the basis of several recombination events encountered in members of the CP family (Norton *et al.* 1993). Thus, their results indeed indicated the existence of a hitherto unknown gene defect causing CP.

Searching genomic databases, we detected two genes, *FDX1* and *SDHD*, residing on chromosome 11q22 and 11q23.1, respectively, which we considered good candidates for CP. Thus, we subsequently cloned these genes *in silico*, established their exon-intron-boundaries using methods previously described in detail (Frank *et al.* 2001), and screened both genes for disease causing mutations by automated sequencing (data not shown).

The *FDX1* gene (OMIM 103260) encodes an iron-sulfur protein, ferredoxin, also referred to as adrenodoxin. Ferredoxin serves as an electron transport intermediate for mitochondrial cytochrome P450. Electrons are transferred from NADPH through a flavin-containing protein (ferredoxin oxidoreductase) and ferredoxin to the terminal cytochrome P450 for oxidation/reduction reactions (Jefcoate *et al.* 1986). The gene contains 4 exons and 3 introns with a 540 bp coding region (Morel *et al.* 1988) and the results of our screening efforts excluded *FDX1* as the disease-causing gene.

The *SDHD* gene (OMIM 602690) encodes a protein that belongs to the mitochondrial succinate dehydrogenase complex II (succinate-ubiquinone oxidoreductase) which represents an important enzyme complex in both the tricarboxylic acid cycle and the aerobic respiratory chains of mitochondria in eukaryotic cells and prokaryotic organisms (Hirawake *et al.* 1997). The gene contains 4 exons and 3 introns and was also excluded as the gene harboring mutations causing CP by automated sequencing of its entire coding region. While this work was in progress, different authors reported mutations in the *SDHD* gene underlying familial paragangliomas of the central nervous system (OMIM 168000) (Baysal *et al.* 2000) and familial pheochromocytoma (OMIM 171300) (Astuti *et al.* 2001), thereby making it an even more unlikely candidate in a disorder of heme biosynthesis.

Although the original linkage report from Norton and coworkers actually excluded both the *PBGD* and *PPO* gene (Norton *et al.* 1993), we nevertheless decided to screen both genes by automated sequencing because the biochemical and enzymatic data published by McColl and colleagues indicated deficiencies of both PPOX and PBGD (McColl *et al.* 1985).

In accordance with the report of Norton and colleagues, sequencing analysis of the coding regions of the *PPOX* gene and its promoter region revealed no mutation.

Surprisingly, however, in exon 9 of the *PBGD* gene we detected a sequence variation in four of the five individuals classified as being affected by the disease. The mutation consisted of a C-to-T transition in exon 9 of the *PBGD* gene, resulting in the change of an arginine residue to a premature termination codon, designated R149X. This mutation most likely will lead to disturbance of protein function by nonsense mediated messenger RNA decay of the mutated transcript (Hentze and Kulozik 1999). R149X was the only sequence variant detected in the CP family and was not found in 200 chromosomes from healthy unrelated Caucasian control individuals.

Mutation R149X has already been previously described by Kauppinen and colleagues in the Finnish population (Kauppinen et al. 1995). The sequence variation occurs at a putatively hypermutable CpG dinucleotide leading to the substitution of arginine (**C**GA) by a premature termination codon (**T**GA). CpG dinucleotides were shown to represent hotspots for mutations in vertebrate genomes (Cooper and Krawczak 1990), and 35 % of all single basepair substitutions causing human genetic diseases were found to occur within CpG dinucleotide motifs (Copper and Youssoufian 1988). In further support of the notion that codon 149 apparently represents a hotspot for the occurrence of mutations within the *PBGD* gene two other sequence

variations at the same amino acid position have been previously reported, designated R149Q and R149L, respectively (Delfau *et al.* 1991, Gu *et al.* 1994).

Of note, only four of the five individuals from the original CP family who were classified as suffering from the disease on the basis of previous biochemical and enzymatic studies indeed carried the disease causing mutation R149X, thereby possibly suggesting that the original classification into affected and non-affected individuals in the publications from McColl, Qadiri, Norton, and colleagues might have already been somewhat imprecise [McColl *et al.* 1985, Qadiri *et al.* 1986, Norton *et al.* 1993). This could also explain why the linkage data from Norton et al. erroneously indicated a novel chromosomal locus for CP and why the *PBGD* gene locus was excluded on the basis of recombination events in certain family members (Norton *et al.* 1993), if these family members revealing the crucial recombination events were perhaps wrongly classified prior to linkage.

Our data demonstrate that the individuals from the CP family studied herein do not suffer from a novel type of porphyria, but rather from a variant of AIP. It still remains elusive why some family members revealed the characteristic porphyrin excretion patterns of VP and reduced enzymatic activities of PPOX, likewise indicative of VP.

Interestingly, however, other authors have previously pointed out that biochemical analyses and even the measurement of enzymatic activities in fibroblasts or lymphocytes are somewhat imprecise, since a certain overlap between the values measured in patients, clinically unaffected mutation carriers (so called "silent" carriers), and normal control individuals can be found. Thus, the results of biochemical and even enzymatic studies in the porphyrias are not always conclusive, sometimes making an accurate diagnosis of the respective type of porphyria impossible (Da Silva *et al.* 1995, Grandchamp *et al.* 1996). In support of this notion, several authors have previously reported that a coexistent decrease in PBGD activity can be frequently detected in patients suffering from VP who usually exhibit a catalytic deficiency of PPOX solely (Meissner *et al.* 1986, Doss *et al.* 1996, Weinlich *et al.* 2001). Still, in none of these individuals the existence of a dual porphyria could be confirmed on the genetic level. Even in a family who revealed a concomitant decrease in PBGD activity being as high as 50 % of the normal range no underlying mutation in the *PBGD* gene was detected and, furthermore, this catalytic deficiency apparently had no clinical consequences (Weinlich *et al.* 2001). In light of these reports, it seems admissible to speculate that the accompanying decrease in PPOX activity observed in some members the CP family can most likely be attributed to a phenomenon secondary to the disease causing genetic defect in the *PBGD* gene and, thus, has no consequences on the clinical course of the disease.

The results of our studies in the CP family indicate that CP is neither a dual porphyria, nor a separate type of porphyria but rather a variant of AIP. Our data also largely exclude the possibility that a hitherto unknown gene is involved in the pathogenesis of the porphyrias. We further suggest that, whenever possible, all so-called double porphyrias should be confirmed on the genetic level first to prove the hypothesis of their existence.

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Reprint requests

Jorge Frank, Afdeling Dermatologie, Academisch Ziekenhuis Maastricht, P. Debyelaan 25, Postbus 5800, 6202 AZ Maastricht, The Netherlands. Phone: 0031-43-3875292. Fax: 0031-43-3877293. E-mail: jfra@sder.azm.nl