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# Hemochromatosis Gene Sequence Deviations in German Patients with Porphyria Cutanea Tarda

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## Summary

Patients with porphyria cutanea tarda (PCT) reveal a susceptibility to reversible inactivation of hepatic uroporphyrinogen decarboxylase, which might be triggered by alcohol, hepatitis C virus infection, and iron overload. Inherited factors that may predispose to clinically overt PCT also include sequence deviations in the *HFE* gene that is mutated in classical hemochromatosis. Here, we studied the prevalence of both common and rare hemochromatosis gene variations in 51 PCT patients and 54 healthy controls of German origin. The frequency of the common *HFE* gene mutation C282Y was 15.7 % in PCT patients and 2.8 % in healthy control individuals ( $P < 0.001$ ). By contrast, the frequencies of the common H63D mutation did not differ, and the allele frequencies of the less frequently observed sequence deviations as substitution S65C in the *HFE* gene and mutation Y250X in the *TFR2* gene underlying hemochromatosis type 3 (*HFE3*) were  $< 0.02$  both in PCT patients and controls. Our results comprise the first molecular studies of both common and rare hemochromatosis gene variants in German PCT patients, indicating a significant role of the C282Y mutation in the pathogenesis of PCT.

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## Key words

Porphyria cutanea tarda • Iron overload • Hemochromatosis • *HFE1* gene • *TFR2* gene • Transferrin receptor

## Introduction

The porphyrias are clinically and genetically heterogeneous metabolic diseases arising from predominantly inherited catalytic deficiencies of specific enzymes involved in heme biosynthesis. Porphyria cutanea tarda (PCT) (OMIM 176100), the most frequent

type of porphyria worldwide, results from a decreased catalytic activity of uroporphyrinogen decarboxylase (UROD), the fifth enzyme in heme biosynthesis (Bickers *et al.* 2003).

The disorder represents the only type of porphyria that is not exclusively inherited monogenetically and at least two different types can be

distinguished: acquired/sporadic (type I) PCT, in which the enzymatic deficiency is limited to the liver and inherited/familial (type II) PCT, which is inherited as an autosomal dominant trait with a decrease of enzymatic activity in all tissues (De Verneuil *et al.* 1978, Grossman *et al.* 1979). Currently, the ratio between type I and type II PCT is estimated to be approximately 3:1 to 4:1, although recent data suggest that, in some countries, the frequency of type II PCT might be much higher than previously thought (Bickers *et al.* 2003, De Verneuil *et al.* 1978, Poblete-Gutierrez *et al.* 2004).

The diagnosis of PCT is made on the basis of cutaneous manifestations, a characteristic urinary porphyrin excretion profile, and, in some laboratories, by measuring UROD activities in red blood cells. The skin findings include increased photosensitivity due to photosensitization by porphyrins and skin fragility as well as blistering, erosions, crusts, and miliae on the sun-exposed areas of the body (Fig. 1). Additionally, hyperpigmentation, hypertrichosis, sclerodermoid plaques, and scarring alopecia can be observed. Biochemically, an increased excretion of uroporphyrin (type I isomers > type III isomers), 7-carboxyl porphyrins (type III isomers > type I isomers), and coproporphyrin in the urine can be found. Enzymatically, UROD activity is decreased by approximately 50 % in red blood cells of individuals suffering from type II PCT (Bickers *et al.* 2003, De Verneuil *et al.* 1978, Grossman *et al.* 1979).

A wide range of triggering factors has been reported to precipitate the clinical manifestation of PCT, among them alcohol, estrogens, polychlorinated hydrocarbons, hemodialysis in patients with renal failure, viral infections such as hepatitis C and HIV, hepatic iron, and the inheritance of specific mutations in the *HFE* gene underlying classic hemochromatosis. Interestingly, homozygosity for the *HFE* gene mutation C282Y was found to be associated with an earlier onset of cutaneous lesions in both sporadic and familial PCT, the effect being more marked in familial PCT (Brady *et al.* 2000).

Histopathologically, 60 - 70 % of those individuals affected by PCT will develop hepatic siderosis (Uys and Eales 1963, Elder and Worwood 1998). Clinical and experimental studies suggest that UROD is reversibly inhibited by high iron concentrations (Sampietro *et al.* 1999). This is consistent with the observation that phlebotomy can lead to clinical remission and reversal of the metabolic defect (Lundvall and Weinfeld 1968, Sampietro *et al.* 1998). Several groups have studied the common *HFE* gene sequence deviations C282Y and H63D, providing evidence for increased frequencies of these molecular alterations in PCT patients from distinct ethnic origin (Hift *et al.* 1997, Roberts *et al.* 1997a, b, Santos *et al.* 1997, Stuart *et al.* 1998, Bonkovsky *et al.* 1998, Bulaj *et al.* 2000, Tannapfel *et al.* 2001, Furuyama *et al.* 1999).



**Fig. 1.** Blisters and erosions on the fingers and the back of both hands in an individual suffering from overt PCT.

Recently though, another mutation in the *HFE* gene, designated S65C, was shown to be associated with a mild form of iron overload (Mura *et al.* 1999), as indicated by significantly higher mean serum transferrin saturation in S65C heterozygotes (Beutler *et al.* 1999, 2000). In addition, a further novel mutation, designated Y250X, in the *TFR2* gene, encoding the transferrin receptor 2, was detected in a subgroup of hemochromatosis patients suffering from hemochromatosis type 3 (Camaschella *et al.* 2000).

To date, the occurrence of mutation S65C has only been reported in two PCT patients (Barton *et al.* 1999, von Ahsen *et al.* 2001), whereas the frequency of mutation Y250X in the *TFR2* gene has not been defined in PCT. Therefore, we herein sought to delineate the incidence of common and rare hemochromatosis mutations in PCT patients from Germany.

## Methods

### Patients

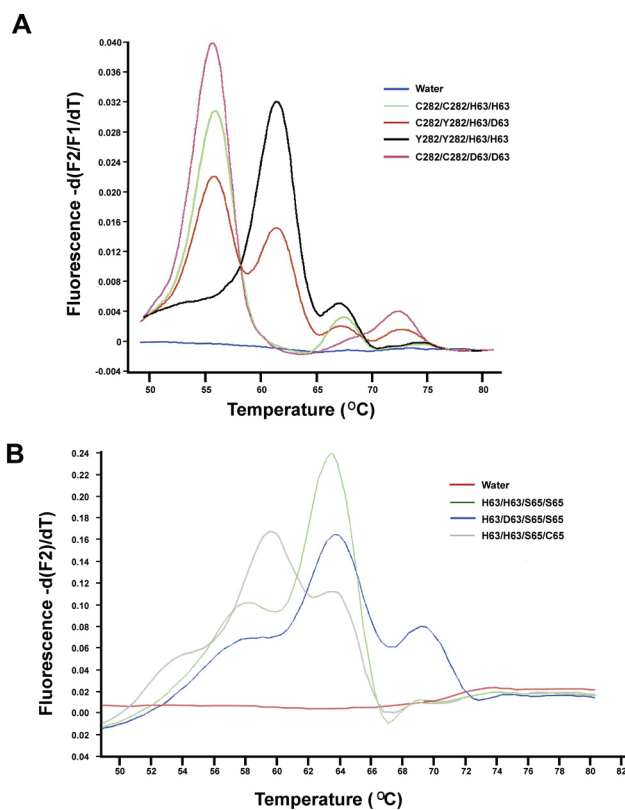
Diagnosis of PCT was established on the basis of cutaneous photosensitivity and typical skin symptoms on the sun exposed areas like hands, forearms and face in combination with a characteristic porphyrin excretion profile in the urine consisting of increased values for urinary uroporphyrin (ten times higher than the upper normal range, type I isomers > type III isomers), 7-carboxyl porphyrins (type III isomers > type I isomers), and coproporphyrin. Urine levels of the porphyrin precursors  $\delta$ -aminolevulinic acid and porphobilinogen were within normal ranges. Hemochromatosis genotypes were analyzed in 51 German PCT patients diagnosed and registered in the Porphyrin Center Aachen, Germany, the majority of them residing in the federal state of North-Rhine-Westphalia. For control purposes, we analyzed 54 healthy individuals without porphyria residing in the same geographical region for the occurrence of these mutations. Blood samples were collected in tubes containing ethylenediamine tetraacetic acid (EDTA). All individuals provided informed consent for inclusion in the investigation.

### Genotyping

Genomic DNA was isolated from EDTA-anticoagulated whole blood using a standard technique (QIAamp DNA Blood Kit, Qiagen, Hilden, Germany). For detection of the C282Y and H63D polymorphisms in the *HFE* gene, we employed LightCycler technology (Roche, Mannheim, Germany) essentially as described previously (Mangasser-Stephan *et al.* 1999, Tag *et al.* 2001), resulting in the genotype specific melting curves (Fig. 2A).

Likewise, the S65C mutation was detected by melting point analysis with fluorescent hybridization probes using the *HFE* H63D+S65C ToolSet for LightCycler (Genes-4U, Neftenbach, Switzerland). The assay allowed simultaneous detection of mutations H63D and S65C in the *HFE* gene by their typical melting temperatures ( $T_m$ ) (Bollhalder *et al.* 1999) (Fig. 2B).

For genotyping of mutation Y250X mutation in the *TFR2* gene, we employed direct sequencing. Primers for PCR amplification were 5'-CCC TAC AGC GCC ATC GGC-3' and 5'-GGC CCA CTG GAT CCA CGC-3' (Bulaj *et al.* 2000). For cycle sequencing, DNA (50 ng) was amplified together with 4  $\mu$ l Terminator Ready Reaction Mix (Applied Biosystems, Weiterstadt,



**Fig. 2.** Molecular analysis of the H63D, C282Y, and S65C *HFE* gene polymorphisms. (A) The derivative melting curves of the following genotypes are shown: C282/C282/H63/H63 (green), C282/Y282/H63/D63 (red), Y282/Y282/H63/H63 (black), C282/C282/D63/D63 (pink). (B) The derivative melting curves of the following genotypes are shown: H63/H63/S65/S65 (dark green), H63/D63/S65/S65 (blue), H63/H63/S65/C65 (grey). Negative controls (water) without DNA were run in parallel. The values for the respective melting temperatures varied for  $\pm 2.0$  °C between different experiments.

Germany) and 0.5 mmol primer in a total reaction volume of 20 µl for 25 cycles (96 °C/20 sec, 50 °C/20 sec, 60 °C/4 min). Products were precipitated, dissolved in 24 µl Template Suppression Reagent (Applied Biosystems), and denatured at 95 °C for 2 min. Capillary electrophoresis and data analysis were performed on the ABI PRISM 310 automated sequencer (Applied Biosystems).

### Statistics

As tests for association, we compared the distribution of alleles and genotypes in contingency tables by Pearson's goodness-of-fit  $\chi^2$  test and Armitage's trend test, respectively, using software developed by T. Wienker and T. Strom (<http://ihg.gsf.de/>). Consistency of genotype frequencies with Hardy-Weinberg equilibrium was confirmed using an exact test.

**Table 1.** Allele and genotype frequencies of common *HFE* gene mutations in 51 PCT patients and 54 controls

Mutation <sup>a</sup>		PCT Number (frequency)	Controls Number (frequency)	Tests for association Contingency table statistics
H63D	C282Y			
<i>Alleles</i>	187 C	80 (0.78)	88 (0.81)	P = 0.581
	187 G	22 (0.22)	20 (0.19)	
	845 G	86 (0.84)	105 (0.97)	
	845 A	16 (0.16)	3 (0.03)	
<i>Genotypes <sup>b</sup></i>	187 CC	20 (0.39)	34 (0.63)	OR = 6.5, 95 % CI = 1.8 - 23.1, P = 0.001
	187 CC	7 (0.14)	2 (0.04)	
	187 CC	4 (0.08)	0 (0.00)	
	187 CG	17 (0.33)	15 (0.28)	
	187 CG	1 (0.02)	1 (0.02)	
	187 GG	2 (0.04)	2 (0.04)	
	187 CC	31 (0.61)	36 (0.67)	
	187 CG	18 (0.35)	16 (0.30)	
	187 GG	2 (0.04)	2 (0.04)	
	845 GG	39 (0.76)	51 (0.94)	
	845 GA	8 (0.16)	3 (0.06)	
	845 AA	4 (0.08)	0 (0.00)	
				P = 0.578
				OR = 11.7, P = 0.005 (Armitage's trend test)

<sup>a</sup> cDNA nucleotide variant c.187C→G corresponds to amino acid substitution H63D and c.845G→A corresponds to C282Y.

<sup>b</sup> Genotype combinations not listed were not observed.

## Results

### PCT patients

Of the 51 PCT patients studied, 8 (15.7 %) were heterozygous for mutation C282Y in the *HFE* gene and 4 (7.8 %) were homozygous for this mutation (Table 1). Eighteen patients (35.3 %) were heterozygous for the H63D mutation and 2 (3.9 %) were homozygous carriers of this mutation. One patient was a compound heterozygote (C282Y/H63D).

A single PCT patient carried mutation S65C in

the heterozygous state, but neither mutation C282Y nor H63D. Automated sequencing revealed that none of the PCT patients carried mutation Y250X in the *TFR2* gene.

### Control individuals

Of the 54 healthy control individuals, 3 (5.6 %) were heterozygous for mutation C282Y, 16 (29.6 %) were heterozygous for H63D (1 compound heterozygote), and 2 (3.7 %) were homozygous for the latter mutation. One of the controls was shown to be heterozygous for mutation S65C in the *HFE* gene, and another control

carried mutation Y250X in the *TFR2* gene in the heterozygous state.

#### Statistical analysis

All genotypes were in Hardy-Weinberg equilibrium, except for the C282Y mutation in PCT patients ( $P < 0.05$ ).

Accordingly, C282Y allele frequencies were significantly higher in PCT patients compared to controls (OR 6.5, 95 % CI 1.8 - 23.1,  $P < 0.01$ , Table 1). Homozygous C282Y genotypes increased the PCT risk significantly (OR 5.2, 95 % CI 1.3 - 19.8,  $P < 0.05$ ). As determined by Armitage's trend test, the common odds ratio of the C282Y mutation was 11.7 ( $P < 0.01$ ).

Allele and genotype frequencies of the H63D, S65C, or Y250X mutations did not differ significantly between PCT patients and control individual.

## Discussion

The aim of this study was to assess the frequency of common and rare genetic alterations in the *HFE* and *TFR2* genes in association with PCT in German patients suffering from this disorder rather than evaluating the relative importance of these factors in disease pathogenesis or determining disease-specific mechanisms contributing to overt disease. Therefore, we studied the occurrence of specific sequence deviations that have all been previously reported to be associated with hemochromatosis and disturbance of iron metabolism, as PCT is an iron-dependent disease and up to 65 % of patients with PCT show an increase in total body iron stores as well as hepatic iron concentrations (Lundvall *et al.* 1970, Edwards *et al.* 1989, Fargion *et al.* 1996).

In several previous reports the association of common *HFE* gene mutations with PCT has been studied, indicating that major differences exist between allele frequencies and distributions of these sequence variants when comparing patients from different geographical background within Europe. Whereas C282Y was detected with relatively high frequencies in PCT patients from Germany, France, Hungary, Southern Italy, the Netherlands, Spain, and the United Kingdom (Roberts *et al.* 1997a, Santos *et al.* 1997, Tannapfel *et al.* 2001, Stölzel *et al.* 2003, Feder *et al.* 1996, D'Amato *et al.* 1998, Enriquez de Salamanca *et al.* 1999, Nagy *et al.* 2004, Chiaverini *et al.* 2003), a single investigation from Northern Italy revealed that the occurrence rate of this

mutation showed no differences when comparing patients suffering from PCT and healthy control individuals, whereas the frequency of H63D was significantly increased (Sampietro *et al.* 1998). Taking these data into consideration, particularly the previous studies performed on German PCT patients (Tannapfel *et al.* 2001, Stölzel *et al.* 2003), the high frequency of the C282Y *HFE* gene alleles observed in our and previous investigations in German PCT patients strongly suggest that this mutation is associated with the manifestation of overt disease in PCT, thus emphasizing that liver iron content is an important pathogenic factor in this disease. Apparently, the penetrance of mutation C282Y is much stronger than that of H63D, as reflected by the fact that individuals carrying genotype C282Y/C282Y are at much higher risk for iron overload than those with genotypes C282Y/H63D or H63D/H63D (Egger *et al.* 2002, The UK Haemochromatosis Consortium 1997, Moirand *et al.* 1999).

Beside the avoidance of well known triggering factors such as alcohol and estrogens, two major therapeutic regimens for PCT have been described: phlebotomy and low-dose chloroquine therapy. Phlebotomy leads to resolution of skin fragility and blistering within 2 - 4 months. However, normalization of urinary porphyrin concentrations usually takes longer (about 12 months). Chloroquine is thought to work by accelerating the secretion of porphyrins and may also inhibit porphyrin synthesis. The standard therapy consists of 125 mg chloroquine twice weekly, and complete remission can be expected within 6 - 9 months. Chloroquine and phlebotomy can also be used in combination in order to induce remission faster (Bickers *et al.* 2003, Lecha *et al.* 2003).

Of note, however, a recent report indicates that the genetic background of PCT patients with regard to the presence of the common *HFE* gene mutations C282Y and H63D might play a critical role in the outcome of chloroquine treatment. Whereas heterozygosity for mutation C282Y and compound heterozygosity for C282Y and H63D did not compromise the therapeutic response to chloroquine, PCT patients homozygous for C282Y seemed to retain high serum ferritin and transferrin saturation and, most importantly, failed to respond to chloroquine therapy (Stölzel *et al.* 2003).

In contrast to the two common *HFE* gene mutations C282Y and H63D, very little is known to date about the association of other mutations causing hemochromatosis and PCT. Our data indicate that the



overall prevalence rate of mutation S65C in the *HFE* gene within the German PCT population studied herein is 2.0 %, consistent with the results of previous studies performed in Denmark (Simonsen *et al.* 1999), France (Chiaverini *et al.* 2003), and the United States (Mura *et al.* 1999, Beutler *et al.* 1999), in which the prevalence ranged from 1.5 to 2.5 %. These numbers indicate that this sequence deviation does not play a crucial role in the pathogenesis of PCT, a notion that is further supported by a similar study on 47 PCT patients from Bulgaria who likewise did not display an increased frequency of mutation S65C when compared to healthy control individuals (Ivanova *et al.* 1999).

In a recent study, Lamoril and colleagues sought to evaluate a possible association between type I PCT and five different single nucleotide polymorphisms (SNPs) in the human transferrin receptor (TFRC) gene. Independent of *HFE* gene mutations C282Y, H63D, and S65C they reported an association between the IVS4+198T-allele in the TFRC gene and sporadic PCT, suggesting that type I PCT has to be considered as a multi-factorial (complex) disease, in which not only alterations in the *HFE* gene might confer susceptibility to iron overload but also other gene variants known to influence intracellular iron metabolism (Lamoril *et al.* 2002).

Based on these observations we searched for a putative association between PCT and a mutation in the *TFR2* gene, encoding the transferrin receptor 2, that has

been recently described in patients with hemochromatosis type 3 (Camaschella *et al.* 2000). This sequence variation occurs in exon 6 of *TFR2* and consists of a C-to-G transversion at nucleotide position 750 of the *TFR2* cDNA, and results in the substitution of a tyrosine residue by a premature termination codon (Y250X). *TFR2* binds transferrin, plays a critical role in cellular uptake of transferrin-bound iron in the liver, and is not down-regulated in the setting of iron overload (Kawabata *et al.* 1999, Fleming *et al.* 2000). The results of our molecular genetic studies revealed that none of the German PCT patients studied herein carried mutation Y250X in the *TFR2* gene, making an association between PCT and alterations in the transferrin receptor 2 unlikely.

In conclusion, our study in a subset of German patients suffering from PCT confirms the association between the common *HFE* mutation C282Y and PCT, and does not support a link between this disorder of heme biosynthesis and the rare *HFE* gene mutation S65C or mutation Y250X in the *TFR2* gene. The complex pathomechanisms affecting the activity of UROD and causing overt disease still remain to be defined in more detail in future studies, particularly the putative contribution of as yet unknown mutations in the *HFE* gene or in other genes involved in iron metabolism, which might promote accumulation of iron and toxic iron species, thereby contributing to inactivation of UROD.

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