SHORT REPORT

His1069Gln and six novel Wilson disease mutations: analysis of relevance for early diagnosis and phenotype

Duc Ha-Hao¹, Harald Hefter¹, Wolfgang Stremmel^{1,2}, Carlos Castañeda-Guillot³, Ana Hernández Hernández³, Diane W Cox⁴ and Georg Auburger¹

¹Department of Neurology, University Hospital, Düsseldorf

²Department of Gastroenterology, Ruprecht Karls University, Heidelberg, Germany

³Department of Pediatric Gastroenterology, Instituto Nacional de Gastroenterología, Havana, Cuba

⁴Research Institute, The Hospital for Sick Children, Toronto, and Department of Medical Genetics, University of Alberta, Edmonton, Canada

In the present study we examined 33 German and 10 Cuban unrelated Wilson disease (WND) index patients and their relatives. The common His1069Gln mutation accounted for 42% of all WND chromosomes in the German series and the haplotype C was found to be highly predictive for this mutation. Six WND gene mutations have not been described previously and involved a splice site at intron 18 (3903 + del1G), a termination codon in the copper-binding region of exon 2 (Cys271X), and missense mutations in transmembrane region 2 (Gly710Ala), in transmembrane region 3 (Tyr741Cys), in the DKTGT motif (Thr1031Ile) and in the ATP loop region (Gly1176Arg). In 15 German WND index patients and three sibs both WND mutations could be determined and a genotype–phenotype correlation was attempted. Patients homozygous for the His1069Gln mutation showed almost the complete range of clinical presentations, and thus in our study this mutation is not associated with a late, neurological presentation.

Keywords: Wilson disease; mutation analysis; haplotypes; single strand conformation polymorphism (SSCP)

Introduction

Copper is an essential trace element in procaryotic and eukaryotic cells where many enzymes such as superoxide dismutase, cytochrome C oxidase, dopamine- β hydroxylase require its specific oxidative capacity to

perform their normal biological roles. However, accumulation of copper can induce the production of highly reactive free radicals with concomitant damage to plasma membranes, peroxisomes, mitochondria, microtubules, enzymes and DNA. Two human genetic disorders, autosomal recessive Wilson disease (WND)¹ and X-lined recessive Menkes disease (MD),² illustrate the systemic effects of copper imbalance.

Wilson disease manifests usually early with chronic liver disease or later with neuropsychiatric impairment, and sometimes with additional kidney malfunction and

Correspondence: G Auburger, Room 2A35, Building 49, NHGR, NIH, Bethesda, MD, USA. Tel: 301 402 2471; Fax: 301 402 2170; E-mail: auburger@nih.gov

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effects on blood, bones, heart, and connective tissue. The prevalence of WND is estimated worldwide between 1/35 000 and 1/100 000 live births.³ The pathological features result from the toxic accumulation of copper mainly in the liver, brain and kidneys, due to a failure of copper secretion from the liver to the bile, and a defective incorporation of copper into ceruloplasmin. Sudden death from haemolytic crisis or liver failure, or lifelong neurological disability can occur when the disease is identified too late for the appropriate removal of copper by chelating agents such as D-penicillamine and trien or by competitive inhibition through oral zinc.⁴

The identification of the WND gene, *ATP7B*,^{5,6} a P-type copper-transporting ATPase with a predicted length of 1465 amino-acids,⁷ made the comparison of different point mutations with differing clinical presentations possible.^{5, 8–14} *ATP7B* consists of 21 exons which span a genomic region of about 80 kb.^{5–8, 15}

In an effort to increase the number of molecularly characterised patients and to aid in genotype–phenotype correlation analysis we endeavoured to characterise WND patients from ethnically different populations and performed molecular analyses in 33 German and 10 Cuban WND index patients and relatives. For haplotype analysis the microsatellites D13S314, D13S301 and D13S316 were chosen, which span the *ATP7B* region on chromosome 13q14.3 and have been used previously by Thomas *et al.*¹⁶ SSCP analysis and subsequent PCR-sequencing were performed to complete the molecular characterisation.

Patients and Methods

Patients

Molecular analysis was performed on 22 German index WND patients with cooperative relatives (43 parents, 10 grandparents, 2 spouses, 3 offsprings, 27 siblings), on 11 German index WND patients without cooperative relatives, and on 10 Cuban index WND patients (with 20 parents and 12 siblings).

Index patients were characterised by H Heftner and W Stremmel at the Department of Neurology and Gastroenterology of the University Hospital, Düsseldorf, and by C Castañedo-Guillot at the Department of Pediatric Gastroenterology of the Instituto Nacional de Gastroenterología, Havana. The diagnosis of Wilson disease was based on low ceruloplasmin and copper serum concentrations, increased urinary copper excretion, and a high liver copper concentration in each patient. Some

details on the clinical investigations have been described elsewhere.^{17–19} Whole venous blood was taken into EDTA and DNA-extracted by a salt-precipitation method (Nucleon II kit, Scotlab®).

Haplotype Analysis

The three microsatellite markers D13S314, D13S301, and D13S316 flanking the WND locus, which have been shown to correlate well with different mutations by Thomas *et al.*,¹⁶ were genotyped in index patients and available relatives and the segregation in haplotypes was established. Absolute allele sizes were determined by comparison with the known sequence of *ATP7B* exon 10 PCR product.

Detection of Mutations

PCR was performed in 40 cycles, each consisting of 30 s denaturation at 94°C, 30 s annealing at appropriate temperature, and 5 s extension at 72°C. The reaction mixture contained 50 ng genomic DNA as template, 25 pmol of each primer, 150 µM of each dNTP, PCR buffer (20 mM Tris-HCl/pH 8.4, 50 mM KCl, 1.5 mM MgCl₂, and 5% formamide), 1.5 units Taq polymerase (Gibco BRL) in a total volume of 50 µl. Primers for the amplification of the exons were taken from Thomas *et al.*¹¹ and Petrukhin *et al.*¹² for the SSCP analysis of exon 2 and 14 additional sets of primers were designed (5' to 3' orientation):

For	exon	2a	gtttcaagggttaaaaaat ⁸	and
			ggcacatatttcacagtgg	
for	exon	2b	ggccaccagcacagtc	and
			ctgggcaggcaaggac	
for	exon	2c	gaggccagcattgcaga	and
			cacgactccttgacgttt	
for	exon	2d	agctccattgaaggcaaggtc	and
			caatatcaattggtcccaggc	
for	exon	2e	cctgggaccaattgatattgag	and
			gaagggtcatatactttacttgg	
for	exon	2f	gcccaagtaaagtatgaccc	and
			ttcaatggaatggacacagg	
for	exon	2g	gtgtccattccattgaaggc	and
			agggtctacctataccatcc ⁸	
for	exon	14	ttggcagataaagactgtg	and
			gtccacgtacctctttacag.	

The PCR products were then screened for mutations by single strand conformation polymorphism (SSCP) analysis.²⁰ The PCR band were visualised by silver staining.²¹ 5 µl of each PCR product exhibiting a SSCP shift were enzymatically pretreated with exonuclease I (10 units) and shrimp alkaline phosphatase (2 units) for 15 min at 37°C, followed by an inactivation of these enzymes for 15 min at 80°C. The PCR samples were then ready for direct sequencing by the dideoxy chain-termination method with ³⁵S-dATP (5 µCi) (USB, Amersham LIFE SCIENCE) in both directions using the PCR primers again.

Results

Haplotype Analysis

Twenty-four different haplotypes inherited on WND chromosomes were observed in the 22 German index patients with cooperative relatives where phase could be established, and an additional 11 haplotypes in the 10 Cuban index patients. The nomenclature of the haplotypes was as defined by Thomas *et al*,¹⁶ and new haplotypes in our study were named 'O' to 'U'.

One haplotype occurred in 36% of the German series, and was identical with the group C haplotype described

by Thomas *et al*¹⁶ (see Table 1). In four cases asymptomatic sibs showed the same haplotypes as the index patients. In two of these individuals a low level of serum ceruloplasmin was confirmed on preliminary clinical analysis consistent with the subclinical presence of Wilson disease.

The haplotype R was observed in three of the ten Cuban index patients, but also on one normal chromosome. The haplotype U was found in two Cuban patients and one German patient, and was not detectable on 82 normal chromosomes (see Table 1). Only C and U haplotypes were shared among German and Cuban patients, reflecting the importance of *de novo* mutations and ethnic differences.

Mutation Screening

The nomenclature of the mutations identified in this study is defined in Genbank for the mRNA sequence (Acc. No: U03464) and protein sequence (Acc. No.: 1947035), or Swiss-Protein (Acc. No: P35670).

Nine mutations in *ATP7B* were observed, six of which were novel and three of which had been previously described (His1069Gln and 340delC, Tanzi *et al*,⁵ 2531delA, Figus *et al*¹⁰). The novel WND mutations include one splice site alteration 3903 + del1G, which deletes the highly conserved guanin-nucleotide at

Table 1 Haplotypes associated with WND loci identified in German and Cuban WND patients. Only those haplotypes and mutations which occurred in more than one individual were reported

Haplotypes	Markers <i>D13S</i>		German alleles		Cuban alleles	
	314-301-316	Mutations	WD	normal	WD	normal
C	10-4-6	His1069Gln	16	0	1	1
	10-5-6	His1069Gln	1	1	–	–
E	11-4-6	His1069Gln	–	0	1	–
O	8-3-2	3400delC	–	0	1	0
	8-3-3	3400delC	1	0	–	0
P	9-5-2	–	3	0	–	0
Q	9-7-2	–	2	1	–	0
R	11.5-4-6	–	–	0	3	1
S	11.5-4-7	His1069Gln	1	1	–	1
T	11.5-5-7	His1069Gln	2	3	–	0
U	11.5-6-6	–	1	0	2	0
–	7-8-6	2534delA	1	0	–	0
	8-4-4	Gly710Ala	1	0	–	0
	8-5-7	Thr1031Ile	1	0	–	0
	10-6-8	Gly1176Arg	1	0	–	0
	11.5-6-7	Cys271X	1	0	–	0
Sum	16		32	6	9	3
Total			44 ^a	61 ^b	20 ^c	20 ^d

Only 22 German index patients^a had cooperative relatives^b with 43 parents (43 normal haplotypes), 10 grandparents (14 normal haplotypes), and 2 spouses (4 normal haplotypes).

For the 10 Cuban index patients^c all 20 parents^d were available.

Figure 1 Identification of the C271X mutation in exon 2 sequenced with the downstream primer 2e (see Patients and Methods). 1 N 2 indicates as WD9-1 (= 1), normal individual (= N), WD9-2 patient (= 2), respectively. The arrows point to the mutated bases.

position one in the 5' splice site of intron 18, one nonsense mutation Cys271X leading to a premature termination of translation in exon 2 in two sibs (see Figure 1), and missense mutations Tyr741Cys, Thr1031Ile, Gly1176Arg and Gly710Ala (also mutated as Gly710Ser, Waldenström *et al*¹¹). In the German series the His1069Gln (previously His1070Gln) mutation predominated (28 of 66 WND chromosomes, ie about 42%). This common mutation is easily detectable as a shift in SSCP analysis (see Figure 2). The mutations His1069Gln and 3400delC (previously 3403delC) were

also observed among the 20 Cuban WND chromosomes (see Table 1 and 2), although in the case of 3400delC it was a modified haplotype.

From the German series of 33 index patients we could identify the mutations of both alleles in 15 index patients, of one allele in 12 index patients (7 of whom were heterozygous for the His1069Gln mutation); both mutations of 6 index patients are still unknown. In the Cuban series of 10 index patients we could verify the mutations of one allele in only 3 patients; the others were not detectable.

Eight changes in the known sequence of *ATP7B* were identified, which do not modify the amino acid sequence of the gene product or are conservative changes in non-vital residues (see Table 2). These sequence variants were considered as DNA polymorphisms, some were also reported in other studies.^{8,10}

Haplotype-mutation Correlation

Seven out of the nine mutations detected could be correlated to certain haplotypes (see Table 1). The two remaining mutations were found in single WND patients without cooperative relatives, in which haplotypes could not be established. In the German series the haplotype C was highly predictive for WND

Figure 2 SSCP analysis of His1069Gln mutation in exon 14 of the *ATP7B* gene. Probands 1–4 are a family; probands 5–8 are other unrelated WND patients. The homozygous index patient is indicated by a black pedigree symbol, his heterozygous parents are shaded. In addition to the normal SSCP band (N), a lower band (WND) shows the mobility shift representing the His1069Gln mutation.

Table 2 Detected mutations and polymorphisms in *ATP7B* of 33 German and 10 Cuban index patients

Basepair	Codon	Mutation	Effect	Exon	German	Cuban
C810A	TGC → TGC	Cys271X	Ter	2	1	0
G1216T	GCT → TCT	Ala406Ser ¹	P	2	nc	
C1366G	CTG → GTG	Leu456Val ¹	P	3	nc	
G2129C	GGT → GTC	Gly710Ala	MS	8	2	0
C2199A	ATC → ATA	Ile733Ile	P	8	nc	
A2222G	TAT → TGT	Tyr741Cys	MS	8	1	0
G2256A	AAG → AAA	Lys755Lys	P	8	nc	
T2846G	GTT → GGT	Val949Gly	P	12	nc	
G2855A	AGA → AAA	Arg952Lys ¹	P	12	nc	
G3009A	GCG → GCA	Ala1002Ala ¹	P	13	nc	
A3091T	ACC → TCC	Thr1031Ile	MS	14	1	0
C3208A	CAC → CAA	His1069Gln ¹	MS	14	32	2
C3429T	GCC → GTC	Val1140Ala ¹	P	16	nc	
G3526A	GGA → AGA	Gly1176Arg	MS	16	1	0
A2531del	AAG → AxA	2531delA ³	Del	14	1	0
C3400del	CCC → xCC	3400delC ¹	Del	15	2	1
3903+1Gdel	AGA+GT → AGA+xT	3903+del1G	Sp	18–19	1	0

¹Mutation previously described⁶; ²Mutation previously described¹²; ³Mutation previously described¹⁴.

Del: deletion, MS: missense mutation, nc: not characterised, P: polymorphism, Sp: splice-site mutation, Ter: termination.

chromosomes and for the His1069Gln mutation. In the Cuban WND series this haplotype was rare and was also present on one normal chromosome (see Table 1). The His1069Gln mutation was also associated with three other haplotypes (see Table 1), which were rare (found on 1, 1, 2 WND chromosomes, respectively) and much more nonspecific (found on 1, 1, 3 normal chromosomes as well). The mutation 3400delA was associated with haplotype O in German and Cuban patients. Neither variant of this haplotype O was found on normal chromosomes and thus it seemed to be specific for this mutation. All other mutations and haplotypes were too rare or were also detected on normal chromosomes for firm conclusions on their specificity.

Genotype–phenotype Correlation

WND The comparison of patients with identical homozygous or heterozygous constellations and in particular of sibs with identical WND genotype permits estimation of the dependence of the phenotype on additional genetic or environmental factors. In 15 index patients plus three sibs of the German series the mutations on both of their WND chromosomes could be identified (see Table 3).

The patient WD1 was homozygous for the missense mutation Gly710Ala, whose glycine to alanine substitution within transmembrane helix 2 is likely to result in a minor change. This patient showed a late and mild

hepatic presentation with normal serum ceruloplasmin (19.5 mg/dl).

Seven index patients and one sib, WD2 to WD8, were homozygous for the most common mutation, His1069Gln. These patients exhibited a wide range of clinical presentation, as previously observed by Thomas *et al*⁸ Two of them, WD3 and WD7-1, have the hepatic form of disease, but with considerable difference in age at onset (5 years and 24 years, respectively). Four index patients, WD2, WD4, WD5, WD6 and the sib WD7-2 have the neurologic form, with the age at onset ranging from 19 to 35 years. Patient WD8 has the hepatic/neurologic phenotype with an age of onset at 12 years.

The nine German patients WD9-1 to WD15-2 could be demonstrated to be compound heterozygotes of different WND mutations. All of them carried the His1069Gln mutation plus a variable second WND mutation. The patients WD9-1 and WD9-2 were sibs and had the genotype His1069Gln combined with Cys271X. Both patients were subjectively asymptomatic and were diagnosed by a routine blood transaminase assessment. The combination of His1069Gln with 3400delC was observed in the two unrelated patients WD12 and WD13, who both presented with similar neurological symptoms at nearly identical ages. Sibs WD15-1 and WD15-2 had the combination of two missense mutations, His1069Gln with Gly1176Arg, and again both presented with similarly mild hepatic symptoms at around 12 years of age.

Table 3 Correlations of genotypes with phenotypes

German patient	Sex	Age of onset/phenotypes	Serum ceruloph ^a	Genotype: 1 mutation–2 mutation	Affected region on protein/DNA level	Exon on ATP7B
WD 1	F	25/hepatic	19.5 mg/dl	Gly710Ala-Gly710Ala	disrupts Tm2	8+8
WD 2	F	16/neurol	18 mg/dl	His1069Gln-His1069Gln	SEHPL motif	14+14
WD 3	F	5/hepatic	13 mg/dl	His1069Gln-His1069Gln	SEHPL motif	14+14
WD 4	F	25/neurol	24 mg/dl	His1069Gln-His1069Gln	SEHPL motif	14+14
WD 5	M	19/neurol	6 mg/dl	His1069Gln-His1069Gln	SEHPL motif	14+14
WD 6	F	30/neurol	–	His1069Gln-His1069Gln	SEHPL motif	14+14
WD 7–1	F	24/hepatic	30 mg/dl	His1069Gln-His1069Gln	SEHPL motif	14+14
WD 7–2	F	35/neurol	9 mg/dl	His1069Gln-His1069Gln	SEHPL motif	14+14
WD 8	F	12/hep-neur	11 mg/dl	His1069Gln-His1069Gln	SEHPL motif	14+14
WD 9–1	M	–/asymptom	14 mg/dl	His1069Gln-Cys271X	+truncated prot	14+2
WD 9–2	F	–/asymptom	14 mg/dl	His1069Gln-Cys271X	+truncated prot	14+2
WD 10	F	7/hepatic	16 mg/dl	His1069Gln-Tyr741Cys	+disrupts Tm3	14+8
WD 11	M	27/neur-psy	6.5 mg/dl	His1069Gln-Thr1031Ile	+DKTGT motif	14+14
WD 12	M	20/neurol	2 mg/dl	His1069Gln-3400delC	+frameshift	14+15
WD 13	M	20/neurol	4 mg/dl	His1069Gln-3400delC	+frameshift	14+15
WD 14	M	20/neurol	8 mg/dl	His1069Gln-3903+del1G	+intron 18	14+20
WD 15–1	F	13/hepatic	15.4 mg/dl	His1069Gln-Gly1176Arg	+ATP pocket	14+16
WD 15–2	F	10/hepatic	12.7 mg/dl	His1069Gln-Gly1176Arg	+ATP pocket	14+16

^a18–30 mg/dl normal, F: female, M: male.

In Table 4 we have distinguished the German series by three genotype groups – the homozygous index patients for His1069Gln, the heterozygous index patients for His1069Gln, and the index patients with other mutations on both alleles. Patients who were homozygous for His1069Gln mutation were on average older at the age of onset (18.7 years) than were compound heterozygous (16.4 years) and patients with other mutations (18.0 years). Moreover, females seemed to predominate in this homozygous group (6:1) in comparison with the heterozygous group (5:9).

Table 4 Comparison of three genotype groups in the German series

Genotype	Average age of onset	Phenotype	Sex
Homozygous for His1069Gln	18.7 years	4 neurologic 2 hepatic 1 neur-hepa	3 F, 1 M 2 F 1 F
Heterozygous for His1069Gln	16.4 years	9 neurologic 3 hepatic 2 asymptomatic	2 F, 7 M 3 F –, 2 M
Other mutations, no His1069Gln	18.0 years	5 neurologic 5 hepatic 1 neur-hepa 1 asymptomatic	4 F, 1 M 4 F, 1 M 1 F 1 F

F: female, M: male.

Discussion

In this study of 33 German and 10 Cuban unrelated Wilson disease patients, we identified the mutation on 47 WND chromosomes (55%). This is in good agreement with other studies using SSCP-based mutation analysis.^{8,10,13} Since WND locus homogeneity in the *ATP7B* region has been shown by multipoint linkage analysis of Wilson patients with widely differing clinical presentations,^{6,15} almost half of WND mutations remained undetected by the methods used or may lie outside the coding region, possibly in the regulatory regions. In agreement with other European studies the His1069Gln mutation predominates by far and accounts for 42% of German WND chromosomes. Because of the importance of this mutation we developed an SSCP system to facilitate its detection (see Patients and Methods), which would be an equivalent alternative to the previously described PCR diagnosis.²² The investigation of His1069Gln in different ethnic groups underlines the predominance of this mutation for Wilson disease particularly in Central Europe, with frequencies of 61% among Austrian,²² 39% among Russian, 38% among North American and Swedish,¹³ 34% among Dutch,²³ 28% among Canadian,⁸ 17% among Turkish, 12% among Italian¹⁰ and 5% among Cuban Wilson patients being reported. A single ancestral event has been assumed¹⁰ for this mutation on the basis of its association with one haplotype, and indeed

the haplotype C is highly predictive for the mutation among Germans. Our observation of four different haplotypes associated with the His1069Gln mutation could be due to at least two independent historic events or to microsatellite slippage.

In 7 German index patients and one sib the His1069Gln mutation was observed in a homozygous state, but in contrast to Houwen *et al*²³ who found a late, neurological presentation in 10 homozygous His1069Gln patients, one patient in our sample developed hepatic symptoms at 5 years of age, and two more patients at 12 and 24 years. The His1069Gln homozygotes showed early and severe hepatic manifestations as well as a case of mild neurological presentation at age 30. On the whole we observed no significant difference in clinical picture between Wilson patients world-wide (where about three-quarters present with neurological symptoms at an average age of 20 years¹), our German Wilson disease population and this homozygous His1069Gln sample. Similar observations were also noted in an Austrian study, where seven of 15 homozygous patients with His1069Gln mutation presented with liver disease,²² and in a North American study, where ten of 20 homozygous patients had hepatic presentation.¹³ It seems, therefore, that the His1069Gln mutation is permissive for a wide range of Wilson phenotypes, which are determined by additional factors.

The observed uneven distribution of the His1069Gln mutation according to sex in homozygotes indicates that female sex hormones could influence the clinical manifestation.²²

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