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Haplotype and Mutation Analysis in Japanese Patients with Wilson Disease

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Summary

Wilson disease (WD), an autosomal recessive disorder of copper transport, is characterized by impaired biliary excretion and by impaired incorporation of copper into ceruloplasmin. Toxic accumulation of copper causes tissue damage, primarily in the liver, brain, and kidneys. The gene for WD (*ATP7B*) has been cloned, and the protein product is predicted to be a copper-transporting P-type ATPase with high amino acid identity with that for Menkes disease, an X-linked disorder of copper transport. Mutation screening in WD patients has led to the identification of at least 40 mutations. In addition, haplotype analysis using three dinucleotide-repeat markers, D13S314, D13S301, and D13S316, has been a useful indicator of specific mutations. We have determined haplotypes for the patients and their parents and sibs, in 21 unrelated WD families from Japan. Twenty-eight different haplotypes were observed on 42 WD chromosomes. In all the patients, the *ATP7B* coding sequence, including the intron-exon boundaries, was screened for mutations, by SSCP, followed by direct-sequence analysis of the shifted fragments. We identified 13 mutations, of which 11 mutations are novel, including 7 mutations—1 insertion, 4 deletions, and 2 missense mutations—in the coding region. The mutations reported in previous studies are 2299insC and Arg778Leu. Two patients were shown to have the 2299insC mutation, which has occurred in many different haplotypes in several populations, indicating a mutation hot spot. Primer-extension analysis of *ATP7B* mRNA has revealed multiple transcription start sites. Four of the novel mutations (three 1-bp changes and one 5-bp deletion) occur in the 5′ UTR and may result in altered expression of the WD gene.

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Introduction

Wilson disease (WD) is an autosomal recessive disorder of copper transport, characterized by impaired incorporation of copper into ceruloplasmin and by impaired excretion via the bile. The disease phenotype includes progressive liver degeneration and/or neurological impairment, frequently with kidney malfunction, as a result of toxic effects of accumulated copper in several tissues, principally the liver, brain, and kidneys (Danks 1995). In most populations, WD has a prevalence of ~1 in 30,000 and a corresponding carrier frequency of ~1 in 90 (Scheinberg and Sternlieb 1984). Treatment involves removal of excess copper by chelating agents, such as penicillamine or trientine, or by the blocking of intestinal copper absorption with zinc salts.

The gene defective in WD (*ATP7B*) has been isolated by use of YAC mapping on 13q14.3 (Bull et al. 1993), on the basis of homology with the Menkes disease gene (*ATP7A*), which is defective in an X-linked disorder of copper transport (Chelly et al. 1993; Mercer et al. 1993; Vulpe et al. 1993), and by use of data from a brain-cDNA library that was being screened for amyloid-related genes (Tanzi et al. 1993). The 7.5-kb mRNA transcript is expressed predominantly in the liver and kidneys, and a trace amount of message was detected in the heart, brain, lungs, muscle tissue, placenta, and pancreas (Bull et al. 1993). Characterization of the exon-intron structure reveals that *ATP7B* is organized into 22 exons spanning a DNA region of ~100 kb (Petrukhin et al. 1994; Thomas et al. 1995*a*). The predicted protein product of *ATP7B* (designated as "ATP7B") belongs to a group of cation-transporting P-type ATPases and is homologous to the predicted protein product of the Menkes disease gene (*ATP7A*) (Chelly et al. 1993; Vulpe et al. 1993) and to bacterial heavy-metal-transporting ATPases (Solioz et al. 1994). The functionally important regions of ATP7B are predicted to be the following: six copper-binding motifs (GMXCXSC) at the N-terminal segment, eight putative transmembrane domains (Tm 1–Tm 8), a phosphorylation site (DKTGT) with a conserved aspartate residue, the TGEA motif located in a conformationally flexible loop, the TGDN motif in the

putative ATP-binding domain, and the AMVGDGVND sequence that connects the ATP-binding domain to transmembrane segments involved in ion binding and translocation (Bull et al. 1993; Tanzi et al. 1993). The description of the intronic sequence flanking each exon (Petrukhin et al. 1994; Thomas et al. 1995*a*) has allowed rapid PCR-based screening to identify new WD mutations. In addition, the identification of closely spaced, highly polymorphic DNA markers that span the WD region has allowed haplotype analysis of WD DNA samples from different human populations (Petrukhin et al. 1993; Tanzi et al. 1993; Thomas et al. 1993, 1994).

Mutation screening in WD patients has led to the identification of at least 40 disease-specific mutations, mostly in populations of European origin (Bull et al. 1993; Tanzi et al. 1993; Figus et al. 1995; Thomas et al. 1995*a,* 1995*b*). The data published to date suggest that WD results from a limited number of frequent mutations that are both common and population specific and from a large number of rare mutations. The most common mutation observed is His1069Gln, which has occurred in the eastern and northern European populations, in which it accounts for ~30% of the WD chromosomes (Tanzi et al. 1993; Thomas et al. 1995*a,* 1995*b*). The other most frequent mutations are Arg778Leu, Arg778Gly, and 2299insC. Arg778Leu and Arg778Gln have occurred at a higher frequency in the East Asian population (Chuang et al. 1996). The mutation Arg778Gln has not been reported in a European population. 2299insC has occurred in many populations and in several different haplotypes, suggesting a mutation hot spot (Figus et al. 1995; Thomas et al. 1995*b*).

In Japanese WD patients, a point mutation of a splice acceptor site that is 5 bp upstream of the junction of intron 4 and exon 5, resulting in the skipping of exon 5, has been reported (Shimizu et al. 1995). This study reports the results of haplotype analysis and mutation screening performed on WD patients of Japanese descent and the mapping of the transcription start site of *ATP7B*.

Subjects and Methods

Subjects

DNA was isolated from peripheral blood (Miller et al. 1988) collected from 21 unrelated Japanese families including 21 unrelated patients, 21 parents (from 11 families), and 7 unaffected sibs (from 6 families). There was no known consanguinity between the parents of each patient. These patients were mainly from the Kumamoto, Osaka, and Sendai regions of Japan. Diagnosis of WD was based on low ceruloplasmin and copper serum concentrations, high urinary copper, and high hepatic copper content. The age at onset of each patient was within the range of 4–21 years. Seventy-six percent of the patients had an age at onset of <16 years (81% of these patients had liver disease), and 24% had an age of onset of >16 years (20% of these patients had liver disease). The normal chromosomes analyzed were those from the heterozygous parents of the patients.

Haplotype Analysis

DNA from the parents and patients within each family was analyzed to determine the marker haplotypes present on each WD and each normal chromosome. Haplotypes were derived by use of three microsatellite markers (D13S301, D13S314, and D13S316) flanking the WD locus. These markers have been found to be close to the WD gene, in previously published studies (Petrukhin et al. 1993; Thomas et al. 1993, 1994). The amplification of CA repeats in patient and in parent DNA was performed by use of PCR with pairs of specific primers, as described in previous studies (Thomas et al. 1994, 1995*b*). The analysis was carried out by PCR in 10-μl volumes containing 50 mM KCl; 10 mM Tris, pH 8.0; 10 mg BSA/ml; 1.5 mM $MgCl_2$; 200 μM each of dCTP, dGTP, and dTTP; 25 μM dATP; 0.2 μCi [α-^{35}S]-dATP; and 0.5 units AmpliTaq (Perkin Elmer). Amplification was performed in a programmable thermal controller (MJ Research PTC-100-96V), for 30 cycles of 30 s of denaturation at 94°C, 30 s of annealing at 58°C, and 30 s of extension at 72°C. The samples then were electrophoresed through 6% denaturing polyacrylamide gels, which were dried and exposed to x-ray film (X-omat; Kodak) at room temperature for 1–5 days.

Detection of Mutations

Exons 1–21 of *ATP7B* were amplified by use of primers complementary to the DNA sequences flanking the exon-intron boundaries. The primers used for amplification of exons 2–21 and their product length are described elsewhere (Thomas et al. 1995*a*). The primers used for amplification of exon 1 were as described elsewhere (Petrukhin et al. 1994). Exons were amplified under conditions identical to those used for CA repeats and were digested for 2 h with the appropriate restriction enzyme (Thomas et al. 1995*a*). The samples then were diluted with one volume of SSCP buffer (0.2 M NaOH and 1% SDS) and three volumes of loading buffer (95% formamide, 15 mM EDTA, and 0.03% each of xylene cyanol and bromophenol blue), were denatured at 95°C for 5 min, and were electrophoresed through 6% nondenaturing polyacrylamide gels, under two different conditions—with 8% glycerol and without glycerol—at room temperature for 18–24 h at 10 W before drying and exposure to x-ray film (X-Omat; Kodak). Patient DNA exhibiting shifts relative to normal samples, as detected by SSCP analysis, was directly sequenced to identify the mutation. Patient DNA samples that did not exhibit shifts relative to normal samples, as detected by SSCP analysis, for exons 5, 7, and 8 were directly sequenced for detection of mutations.

DNA from patients was amplified as described above for 35 cycles with 200 μM of cold dATP and without [α-^{35}S]-dATP. Products were purified with a Qiaquick spin column (Qiagen), were cycle sequenced (with Thermosequanase; Amersham) by use of each of the PCR primers, and were electrophoresed through 6% denaturing polyacrylamide gels.

RNA Preparation and Primer-Extension Analysis

Total RNA was prepared from a human liver sample by use of TRIzol Reagent (Gibco, BRL), according to the manufacturer's recommendations. Yeast RNA (Ambion) was used as a negative control in this experiment. Ten μg of total RNA were mixed with [γ-^{32}P]-ATP end-labeled primers, and the mixtures were heated at 70°C for 10 min and were snap chilled on ice. The primers then were extended for 50 min at 42°C with SuperScriptII reverse transcriptase, according to the manufacturer's recommendations (Gibco, BRL). The antisense primers, Ex1a and Ex1b (fig. 1*B*), were derived from sequence analysis of the *ATP7B* cDNA (Petrukhin et al. 1994). After cDNA synthesis, the DNA:RNA hybrid was digested with RNAse A (Boehringer Mannheim), and the primer-extended products were analyzed on a 6% denaturing polyacrylamide gel. A dideoxy chain termination sequence ladder derived from direct sequencing, by use of primer Ex1a, of a cosmid clone containing the 5′-most region of *ATP7B*, including exon 1, was loaded next to the extension products in order to map precisely the transcription initiation sites.

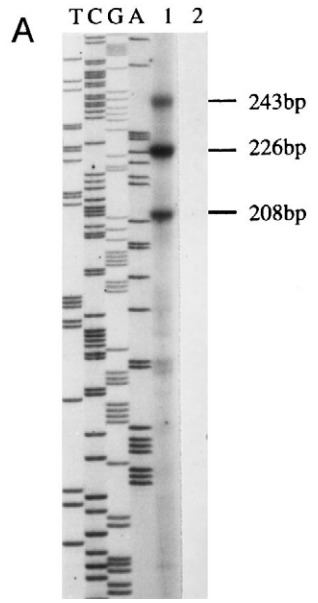
Results

Haplotype Analysis

Haplotypes of the markers D13S301–D13S314–D13S316 were constructed for 21 normal and 42 WD chromosomes (table 1). We detected 28 different haplotypes associated with WD chromosomes. Haplotypes 5–7–5.5 and 11–4–7 are the most common, each being observed in 9.5% of the WD chromosomes. Haplotype 5–7–5.5 was also observed in 9.5% of the normal chromosomes analyzed.

Mutation Screening

We identified 13 mutations, of which 11 are novel and 2 have been described elsewhere (Thomas et al. 1995*a*) (table 2). The mutations reported elsewhere are 2299insC and Arg778Leu. The novel mutations include seven mutations—one insertion, four deletions, and two missense mutations—in the coding region, and four mutations (three 1-bp changes and one 5-bp deletion) are in the 5′ UTR of *ATP7B*. The 13 mutations identified account for 60% of the WD chromosomes analyzed by SSCP. We did not detect additional mutations, with direct sequencing of exons 5, 7, and 8, in patient DNA samples that did not exhibit shifts relative to normal samples, as detected by SSCP analysis. The insertion and deletion mutations cause a frameshift and are predicted to alter the reading frame of *ATP7B*, which would result either in the absence of the protein product or in the production of a shortened nonfunctional protein. The identified missense mutations reside in the Tm 4 and Tm 6 regions and in the phosphorylation domain. All of these mutations are nonconservative and change amino acid residues as follows: a basic for a neutral hydrophobic residue (Arg778Leu), a neutral cyclic for a neutral hydrophobic residue (Pro992Leu), and a neutral



B

1 CCCCGTGCCCCCAGGTCGGGAGGACGGCGGCGCGCAACTTTGAATCATCCGTGTG

56 AAGAGGGCTGCGGCTTCCCCGGTCCCAAATGAAGGGGCGGTTCCCGGACCCCTGT

111 TTGCTTTAGAGCCGAGCCGCGCCGCGCCGATGCCCTCACACTCTGCGCCTCCTCT

156 CCCGGGACTTTAACA*CCCGCTCTCCTCCACCGAC*CAGGTGACCTTTTGCTCTGAG
Primer Ex1b

221 CCAGATCAGAGAAGAATTCGGTGT*CCGTGCGGGACGATGCCTGAGCAGG*AGAGAC
Primer Ex1a

276 AGATCACAGCCAGAGAAGGGGCCAGTCGGAAAgtgagttttgttcccccgcgtcc

Figure 1 *A*, Primer-extension analysis. Ten μg of total RNA from a human liver sample (lane 1) and from yeast (lane 2) each were annealed to the end-labeled primer Ex1a and were extended with reverse transcriptase. The primer-extended products were analyzed on a 6% denaturing polyacrylamide gel. The predominant extension products (208 bp, 226 bp, and 243 bp) are indicated by horizontal lines to the right of the figure, and a dideoxy chain termination sequence ladder is marked "TCGA." *B*, Nucleotide sequence of the 5′ end of *ATP7B*. Transcription start sites are indicated by bent arrows. The major start site is also indicated by "+1." Primers Ex1a and Ex1b are the antisense sequences of the italicized bases. The boldface TTC triplet of this sequence corresponds to the first triplet of the previously reported *ATP7B* cDNA sequence (Petrukhin et al. 1994). The translation start site is underlined. The intronic sequence is shown in lowercase letters.

Table 1

Haplotypes of WD and Normal Chromosomes in Japanese Families

Haplotype, by Marker Locus			No. (%) of Chromosomes	
D13S314	D13S301	D13S316	WD [n = 42]	Normal [n = 21]
1	3	4	1 (2.4)	...
1	4	7	1 (2.4)	...
1	5	7	2 (4.8)	...
1	7	4	2 (4.8)	3 (14.3)
3	3	4	1 (2.4)	...
3	5	7	...	1 (4.8)
3	6	5.5	1 (2.4)	...
3	7	7	1 (2.4)	...
3	7	9.5	1 (2.4)	...
3	8	3	...	1 (4.8)
3	8	4	1 (2.4)	1 (4.8)
3	9	4	...	1 (4.8)
5	5	6	1 (2.4)	...
5	7	4	2 (4.8)	...
5	7	5	1 (2.4)	...
5	7	5.5	4 (9.5)	2 (9.5)
5	7	7	1 (2.4)	...
5	7	8	1 (2.4)	...
5	8	7	...	1 (4.8)
6	3	10	...	1 (4.8)
6	4	4	...	1 (4.8)
6	9	4	3 (7.1)	1 (4.8)
6	11	4	...	1 (4.8)
7	7	7	2 (4.8)	...
7	8	7	1 (2.4)	...
10	5	5.5	1 (2.4)	...
11	3	7	1 (2.4)	...
11	4	4	2 (4.8)	1 (4.8)
11	4	6	1 (2.4)	...
11	4	7	4 (9.5)	...
11	5	7	1 (2.4)	3 (14.3)
11	5	9	...	1 (4.8)
11	6	5.5	1 (2.4)	...
11	7	5.5	1 (2.4)	...
11	7	7	2 (4.8)	1 (4.8)
11	8	4	1 (2.4)	...

polar for a neutral hydrophobic residue (Gly1035Val). None of the missense mutations were seen among the 21 normal chromosomes analyzed.

DNA Polymorphisms

We have identified four sequence changes in the WD gene that do not modify the amino acid sequence of the protein product or that are detected in normal chromosomes of the same population or in chromosomes with defined disease-causing mutations (table 3). These variants have been considered polymorphisms. The variant 3903+6T→C has been described elsewhere (Thomas et al. 1995*a*).

Haplotype Association of WD Mutations

Most of the patients investigated are presumed to be compound heterozygotes for an identified mutation and an unknown mutation. Homozygotes were detected for the following mutations: 2299insC, Pro992Leu, and delAGCCG 138 bp upstream of the translation start site. Mutations usually were linked to a single specific haplotype. However, mutations 2299insC and Arg-778Leu were found on several different haplotypes (table 2). Arg778Leu is the most common mutation, accounting for 12% of the WD chromosomes. Homozygotes were identified for haplotypes 1–7–4, 6–9–4, and 7–7–7, which were associated with the following mutations: delAGCCG 138 bp upstream of the translation start site, Pro992Leu, and 2299insC, respectively.

Mapping of Transcription Start Sites of ATP7B

Primer-extension analysis of the total RNA obtained from a human liver sample (fig. 1*A*, lane 1) and of the total RNA from yeast (fig. 1*A*, lane 2) were annealed to end-labeled primer Ex1a (fig. 1*B*), and the primer was extended with reverse transcriptase. The predominant extension products were 208 bp, 226 bp, and 243 bp. In addition to these extension products, smaller products were also observed. These smaller products may reflect either multiple start sites that are often observed in the GC-rich promoters or artifacts resulting from reverse-transcriptase termination in the GC-rich region. The positions of transcription start sites were confirmed by use of a second primer, Ex1b (fig. 1*B*), for which the sequence is located 64 bp 5′ of primer Ex1a. This primer generated three predominant extension products that map to positions identical to those generated with primer Ex1a (data not shown).

Discussion

In this study, we have characterized mutations and defined respective haplotype associations, in 21 WD patients of Japanese origin. In WD, haplotype data has been useful in mutation detection, since each haplotype generally is associated with a specific mutation (Figus et al. 1995; Thomas et al. 1995*b;* Loudianos et al. 1996). We have detected 28 different haplotypes associated with the 42 WD chromosomes. Haplotypes 5–7–5.5 and 11–4–7 are the most common, each being observed in 9.5% of WD chromosomes. Haplotype 5–7–5.5 was also observed in 9.5% of the normal chromosomes analyzed. The haplotype data suggest the presence in Japanese patients of many uncommon mutations. The mutation analysis detected 13 mutations, of which 11 are novel and 2 have been described elsewhere (Thomas et al. 1995*a*). The novel mutations include five frameshift and two missense mutations in the coding region and four mutations (three 1-bp changes and one 5-bp deletion) in the 5′ UTR, of the WD gene. No analysis of mutations in this region has been reported previously. The frameshift mutations are predicted to alter the reading frame of the WD gene, which would result

Table 2

Mutations Detected in WD Chromosomes

Mutation	Sequence[a]	Haplotype(s)	Exon	Domain	Frequency of Associated WD Chromosomes (%)	Patient Location
Frameshift:						
1292delGT	CTGTT	11–4–4	3	Copper 5	2.4	Osaka
2004delGAT	TGATC	11–4–7	7	Tm 1	2.4	Osaka
2164insT	CTCTG	. . .[b]	8	Tm 3	2.4	Japan
2203del29	CT..TTC	. . .[b]	8	Tm 3	2.4	Kumamoto
2299insC	CGCCC	7–7–7, 7–8–7	8	Tm 4	7.1	Kumamoto
2871delC	CCCAA	11–5–7	13	Cation channel/Tm 6	2.4	Tokyo
Missense:						
Arg778Leu	CGGTG	5–5–6, 5–7–4	8	Tm 4	12	Kumamoto
		5–7–5, 5–7–5.5				Osaka
		5–7–7				Niigata
Pro992Leu	GCCCA	6–9–4	13	Cation channel/Tm 6	4.8	Osaka
Gly1035Val	TGGCG	. . .[b]	14	Phosphorylation	2.4	Japan
Putative regulatory:[c]						
75A→C	ACACC	11–7–5.5	1	5′ UTR	2.4	Niigata
78A→C	CACGC	1–7–4	1	5′ UTR	7.1	Miyagi
128C→A	CGCGC	1–7–4	1	5′ UTR	7.1	Osaka
138delAGCCG	AGCCGA	1–7–4	1	5′ UTR	4.8	Osaka

[a] Altered sequences are underlined.
[b] No parental DNA samples were available to associate the mutations with the haplotypes.
[c] Nucleotides are numbered as 5′ upstream of the translation start site.

either in the absence of the protein product or in the production of a shortened nonfunctional protein. Evidence for the morbidity of the missense mutations came from several sources—the recurrence among WD patients, the failure to observe these mutations in normal chromosomes, the nonconservative nature of the substitution, and the occurrence of mutations at conserved residues in the regions of predicted functional importance. The mutations Gly1035Val and Pro992Leu occur at highly conserved amino acid residues in the Menkes and in other P-type ATPases (Bull et al. 1993). The Gly1035Val mutation is located four amino acids 5′ to the phosphorylation site DKTGT. The mutation Pro992Leu occurs in the Tm 6 domain and in the predicted cation channel. This mutation is predicted to disrupt the function of the cation channel and/or the formation of the transmembrane domain. For the Arg778Leu mutation, the conclusion in favor of a disease-causing defect is supported by the description of another amino acid substitution, Arg778Gln, at the same position in patients of Taiwanese origin (Chuang et al. 1996) and of Arg778Gly in Sardinian patients (Figus et al. 1995). These findings indicate the critical role of arginine at position 778 for the function of Tm 4. We have identified Arg778Leu and 2299insC to be the most common mutations in our study group, representing 12% and

Table 3

Probable Polymorphisms

Polymorphism	Exon	Domain	Evidence	Frequency of Associated Chromosomes (%) WD	Normal
Leu770Leu	8	Tm 4	No amino acid change	7.1	4.8
Lys831Arg	10	Transduction	Found in normal chromosomes	2.4	9.5
Ala1063Val	14	Phosphorylation	Found in normal chromosomes	2.4	4.8
3903+6T→C	19	Intronic sequence	Found in normal chromosomes	4.8	4.8

7.1%, respectively, of the mutations in the WD chromosomes. Mutation 2299insC has been observed on two different haplotypes (10–3–8 and 12–4–7) in the northern European population (Thomas et al. 1995*b*) and on several other haplotypes, including 10–17–5 and two other haplotypes in the Mediterranean population (Figus et al. 1995; Thomas et al. 1995*b*). This indicates multiple origins and a hot spot for mutation, probably owing to replication slipping in the series of cytosines. We did not detect the His1069Gln mutation, the most common WD mutation found in eastern and northern European populations, in which it accounts for ~30% of the WD chromosomes (Tanzi et al. 1993; Thomas et al. 1995*a,* 1995*b*). Mutations detected in our study account for 60% of the WD chromosomes analyzed. Failure to detect the remaining mutations could be due to the limitations of SSCP analysis, under the conditions used in our study, and/or the presence of mutations in the promoter, the introns, or other DNA control regions outside of the exons and their flanking sequences.

Primer-extension analysis of total RNA from a human liver sample has revealed multiple *ATP7B* transcription start sites. The predominant extension products were 208 bp, 226 bp, and 243 bp. In addition to these extension products, smaller products were also observed. These smaller products may reflect either multiple start sites that are often observed in the GC-rich promoters or artifacts resulting from reverse-transcriptase termination in the GC-rich region. Mutations identified in the 5′ UTR may alter the expression of the WD gene. Regions of mRNA sequence between translation and transcription start sites are known to be crucial for ribosome binding. In addition, in some genes, there are protein binding sites between translation and transcription start sites, which are required for gene transcription (Pain 1996). Fresh blood samples or liver-biopsy samples were not available from patients, for analysis of *ATP7B* mRNA for illegitimate transcription. The analysis of the effects of sequence changes, observed in the 5′ UTR of *ATP7B,* on the expression of a transfected reporter gene will be a useful way to show that the mutations identified in this region result in disease. The data presented in this study have increased our knowledge of the spectrum of mutations in functionally important sequences of the WD gene and should aid in the diagnosis of WD in Japanese patients.

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