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RESEARCH ARTICLE

Mutation Analysis of Wilson Disease in Taiwan and Description of Six New Mutations

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Wilson disease is an autosomal recessive disorder of copper metabolism. Mutation screening in Wilson disease has led to the detection of at least 89 disease-specific mutations. Some mutations appear to be population specific, while others are common to many populations. In this study, 38 Taiwanese patients with Wilson disease were screened using single-strand conformation polymorphism analysis, followed by direct DNA sequencing. We found 12 different mutations, six of which were novel. All our detected mutations were found to be in eight exons. Four mutations in three loci (Arg778Gln, Arg778Leu, Gly943Asp, and Pro992Leu) accounted for about 58% of the mutant alleles we detected. Using an RNA transcriptional assay, we confirmed that both of our detected splice-site mutations resulted in exon skipping. Hum Mutat 12:370–376, 1998.

KEY WORDS: **Wilson disease; mutation screen; single-strand conformation polymorphism; RNA transcriptional assay; polymorphism; Taiwanese**

INTRODUCTION

Wilson disease (WD; MIM# 277900) is an autosomal recessive disorder of copper metabolism (Scheinberg and Sternlieb, 1984). The worldwide frequency is 1 in 35,000 to 1 in 100,000 live births (Danks, 1995). The two most fundamental disturbances of copper metabolism in Wilson disease are a reduction in the rate of incorporation of copper into ceruloplasmin and reduction in biliary excretion of copper. Patients with WD most often present with either progressive liver degeneration or neurologic symptoms, or both (Brewer and Yuzbasiyan-Gurkan, 1992; Yarze et al., 1992). The clinical features of WD are attributable to the toxic accumulation of copper in the liver and other tissues, such as kidney, brain, and cornea. Copper is the third most abundant trace element in the body, after iron and zinc, acting as a cofactor for a number of important copper-dependent enzymes (Bull and Cox, 1994). Copper-dependent enzymes are required for diverse processes of oxidative metabolism, including respiration, free-radical detoxification, neurotransmitter synthesis, and maturation of connective tissue, as well as for iron uptake (Yuan et al., 1995).

The WD locus (ATP7B; MIM# 277900), consisting of 21 exons, has been isolated through use of YAC mapping on 13q14.3 (Bull et al., 1993), on the basis of homology with the Menkes disease gene (ATP7A; MIM #300011), which is defective in an X-linked disorder of copper transport (Chelly et al., 1993; Vulpe et al., 1993). The gene defective in WD has been identified and encodes a putative copper-transporting P-type ATPase

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(Bull et al., 1993; Chelly and Monaco, 1993; Tanzi et al., 1993).

Mutation screening in WD patients has led to the detection of at least 89 disease-specific mutations, mostly in populations of European origin (Bull et al., 1993; Tanzi et al., 1993; Figus et al., 1995; Thomas et al., 1995a,b). The published data suggest that some mutations appear to be population specific, while others are common to many populations. His1069Gln has been identified as the most common mutation in the eastern and northern European populations, accounting for 30% of WD chromosomes (Tanzi et al., 1993; Thomas et al., 1995a,b). The codon 778 mutation (Arg778Leu or Arg778Gln) has been reported to have a high allele frequency in the Taiwanese population (Chuang et al., 1996). However, the Arg778Gln mutation has not been reported in the European population. Among the Japanese population, the Arg778Leu mutation was reported to occur with a frequency of 12%, but the His1069Gln mutation is absent (Nanji et al., 1997).

Although mutations of codon 778 have been reported to be most common in Taiwanese patients with WD (Thomas et al., 1995a; Chuang et al., 1996), mutation analysis in the Taiwanese population has not been fully investigated. In this study, mutation screening of WD patients in Taiwan was performed using single-strand conformation polymorphism (SSCP) analysis, followed by direct DNA sequencing. Twelve different mutations, accounting for more than 70% of the patients' chromosomes, were found, six of which were novel. Through RNA transcriptional assay, we confirmed that both of our detected splicing mutations resulted in exon skipping.

Thus far, this study provides the most complete data for the Taiwanese WD mutation. When compared with the WD mutation studies done in Japan, our study showed that the allele frequency of some common mutations in Taiwanese and Japanese WD chromosomes is similar. Those common mutations, however, are either relatively rare or absent in European WD patients.

MATERIALS AND METHODS

Subjects

DNA was isolated from peripheral blood collected from 38 unrelated patients, by the use of a DNA Extractor WB kit (Wako, Japan). The diagnosis of WD was made on the basis of low ceruloplasmin and low serum copper concentrations, and high urinary and high hepatic copper content.

SSCP Analysis and DNA Sequencing

Exons 1–21 of ATP7B were polymerase chain reaction (PCR) amplified and subjected to mutation analysis by SSCP using the GenePhor DNA Electrophoresis System (Pharmacia, Sweden). PCR amplification conditions and primers used were identical to those described previously (Thomas et al., 1995a) for all exons, except exon 1 (Petrukhin et al., 1994). The PCR products were first diluted with a sample buffer (10 mM Tris-HCl, 1 mM EDTA, xylene cyanol 0.05%, bromophenol blue 0.04%, adjusted to pH 7.5 with acetic acid) to about 2 ng/μl and then denatured 1:1 in a denaturing solution (formamide 94%, xylene cyanol 0.05%, and bromophenol blue 0.04%) at 95°C for 5 min; thereafter, they were placed directly on ice to prevent reannealing of the single-stranded products. The samples were then applied to a GeneGel Exel 12.5/24 kit (Pharmacia, Sweden), following the manufacturer's recommendation. Exons that exhibited an irregular shift by SSCP were subjected to direct sequencing for mutation identification. Before direct sequencing, PCR fragments were purified from agarose gel, using QIAEX II (Qiagen, Germany). Direct sequencing was performed using a Taq DyeDeoxy Terminator sequencing kit (PE Applied Biosystems, Foster City, CA) with an ABI Prism 377 DNA sequencer (PE Applied Biosystems, Foster City, CA).

Allele Frequency Analysis of –120delGCGCC

To determine the allele frequency of –120delGCGCC, gene scan analysis was used. Exon 1 was amplified from 50 normal control genomic DNA. The primers and amplification condition were identical to those described previously. Fluorescent rhodamine dyes labeled dUTP (PE Applied Biosystems), such as 0.2 μM R6G (green)-labeled dUTP or 1 μM TAMRA (yellow)-labeled dUTP, were added to the reaction mixture. GeneScan-350 ROX (red) (PE Applied Biosystems) was run at the same time as an internal molecular weight standard and the data were analyzed using software GeneScan Analysis 2.1 (PE Applied Biosystems).

RNA Preparation and Exon Amplification

To investigate the effect of the splice-site mutation, RNA transcription assays were performed. Lymphocytes were isolated from the probands that had the splice-site mutation. Total RNA was prepared using a Trizol reagent (Life Technologies, Gaithersburg, MD), according to the manufacturer's protocol. First-strand cDNA was then

synthesized using the Advantage RT-for-PCR kit (Clontech, Palo Alto, CA) with random hexamer as primer. PCR amplification was then performed. For the 1708-1G→C splice-site mutation, the primers used were WD1703(+) 5′-CTGGTGTT-CTCTCCGTGTTGGT-3′ and WD2177(–) 5′-TGGGGCTCGTTGCTGGGTATCA-3′. The amplification condition was set as follows: one cycle at 94°C for 2 min, 35 cycles of 94°C for 20 s, 50°C for 20 s, and 72°C for 30 s, with use of a standard PCR reaction mixture. For the 3700–2A→T splice-site mutation, the primers used were WD3669(+) 5′-GACAGACCACGAGATGA-AAGGAC-3′ and WD4067(–) 5′-AGCCACCA-CATCCAGCAAATCAT-3′. The amplification condition was set as follows: one cycle at 94°C for 2 min, 35 cycles of 94°C for 20 s, 52°C for 20 s, and 72°C for 30 s, with use of a standard PCR reaction mixture. PCR fragments were isolated from the agarose gel, and direct sequencing was performed as described above.

RESULTS

Mutation Analysis

We identified 12 different mutations; six were novel and six had been reported elsewhere (Table 1). Novel mutations include three missense (Gly943Asp, Gln1142His, Trp1153Cys), one nonsense (Cys490ter), one insertion (523insA), and one splice-site alteration (3700-2A→T). Those three missense mutations were not found in 100 normal individuals. The amino acid changes involved are nonconservative. Gly943 is located at the Tm5 domain of the copper transport polypeptide. Waldenstrom et al. (1996) reported that replacement of glycine with a larger serine residue would disrupt the Tm5 domain Therefore, replacing glycine with acidic residue of aspartic acid would definitely disrupt the Tm5 domain and cause a mutation. Gln1142 is located at the ATP pocket of the polypeptide. Replacing acidic glutamine with basic histidine residue would disrupt ATP binding. Trp1153 is located at the ATP pocket of the polypeptide. Replacing tryptophan with cystein residue would also disrupt ATP binding. The nonsense mutation of Cys490ter would result in a truncated polypeptide of 489 amino acid residues. The insertion mutation (523insA) would lead to an early stop codon for protein translation, resulting in a truncated polypeptide with 202 amino acid residues.

DNA polymorphism

We identified 12 nucleotide changes that either did not modify the amino acid sequence of the polypeptide, or that resulted in conservative changes in the amino acid residues, or that were found in normal chromosomes or chromosomes

TABLE 1. Mutations Identified in Wilson Disease Chromosomes*

Mutation	Sequence	Exon	Predicted effect	Frequency of associated WD chromosomes (%)
Missense				
Arg778Leu[a]	CGG→CTG	8	Disrupts Tm4	28.9
Arg778Gln[b]	CGG→CAG	8	Disrupts Tm4	6.6
Gly943Asp	GGT→GAT	12	Disrupts Tm5	7.9
Pro992Leu[c]	CCC→CTC	13	Disrupts cation channel and Tm6	14.5
Gln1142His	CAG→CAC	16	Disrupts ATP binding	1.3
Trp1153Cys	TGG→TGT	16	Disrupts ATP binding	1.3
Asn1270Ser[d]	AAT→AGT	18	Disrupts ATP hinge	2.6
Nonsense				
Cys490ter	TGC→TGA	3	Truncate protein	1.3
Insertion				
523insA	AGTC<u>A</u>AAAGTCT	2	Frameshift	1.3
2304insC[a]	ACGCCCCCC<u>C</u>ATG	8	Frameshift	2.6
Splicing				
1708-1G→C[a]	gttgcag<u>A</u>TCACA	5	Skips exon 5	1.3
3700-2A→T	gtctt<u>ag</u>GTTGG	18	Skips exon 18	1.3

ter, termination; Tm, transmembrane.
*Mutations sites re underlined. Numbering of base pairs and amino acids begins at the ATG initiation codon published by Petrukhin et al. (1994)
[a]Mutation previously described (Thomas et al. 1995a).
[b]Mutation previously described (Chuang et al., 1996).
[c]Mutation previously described (Nanji et al., 1997).
[d]Mutation previously described (Tanzi et al., 1993).

TABLE 2. Polymorphism Identified at the ATP7B Locus*

Polymorphism	Sequence	Exon	Evidence of polymorphism
–120delGCGCC	TAA$(GAGCC)_2$($\underline{GCGCC}$)$_2$GAT	5′UTR	Found in normal chromosomes
A(–75)→C[a]	ACACC$\underline{A}$CGCT	5′UTR	Found in normal chromosomes
Thr230Thr	AC$\underline{T}$→AC$\underline{G}$	2	No amino acid change
Ile390Val	$\underline{A}$TA→$\underline{G}$TA	2	Conservative amino acid change
Ala406Ser[b,c,e]	$\underline{G}$CT→$\underline{T}$CT	2	Found in normal chromsome
Leu456Val[b,c,e]	$\underline{C}$TG→$\underline{G}$TG	3	Conservative amino acid change
Leu770Leu[d]	CT$\underline{C}$→CT$\underline{G}$	8	No amino acid change
Thr935Met	A$\underline{C}$G→A$\underline{T}$G	12	Conservative amino acid change
Lys952Arg[b,c,e]	A$\underline{A}$A→A$\underline{G}$A	12	Conservative amino acid change
Val1140Ala[a,b,c,e]	G$\underline{T}$C→G$\underline{C}$C	16	Conservatinve amino acid change
Ile1148Thr	A$\underline{T}$T→A$\underline{C}$T	16	Found in normal chromosomes
Leu1154Leu	$\underline{C}$TG→$\underline{T}$TG	16	No amino acid change

*Mutations sites are underlined. Numbering of base pairs and amino acids begins at the ATG initiation codon published by Petrukhin et al. (1994). 5′ UTR 5′ untranslated region.
[a]Polymorphism previously described (Shah et al., 1997).
[b]Polymorphism previously described (Thomas et al., 1995a).
[c]Polymorphism previously described (Waldenstrom et al., 1996).
[d]Polymorphism previously described (Nanji et al., 1997).
[e]Polymorphism previously described (Figus et al., 1995).

with known disease-causing mutant alleles. These variants were considered polymorphisms. Six of them had been reported elsewhere (Table 2). In comparing the polymorphism found in the 5′ untranslated region of ATP7B, we found four pentanucleotide repeats with sequence of –$(GAGCC)_2(GCGCC)_2$– starting from – 139 to – 120 relative to the ATP7B starting codon. One polymorphism (–120delGCGCC) listed in Table 2 happened to be in the region. With the above-mentioned pentanucleotide repeat in mind, we found that a previously reported Japanese WD mutation, –138delAGCCG (Nanji et al., 1997), could be assigned as –120delGCGCC. To verify that –120delGCGCC is a polymorphism, rather than a mutation, exon 1 from 50 normal individuals was amplified and analyzed by GeneScan analysis. A normal allele will show a band size of 267 bp, while the –120delGCGCC allele will display a band size of 262 bp (data not shown). In our study of 100 normal alleles, 25 were of the 262-bp type, while the remainder were of the 267-bp type. On the basis of the GeneScan analysis study, we verified that the –120delGCGCC is indeed a polymorphism, rather than a mutation.

Transcriptional Assay for WD Splice-Site Mutation

We identified two splice-site mutations (Table 1), both of which involved a splice-acceptor site. Each splice-site mutation was found in only one allele among all WD chromosomes investigated. Both probands carrying the splice-site mutant allele were found to be heterozygous, with Arg-778Leu as the other disease-causing mutation, the most common allele among the Taiwanese WD patients. To analyze the effect of splice-site mutations, transcriptional assay was performed as described under Materials and Methods. As for the 1708-1G→C mutation, a normal individual produced the full-length 496-bp fragment, while the compound heterozygous proband produced a 496-bp fragment along with a 334-bp fragment (Fig. 1A,C). Both the 334- and the 496-bp fragments were isolated and sequenced. Direct sequencing confirmed that the 496-bp fragment was the authentic transcript (data not shown), and the 334-bp fragment was free of exon 5 nucleotide sequences (Fig. 1D). As for the 3700-2A→T mutation, a normal individual produced a full-length 421-bp fragment, while the proband with the 3700-2AT→T mutation produced a 421-bp fragment along with a 217-bp fragment (Fig.1B,C). Both the 421- and 217-bp fragments were isolated and sequenced. Direct DNA sequencing confirmed that the 421-bp fragment was the authentic transcript (data not shown), and the 217-bp fragment was free of exon 18 nucleotide sequences (Fig. 1E).

DISCUSSION

Mutation detection in WD is challenging because of the presence of a large number of mutations in a 4.4-kb coding region in 21 exons spread over 80 kb of genomic DNA. Haplotype analysis can provide us with specific information for each different mutation. In the previous Taiwanese WD study, haplotype analysis was used to identify a codon 778 mutation in exon 8 of the ATP7B gene (Chuang et al., 1996). In this study, haplotype analysis was not employed, but SSCP combined

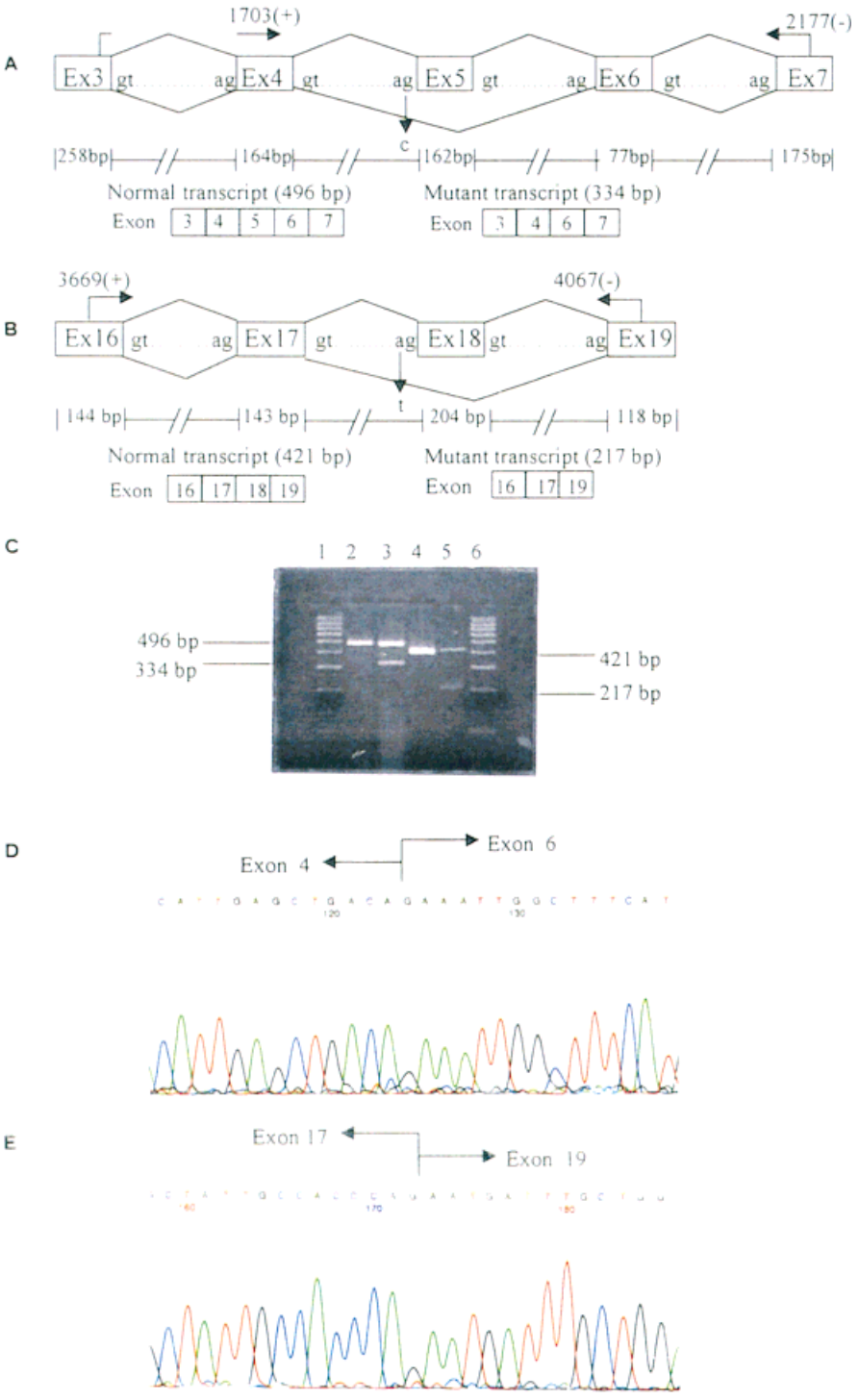

FIGURE 1. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis for splice-site mutations. **A:** Genomic DNA structure of exons 3–7 of the ATP7B gene. Primers 1703(+) and 2177(–) used for RT-PCR are shown above. **B:** Genomic DNA structure of exons 16–19 of the ATP7B gene. Primers 3669(+) and 4067(–) used for RT-PCR are shown above. **C:** Gel electrophoresis of fragments produced by RT-PCR in samples from the proband *(lane 3)* heterozygous for 1708-1G→C mutation, from the proband *(lane 5)* heterozygous for 3700-2A→T mutation, and from normal individuals *(lanes 2 and 4)*. *Lanes 1 and 6*, 100-bp ladder marker. **D:** Electrophoretogram showing raw data for the partial sequence of 334-bp fragment. The nucleotide sequence demonstrates the skipping of the entire exon 5. **E:** Electrophoretogram showing raw data for the partial sequence of 217-bp fragment. The nucleotide sequence demonstrates the skipping of the entire exon 18.

with direct DNA sequencing was performed with all 21 exons of ATP7B gene. The mutation detection rate among the WD chromosome was greater than 70%, higher than some of other WD mutation detection reports (Thomas et al., 1995a; Chuang et al., 1996; Nanji et al., 1997). This study indicated that most of the mutant alleles had a relatively low allele frequency.

We reported a total of 12 mutations in Taiwanese WD-associated chromosomes. All our detected mutations were found to be in eight exons of ATP7B. The allele frequency for these mutations indicated that the spectrum of WD mutations is heterogeneic, with a small portion of relatively frequent mutations and large portion of rare mutations. As reported in previous Taiwanese WD mutation studies, Arg778Leu is the most common mutation (28.9%). Pro992Leu is the second most frequent allele, with a frequency of 14.5%. Gly943Asp, a novel mutation, is the third most frequent allele (7.9%). Arg778Gln, the other mutant allele found in the Arg778 locus, accounted for 6.6%. The above-mentioned four mutations in the three loci accounted for about 58% of all mutations detected in our study. We did not detect the His1069Gln mutation, the most common WD mutation found in the European populations (Tanzi et al., 1993; Thomas et al., 1995a,b).

We report a total of 12 polymorphisms found in our study of WD mutation. Two polymorphisms, located in the 5′ untranslated regions, were reported as mutations in the Japanese WD mutation study (Nanji et al., 1997). A(–75)→C was one of these polymorphisms. Another WD mutation article recently published by Shah et al. (1997), however, listed A(–74)→C as a polymorphism. By comparing their listed sequences (Nanji et al., 1997; Shah et al., 1997) with both published ATP7B 5′ untranslated sequences (Petrukhin et al., 1994) and ours, we believe that A(–74)→C, reported by Shah et al. (1997), is indeed A (–75)C→C and should be classified as a polymorphism. The other polymorphism, –120delGCGCC, located in the 5′ untranslated region (5′UTR), was verified as a polymorphism, rather than a mutation as described under Results.

The result of the transcriptional study of the splice-site mutation was as expected. Both splice-acceptor site mutations resulted in a deletion of the adjacent exon. The first mutation (1708-1G→C) resulted in the skipping of exon 5. Our data also rule out the possibility of using the potential splice-acceptor site found in exon 5, as was suggested by Thomas et al. (1995a). The resulting mutant transcript, with exon 5 (a total of 162 nucleotides) missing, had an in-frame deletion of 54 amino acids. Although those amino acids contained the last copper-binding site, thought to be nonessential, the lack of these amino acids would probably have disrupted the secondary structure of the polypeptide, thereby causing the mutation. The other mutation (3700-2A→T) resulted in the removal of exon 18. The data presented here also ruled out the possibility of using a potential splice acceptor site found in exon 18. This mutation is also an in-frame deletion of 68 amino acids from the ATP7B polypeptide. The absence of those 68 amino acids might have disrupted the hinge of the ATP region, causing a mutation.

Mutation analysis in WD is highly significant, especially in diagnosing potential patients with no family history of WD, and in the early diagnosis of the sibs of affected probands, as well as in prenatal diagnosis. The data presented in this paper provide us with knowledge of the spectrum of mutations of the Taiwanese WD gene and can aid in the diagnosis of and possible gene therapy for WD in the future.

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