Mutation Analysis and Characterization of Alternative Splice Variants of the Wilson Disease Gene *ATP7B*

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Wilson disease is a copper metabolism disorder caused by mutations in ATP7B, a copper-transporting adenosine triphosphatase. A molecular diagnosis was performed on 135 patients with Wilson disease in Taiwan. We identified 36 different mutations, eight of which were novel: five missense mutations (Ser986Phe, Ile1348Asn, Gly1355Asp, Met1392Lys, and Ala1445Pro), one deletion (2810delT) in the coding region, and two nucleotide substitutions (−133A→C and −215A→T) in the promoter region. These mutations were not observed in 100 control subjects and reduced the activity of the mutated protein by at least 50% when compared with wild-type ATP7B. In addition to exon 8, our data indicate another mutation hotspot in exon 12 where 9.62% of all mutations occurred. An alternative splice variant of *ATP7B* lacking exon 12 was observed in one patient who had a homozygous 2810delT mutation and very mild clinical symptoms. Clinical examination and functional characterization of alternative splice variants of *ATP7B* lacking exon 12 showed that they retained 80% of their biological activity. The 2810delT mutation increased the expression of these variants, which may have explained the mild symptoms in the patient with the 2810delT mutation. We also discovered that treating liver cancer cells with a Na^+/H^+ exchanger inhibitor, 5-(*N*-ethyl-*N*-isopropyl)-amiloride, significantly enhanced the expression of the alternative splice variant of *ATP7B* lacking exon 12. *Conclusion*: This study suggests a novel therapeutic strategy for patients with mutations in exon 12. (HEPATOLOGY 2010;52:1662-1670)

Wilson disease (WD) is an autosomal recessive copper metabolism disorder with a prevalence of 1 in 35,000 to 100,000 live births.[1-3] It is characterized by impaired biliary excretion and deficient incorporation of copper into ceruloplasmin, leading to toxic accumulation of copper in the liver, brain, cornea, and kidney. The resulting liver cirrhosis and neurological damage are fatal if not treated with copper-chelating agents such as penicillamine. Prompt and appropriate treatment depends on correctly diagnosing WD in the patient and any affected siblings.[4,5]

Patients with WD most often exhibit progressive liver degeneration, neuropsychiatric symptoms, or both. The diagnosis of WD is determined by signs and symptoms in conjunction with laboratory tests that indicate impaired hepatic copper metabolism. However, these standard tests may yield false positive or false negative results. Failure to correctly diagnose a patient with WD can result in lost opportunities for prophylactic therapy or inappropriate administration of potentially toxic drugs.[4-6] Furthermore, standard tests cannot detect heterozygous carriers or be used for presymptomatic diagnosis. Molecular diagnosis is a

Abbreviations: 5′UTR, 5′untranslated region; ATP7B, adenosine triphosphatase, copper transporting, beta polypeptide; cDNA, complementary DNA; CHO, Chinese hamster ovary; Ct, threshold cycle; EIPA, 5-(N-ethyl-N-isopropyl)-amiloride; FRET, fluorescence resonance energy transfer; mRNA, messenger RNA; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; PCR, polymerase chain reaction; PI, propidium iodide; SEM, standard error of the mean; SMN, survival of motor neuron; WD, Wilson disease.

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useful tool to overcome these limitations.[6,7] Although more than 380 disease-causing mutations have been reported, only a few reports have addressed promoter and 5′ untranslated region (5′ UTR) mutations. According to the Wilson Disease Mutation Database (http://www.wilsondisease.med.ualberta.ca/index.asp), only four 5′ UTR mutations have been reported: c.−441_−427del, c.−129_−125del, c.−75A→C, and c.−36C→T.[8] Because mutation analysis has become the diagnostic method of choice, it is important to establish the frequency of mutations in the promoter region of patients with WD.

The WD gene *ATP7B* (adenosine triphosphatase, copper transporting, beta polypeptide) is expressed in the liver and brain. There are more alternatively spliced variants of *ATP7B* in the brain than in the liver. The most abundant form in the liver contains all the exons, whereas splice variants in the brain have several combinations of skipped exons.[9,10] Tissue-specific mechanisms regulate alternative splicing of *ATP7B* in the liver and brain. For example, alternative splicing of exon 12 occurs in the brain but not in the liver.[9,11] It is not known whether these splice variants retain their biological function. Many therapeutic approaches have been explored to modify the splicing pattern of mutant pre-messenger RNA (pre-mRNA) or eliminate mRNA with a disease-causing mutation. For example, skipping exons 6, 7, 8, 12, and 13 maintains the open reading frame of the *ATP7B* gene,[9] and exons 8, 12, and 13 are mutation hotspots in Taiwanese patients.[1,2,12,13] Thus, it would useful to identify the function of alternatively spliced variants to determine whether splice-correction therapy can be used for WD.

In this study, we collected and analyzed blood samples from 135 patients with WD in Taiwan for mutations in the WD gene to increase the accuracy of molecular diagnosis. Because mutation analyses are increasingly important in screening for WD, we also determined the frequency of mutations in the promoter region of *ATP7B* and explored the possibility of using splice-correction therapy in patients with WD.

Patients and Methods

Subjects. Peripheral blood was collected from 135 patients with WD and 100 unrelated healthy subjects in Taiwan. The clinical data for the patients with WD are shown in Supporting Table 1. This study was approved by the ethical committee and institutional review board of the China Medical University Hospital, Taichung, Taiwan. Informed consent forms were signed by all patients or their guardians.

DNA Sequencing of the ATP7B Gene. Genomic DNA was extracted from peripheral blood samples using the MagNA Pure LC system (Roche Applied Science). The 5′ UTR and 21 exons of the WD gene were amplified, and DNA sequencing of the polymerase chain reaction (PCR) products was performed using the Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) with an ABI-Prism 3100 genetic analyzer (Applied Biosystems).

Cloning and Site-Directed Mutagenesis of ATP7B. Wild-type *ATP7B* complementary DNA (cDNA) was obtained from Dr. Svetlana Lutsenko (Oregon Health and Science University, Portland, OR) and cloned into the pcDNA3 vector (Invitrogen). Site-directed mutagenesis was performed using the GeneTailor Site-Directed Mutagenesis System (Invitrogen).

Copper Resistance Assay. The viability of *ATP7B*-transfected Chinese hamster ovary K1 (CHO-K1) cells in the presence of different concentrations of copper was determined. CHO-K1 cells were treated with copper for 72 hours.

Apoptosis Analysis. Apoptosis was detected by staining with Hoechst 33342 and propidium iodide (PI).

Reporter Gene Assay. We used reporter gene assays to evaluate the effect of promoter mutations. The *ATP7B* promoter and the minimal thymidine kinase promoter were cloned into pTAL-SEAP (secreted alkaline phosphatase) (Clontech). Site-directed mutagenesis was performed using the GeneTailor Site-Directed Mutagenesis System.

Copper Accumulation Assay. The concentration of copper in wild-type and exon 12 alternative spliced ATP7B in CHO-K1 cells was determined from acid digests of whole cells and soluble protein fractions.

Expression Levels of Alternative Splice Variants of ATP7B. The expression levels of alternative splice variants of *ATP7B* exon 12 were determined by real-time PCR using the Roche LightCycler 480. We also developed and applied a new method using fluorescence resonance energy transfer (FRET) technology (Supporting Fig. 1).

Results

Mutations in the Coding Region and 5′ UTR of ATP7B. We identified 36 different mutations, eight of which were novel (Table 1). Among the new mutations, five missense mutations (Ser986Phe, Ile1348Asn, Gly1355Asp, Met1392Lys, and Ala1445Pro) and one deletion mutation (2810delT) were found in the coding region of *ATP7B* and two nucleotide substitutions

Table 1. Mutations Identified in Wilson Disease Chromosomes

Mutation	Sequence	Exon	Predicted Effect	Frequency of Associated WD Chromosomes (%)
Missense				
Met729Val	ATG→GTG	8	Disrupts Tm4	0.37
Arg778Leu	CGG→CTG	8	Disrupts Tm4	29.63
Arg778Gln	CGG→CAG	8	Disrupts Tm4	3.7
Lys832Arg	GAA→GGA	10	Disrupts Transduction domain	1.11
Arg875Gly	AGG→GGG	11	Disrupts Transduction domain	0.37
Arg919Gly	CGG→GGG	12	Disrupts Transduction domain	0.37
Thr935Met	ACG→ATG	12	Disrupts Tm5	3.33
Gly943Asp	GGT→GAT	12	Disrupts Tm5	4.81
Ser986Phe	TCC→TTC	13	Disrupts Tm6	0.37
Pro992Leu	CCC→CTC	13	Disrupts cation channel and Tm6	8.89
Arg1041Trp	CGG→TGG	14	Disrupts ATP binding	0.37
Gln1142His	CAG→CAC	14	Disrupts ATP binding	0.74
Trp1153Cys	TGG→TGT	16	Disrupts ATP binding	0.74
Ala1168Pro	GCT→CCT	16	Interfere with ATP binding	0.37
Thr1178Ala	ACA→GCA	16	Interfere with ATP binding	1.85
Ala1193Pro	GCA→CCA	17	Interfere with ATP binding	0.37
Val1216Met	CTG→ATG	17	Interfere with ATP binding	0.74
Thr1220Met	ACG→ATG	17	Interfere with ATP binding	0.37
Asn1270Ser	AAT→AGT	18	Disrupts ATP hinge	2.59
Pro1273Gln	CCG→CAG	18	Interfere with ATP binding	0.74
Pro1273Leu	CCG→CTG	18	Interfere with ATP binding	0.37
Asp1279Gly	GAC→GGC	18	Interfere with ATP binding	0.74
Ile1348Asn	ATT→AAT	20	Disrupts Tm7	0.37
Gly1355Asp	GGC→GAC	20	Disrupts Tm8	1.11
Met1392Lys	ATG→AAG	21	-	0.37
Ala1445Pro	GCA→CCA	21	-	0.37
Nonsense				
Cys490ter	TGC→TGA	3	Truncate protein	0.74
Gln511ter	CAG→TAG	3	Truncate protein	0.74
Insertion				
525insA	AGTCAAAAGTCT	2	Frameshift	1.48
685insA	TTACAAAAGCA	2	Frameshift	0.37
2304insC	ACGCCCCCCCATG	8	Frameshift	1.11
Deletion				
2810delT	TGGTGGT→TGGGGT	12	Frameshift	1.11
Splicing				
1708-1G→C	gttgcagATCACA	5	Skips exon 5	2.22
3700-2A→T	gtcttagGTTGG	18	Skips exon 18	0.74
Promoter				
−133A>C	AGAGCC→AGCGCC	Promoter	Alter the expression level	1.11
−215A>T	TTGAAT→TTGTAT	Promoter	Alter the expression level	4.81

Novel mutations are underlined. Numbering of amino acids begins at the ATG initiation codon published by Petrukhin et al.[9] Ins, insertion; Ter, termination; Tm, transmembrane domain.

(−133A→C and −215A→T) were found in the promoter region. The five missense mutations in the coding region and two nucleotide substitutions in the promoter region of *ATP7B* were not found in the DNA samples from control subjects. In addition to exon 8, the most frequently reported hotspot, our data revealed another hotspot in exon 12, accounting for 9.62% of the patients with WD in this study.

As shown in Table 2, we found that the Arg778Gln homozygotes and Arg778Leu/Pro992Leu heterozygotes were associated with patients with neurological symptoms. In contrast, patients with only the Arg778Leu mutation (not including patients with Arg778Leu/Pro992Leu) were associated with hepatic symptoms.

The effects of these mutations on cell survival were determined by a copper resistance assay. This assay is based on the fact that ATP7B is a copper transporter; therefore, cells with functional ATP7B are more resistant to copper-induced cell death. Four mutations, namely, Ile1348Asn, Gly1355Asp, Met1392Lys, and 2810delT, completely inhibited copper-transporting activity, as indicated by the rapid death of cells expressing the mutant ATP7B when they were exposed to 20 μM copper (Fig. 1A,C). The Ser986Phe and Ala1445Pro mutations decreased enzyme activity by approximately 50% activity (Fig. 1A).

Nucleotide substitutions in the promoter region reduced promoter activity (Fig. 1B). Specifically,

Table 2. Genotype-Phenotype Correlations

	Neurologic Presentation	Hepatic Presentation	Hepato-Neurologic Presentation	Pure Psychiatric Presentation
Number of patients	65 (100%)	27 (100%)	12 (100%)	2
Arg778Gln homozygous	3 (4.6%)	0	1 (8.3%)	0
Arg778Leu/Pro992Leu	5 (7.7%)	1 (3.7%)	1 (8.3%)	0
Arg778Leu heterozygous (exclude Arg778Leu/Pro992Leu)	3 (4.6%)	8 (29.6%)	0	0

promoter constructs having the −133A→C mutation, −215A→T mutation, or both mutations decreased promoter activity by 51%, 25%, and 13%, respectively, suggesting that these nucleotide substitutions affect the expression of ATP7B.

The 2810delT mutation was diagnosed unexpectedly in a 41-year-old female with consanguinous parents. An optometrist first identified signs of her condition after observing an abnormal pigment encircling the irises of both eyes (Supporting Fig. 2). Physical examination was normal; there was no pallor, jaundice, clubbing, cyanosis, or peripheral lymphadenopathy. In addition, her liver size and serum alanine aminotransferase level were normal, and there were no signs of brain atrophy. Her serum copper level was 6.8 μg/dL (normal range: 50-250 μg/dL), 24-hour urine copper output was 28 μg/day, ceruloplasmin was 2.3 mg/dL, and total bilirubin was 0.7 mg/dL, which were all within the normal ranges. Her parents were heterozygous for the 2810delT mutation in the *ATP7B* gene, whereas she was homozygous. This frameshift mutation does not produce functional ATP7B (Fig. 1C).

Alternative Splice Variants of ATP7B Exon 12 Retain Copper Transporting Activity. *ATP7B* exhibits tissue-specific alternative splicing patterns.[9] There are more splice variants in brain cells than in liver cells (Fig. 2A). Moreover, liver cells do not have any alternative splice variants of exon 12. Because alternative splicing of exon 12 maintains the open reading frame of the gene, we investigated the presence and activity of splice variants in liver cells.

Reverse transcriptase PCR with primers spanning exons 11 and 13 produced three bands in liver biopsy sample 2 (total two different biopsies) and in sk-Hep-1,

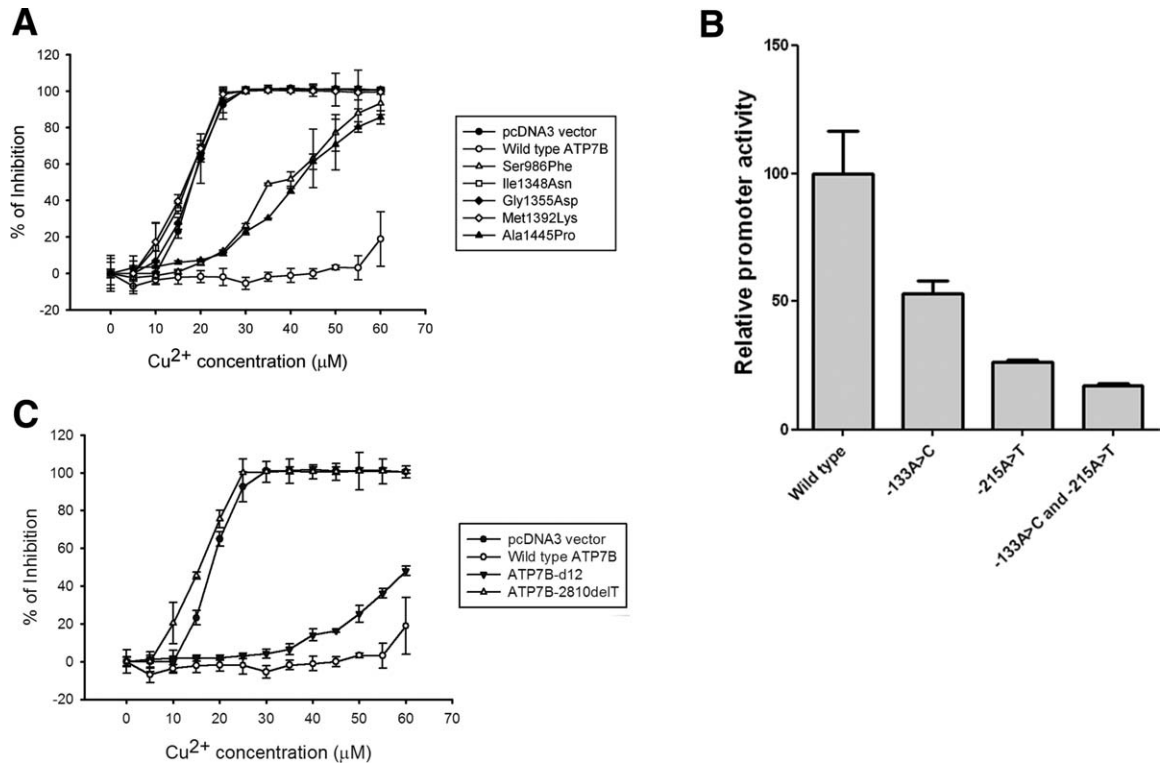

Fig. 1. The function of ATP7B mutants. (A) CHO clones expressing wild-type and mutant ATP7B were grown in media containing the indicated copper concentrations for 3 days, and growth inhibition was then measured by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. The results represent normalized mean ± standard error of the mean (SEM) from three different experiments. (B) Effects of promoter mutations on luciferase induction. Values indicate relative activities and are expressed as mean ± SEM. (C) CHO clones expressing wild-type ATP7B, ATP7B-d12, and ATP7B-2810delT were grown in media containing the indicated copper concentrations for 3 days, and growth inhibition was then measured by the MTS assay. The results represent normalized mean ± SEM from three different experiments.

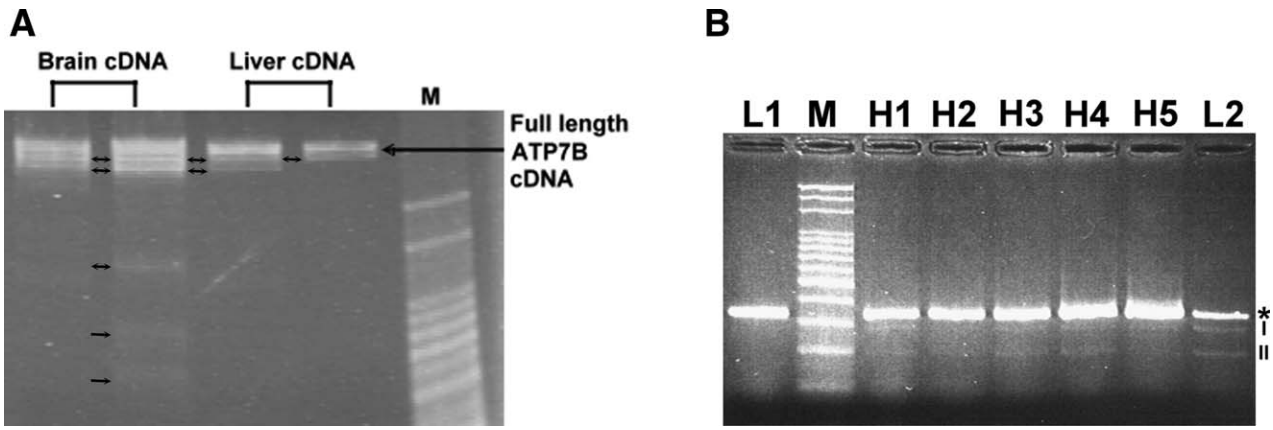

Fig. 2. Alternative splicing of *ATP7B*. (A) Detection of alternative splice variants of *ATP7B* by Reverse transcriptase PCR. PCR was performed on human brain and liver cDNA with Wilson exon 1 (+) and Wilson exon 21 (−) primers. The arrows indicate the increased number of splice variants in the brain compared to that in the liver. (B) PCR analysis of alternative splicing patterns of exons 11-13 of the *ATP7B* gene. PCR analysis of cDNA from liver biopsy 1 (L1), liver biopsy 2 (L2), JHH7 cells (H1), sk-Hep-1 cells (H2), Hep-3B cells (H3), HuH-1 cells (H4), and HuH-7 cells (H5) were performed with alternative exon 12 (+) and alternative exon 12 (−) primers. Exon composition = *, ex11-ex12-ex13; I, unknown sequence; II, ex11-ex13.

Hep-3B, Huh1, Huh7, and JHH7 hepatoma cells (Fig. 2B). Only one band was detected in liver biopsy sample 1. When the PCR products were cloned and sequenced, the largest fragment corresponded to ex11-ex12-ex13, and band II represented ex11-ex13 (Supporting Fig. 3). Band I was a nonspecific amplification of DNA with no homology with any known human DNA sequence.

Because alternative splicing of exon 12 will generate in-frame cDNA, we cloned an *ATP7B* gene without exon 12 (ATP7B-d12) and tested its function. The expression of ATP7B-d12 in CHO-K1 cells was revealed by western blot (Fig. 3A) and immunofluorescence staining (Fig. 3B). In the absence of copper, ATP7B and ATP7B-d12 were located mostly in trans-Golgi networks.[11,14] Cells transfected with ATP7B-d12 retained approximately 80% activity in copper resistance assays compared with wild-type ATP7B (Fig. 1C). In addition, cells with ATP7B-d12 were more resistant to copper-induced cell apoptosis than cells with Gly943Asp ATP7B (Fig. 3C,D). The intracellular copper content of cells expressing ATP7B and ATP7B-d12 was similar to that of CHO-K1 cells transfected with the empty pcDNA3.1 vector in basal media; however, it increased more than four-fold in the presence of 100 μM copper (Fig. 3E). Moreover, similar amounts of intracellular copper were observed in cells with ATP7B and ATP7B-d12, indicating that deletion of exon 12 did not alter the function of ATP7B.

Differential Expression of Alternative Splice Variants of Exon 12. Alternative splice variants were not detected in one of the normal liver biopsy samples (Fig. 2B). This result led us to hypothesize that the expression of alternative splice variants of exon 12 varied among individual patients. To efficiently quantify the expression of exon 12 alternative splice variants, we developed a screening method based on FRET technology using multiplexed fluorescent hybridization probes to detect the relative expression levels of alternative splice variants of exon 12 (Fig. 4). Samples from six patients with hepatoma (including normal and tumor tissues) and one hepatocellular cell line (Huh7 cells) had different expression levels of alternative splice variants of exon 12, ranging from 7%-18% of the wild-type *ATP7B* (Fig. 4A).

Because we could not obtain a liver sample from the patient with the 2810Tdel mutation, we used lymphocyte cDNA to detect the expression of alternative splice variants of exon 12. As shown in Fig. 4B, the expression of these variants in this patient was much higher than in control subjects (approximately equal to the expression of wild-type *ATP7B*), whose expression levels of alternative splice variants of exon 12 were less than 20% of wild-type *ATP7B*.

The 2810delT Mutation Increases the Expression of Alternative Splice Variants of ATP7B Exon 12. To determine whether the 2810delT mutation could influence the expression level of alternatively spliced variants of exon 12, we cloned the wild-type and 2810delT genomic fragments of *ATP7B* containing ex11-in11-ex12-in12-ex13 into the pcDNA3.1 vector, so that the expression of these minigenes was driven by the cytomegalovirus promoter. DNA sequencing confirmed the presence or absence of a thymine at position 2810. Wild-type and 2810delT minigenes were transfected into Hep3B and JHH7 hepatoma cell

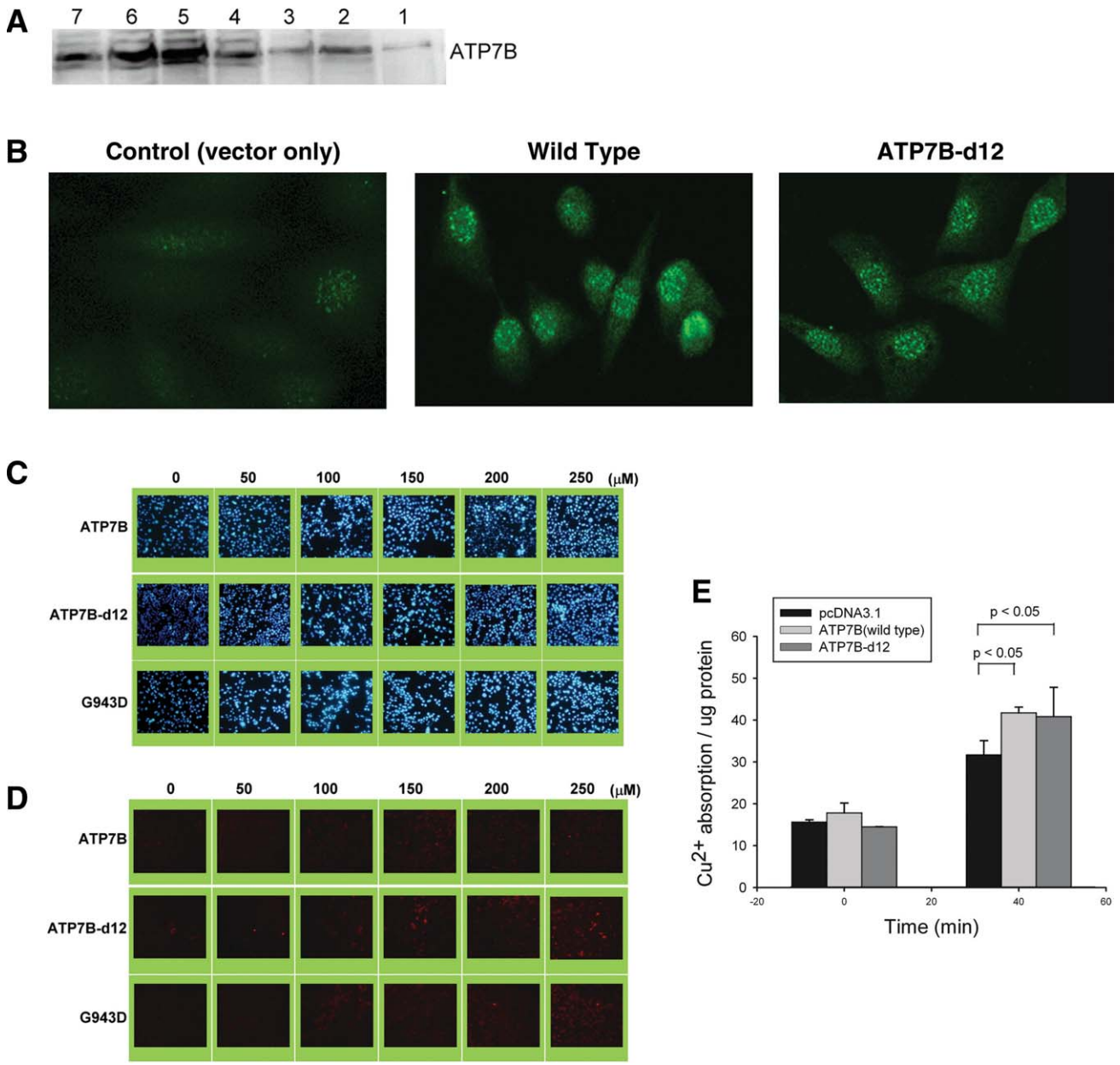

Fig. 3. ATP7B-d12 retained copper-transporting activity. (A) Expression of wild-type, mutant, and truncated ATP7B proteins in CHO-K1 cells. 1, wild-type; 2, ATP7B-d12; 3, Ser986Phe; 4, Ile1348Asn; 5, Gly1355Asp; 6, Met1392Lys; 7, Ala1445Pro. (B) Immunofluorescence microscopy of CHO-K1 cells expressing wild-type ATP7B and ATP7B-d12. Immunofluorescence detection was performed with an anti-ATP7B primary antibody and an anti-rabbit immunoglobulin G–fluorescein isothiocyanate secondary antibody. (C,D) Cells expressing ATP7B-d12 were resistant to copper-induced apoptosis. CHO-K1 cells were transfected with the indicated plasmid and treated with different concentrations of copper. Apoptosis was detected by staining with Hoechst 33342 and PI. CHO-K1 cells were treated with copper for 72 hours and observed using an Olympus XSZD2 fluorescence microscope. G943D indicates Gly943Asp mutation. (E) The intracellular copper content of CHO-K1 cells transfected with or without *ATP7B* was measured after incubation in basal or copper-supplemented (100 μM) medium for 40 minutes. Copper concentration was determined by electrothermal atomic absorption spectroscopy. The results are expressed as normalized mean $\pm$ SEM of three different experiments. The calculated *P* values for the paired comparisons, pcDNA3.1 versus ATP7B or ATP7B-d12, were less than 0.05.

lines, and the expression of alternative splice variants of exon 12 was then determined. As shown in Fig. 5A,B, the expression of these variants was much higher in the 2810delT minigenes ($P < 0.05$). This result agreed with the data shown in Fig. 4B, which showed that the patient may have benefited from this mutation by maintaining enough ATP7B activity to reduce or eliminate symptoms.

Enhanced Exclusion of Exon 12 by an Na^+/H^+ Exchanger Inhibitor. Treatment of cells with 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIPA) has been shown to influence the alternative splicing pattern of the

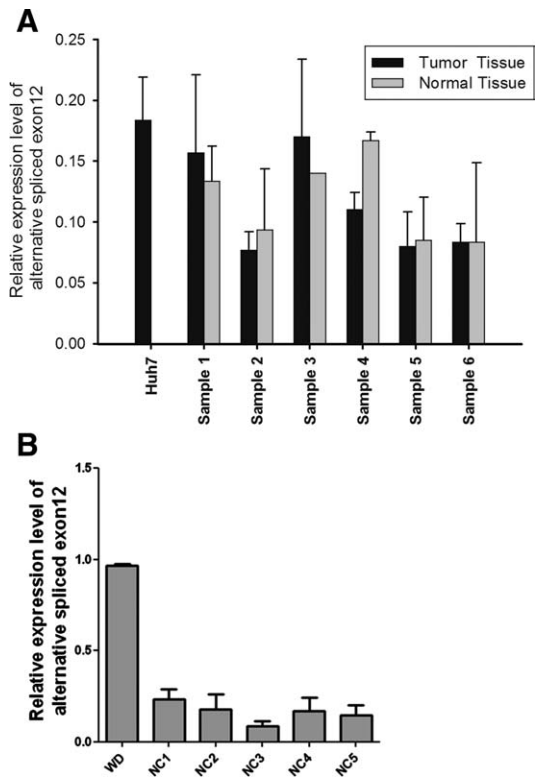

Fig. 4. ATP7B-d12 was differentially expressed among different subjects. (A) Expression levels of ATP7B-d12 in normal and hepatoma tissue samples. (B) Expression levels of alternative splicing variants of exon 12 in the 2810delT patient with WD (indicated as WD) and five normal control (indicated as NC) subjects.

survival of motor neuron 2 (SMN2) gene.[15] Because exon 12 was a mutation hotspot in patients with WD in this study, increasing the expression of alternative splice variants of exon 12 may be a therapeutic approach for treating patients with mutations in this exon. Treatment with EIPA increased the expression of alternatively spliced variants of exon 12 (Fig. 5A). Moreover, this increased expression of splice variants was significantly higher in the 2810delT *ATP7B* minigene than in the wild-type control ($P < 0.005$; Fig. 5B). It should be noted that 2810delT minigene expression of alternatively spliced variants of exon 12 was higher (approximately equal to that of non-spliced mRNA; Fig. 5A,B) than that of endogenous *ATP7B* mRNA (Fig. 4A). To determine whether EIPA could modulate alternative splicing of exon 12 of *ATP7B* mRNA, we treated Hep3B cells with 10 μM EIPA and measured the expression of alternative splice variants of exon 12. EIPA increased the expression level of these variants three-fold (Fig. 5C).

Discussion

Molecular analysis of the *ATP7B* gene is becoming increasingly important in the diagnosis of WD. Currently, this diagnostic method is essential for some cases such as familial screening or when a conventional diagnosis is uncertain. In emergency situations such as acute liver failure, alkaline phosphatase/bilirubin ratios or aspartate/alanine aminotransferase ratios can be used to diagnose WD.[16] Although this diagnosis is quick and accurate, it has not been tested in Asian patients. Therefore, we suggest that molecular diagnosis can strengthen the diagnosis of WD. A newly developed molecular diagnosis method by Lin et al. can diagnose mutations in exons 11, 12, 13, 16, 17, and 18 or the promoter region in 2 hours.[17]

In this study, two mutations were found in 80 patients, one mutation was found in 39 patients, and no mutations were detected in 16 patients with WD. The detection rate of WD mutations was 73.7%. Arg778Leu (29.63%) was the most common WD mutation. Position 778 has a high frequency of mutation among Taiwanese patients, between 27% and

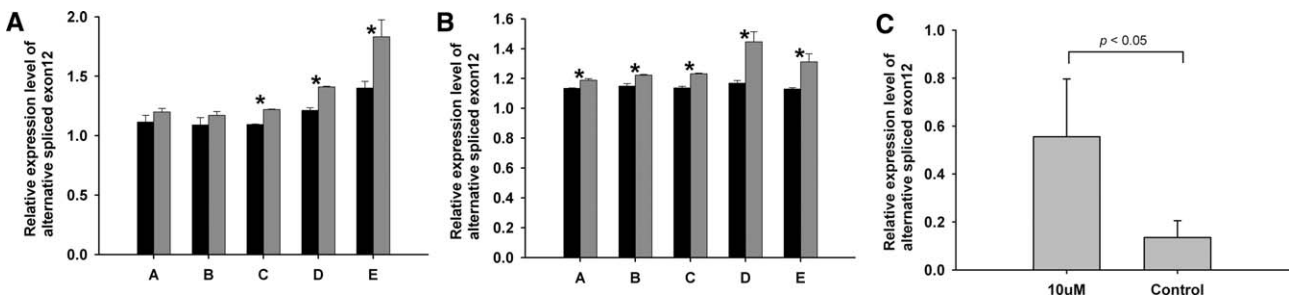

Fig. 5. EIPA promotes alternative splicing of exon 12 of the *ATP7B* gene. The results are expressed as normalized mean ± SEM of three experiments. Asterisks indicate significant *P* values ($P < 0.001$) for paired comparisons between wild-type ATP7B (black bars) and ATP7B-d12 (gray bars). (A) Hep3B or (B) JHH7 cells were transfected with an ATP7B minigene containing exons 11-14 or an ATP7B-d12 minigene, and then treated with increasing concentrations of EIPA (10, 100, and 500 μM) for 24 hours. Paired comparisons between dimethyl sulfoxide–treated wild-type cells (labeled "B", black bars) and 100 μM EIPA (labeled "D", black bars) or 500 μM EIPA (labeled "E", black bars) ($P < 0.05$). A = medium; B = dimethyl sulfoxide; C, D, and E = 10, 100, and 500 μM EIPA, respectively. (C) Hep3B cells were treated with 10 μM EIPA for 24 hours. The expression levels of alternative splice variants of *ATP7B* exon 12 were determined. The paired comparison of 10 μM EIPA and the control was statistically significant ($P < 0.05$).

43.1%.[1,2,12,13] According to several reports, the c.−75A→C substitution in the promoter region may be a single-nucleotide polymorphism.[2,18-20] We also identified this single-nucleotide polymorphism in the control subjects with a minor allele frequency of 35.5%. Because no mutations were detected in the coding region of *ATP7B* in many patients, we performed DNA sequencing to detect promoter mutations in patients with one or no mutation. Two mutations in this region that reduce promoter activity were detected. Three patients had heterozygous mutations in the promoter region, i.e., they had both −133A→C and −215A−T mutations. Because we did not know whether they were on the same chromosome, we cloned a promoter with these two mutations. The promoter activity was even lower when these two mutations were on the same haplotype. The mutations in the 5′ UTR were analyzed using the promoter analysis tool[21] with International Union for Pure and Applied Chemistry consensus strings of the transcription factor binding site. The −215A→T and −133A→C mutations abolished the activator protein 1 (AP1) and specificity protein 1 (SP1) transcription factor binding sites, respectively.[21] These mutations may interfere with normal regulation of the *ATP7B* gene, leading to WD.

Alternative splicing is important in medicine because it is the major source of proteome diversity. Alternatively spliced protein isoforms may have indistinguishable, related, diverse, or antagonistic functions.[22,23] The *ATP7B* gene exhibits a tissue-specific splicing pattern.[9] Most *ATP7B* transcripts in the liver have all the exons found in the genomic DNA, whereas splice variants in the brain have several combinations of skipped exons. Skipping exons 6, 7, 8, 12, and 13 maintains the open reading frame of the gene; however, the function of alternatively spliced variants of *ATP7B* is unknown. We have demonstrated that alternatively spliced variants of exon 12 retained 80% of wild-type function, which likely explained the mild symptoms observed in the patient with the 2810delT mutation.

In mammalian cells, exons constitute only a small part of pre-mRNA transcripts. Accurate splicing requires correct recognition of shorter exonic sequences from longer intronic sequences by spliceosomal components that bind an array of intronic and exonic splicing sequence elements. These elements can either enhance (exonic splicing enhancers) or repress (exonic splicing silencers) splicing at a nearby splice site.[24,25] A higher density of exonic splicing enhancers in authentic exons than in pseudoexons may differentiate recognition of the correct exons, whereas the presence of exonic splicing silencers in pseudoexons may suppress their splicing.[26,27] Thus, both these types of elements may contribute to the specificity of pre-mRNA splicing. A web-based splicing regulatory element recognition program[28] predicted that the 2810delT mutation increases the number of putative exonic splicing enhancer sites from 1 (TGGTGG) to 4 (GTTGGG, TTGGGG, TGGGGT, and GGGGTA), which may explain the increase in alternatively spliced variants of exon 12 and, consequently, the reduction in disease symptoms.[28]

Splice-correction therapy modifies or corrects RNA splicing. Most methods that have been reported use antisense oligonucleotide-based compounds that target key elements in pre-mRNA to control splicing in the nucleus.[29,30] For example, this method has been used to correct the alternative splicing of *SMN2* pre-mRNA to compensate for a defective *SMN1* gene has been used to reduce the severity of spinal muscular atrophy.[31,32] EIPA promotes inclusion of exon 7 in *SMN2* mRNA and the production of SMN protein in motor neurons.[15] EIPA also increases expression of the serine/arginine-rich (SR) splicing factor SRp20, which regulates exon 10 skipping in the tau transcript.[15] EIPA also increased the expression level of alternatively spliced variants of *ATP7B* exon 12, suggesting splice-correction therapy could be used to treat patients with WD. Because skipping exons 6, 7, 8, 12, and 13 produces in-frame *ATP7B* transcripts, it is important to determine the function of these ATP7B variants to determine whether splice-correction therapy can be used for patients with deletions in or mutations on these exons.

Mutation analysis of the *ATP7B* gene from patients with WD around the world revealed more than 380 disease-causing mutations, but only a few common mutations have been identified in specific populations. For example, a mutation in exon 14, His1069Glu, was predominantly detected in 17%-42% of North American, Greek, Polish, Swedish, German, or British patients. In exon 18, another mutation hotspot, Gly1266Lys, has a 10% mutation rate among French and British patients.[33] The most frequent mutation in exon 8, Leu708Pro, was found in the population of the Canary Islands, where it accounts for 50% of all mutations in the exon. For Asian populations in Korea, Japan, China, and Taiwan, Arg778 mutations in exon 8 account for more than 20% of all WD mutations. The Thr935Met in exon 12 has a mutation rate of 10% among Chinese patients. A mutation in exon 13, 2871delC, has a 15.9% mutation rate among Japanese patients.[33] Thus, *ATP7B* mutations in

Caucasian populations are common in exons 8 and 18, whereas mutations in Asian populations tend to occur in exons 8, 12, and 13. Because mutations on exons 8, 12, and 13 account for more than 50% of all WD mutations in Asian patients and exon 8 is a mutation hotspot in Caucasian populations, splice-correction therapy may be a therapeutic option for WD, particularly for patients who cannot receive the standard penicillamine treatment.

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