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RNA Analysis of Consensus Sequence Splicing Mutations: Implications for the Diagnosis of Wilson Disease

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Wilson disease (WD) is an autosomal recessive disorder caused by a defective function of the copper-transporting ATP7B protein. This results in progressive copper overload and consequent liver, brain, and kidney damage. Approximately 300 WD-causing mutations have been described to date. Missense mutations are largely prevalent, while splice-site mutations are rarer. Of these, only a minority are detected in splicing consensus sequences. Further, few splicing mutations have been studied at the RNA level. In this study we report the RNA molecular characterization of three consensus splice-site mutations identified by DNA analysis in WD patients. One of them, c.51 + 4 A → T, resides in the consensus sequence of the donor splice site of intron 1; the second, c. 2121 + 3 A → G, occurred in position + 3 of intron 7; and the c.2447 + 5 G → A is localized in the consensus sequence of the donor splice site of intron 9. Analysis revealed predominantly abnormal splicing in the samples carrying mutations compared to the normal controls. These results strongly suggest that consensus sequence splice-site mutations result in disease by interfering with the production of the normal WD protein. Our data contribute to understanding the mutational spectrum that affect splicing and improve our capability in WD diagnosis.

Introduction

Wilson disease (WD; MIM# 277900) is an autosomal recessive disorder of copper transport, characterized by decreased biliary copper excretion and reduced copper incorporation into ceruloplasmin (Danks, 1989). The WD gene *ATP7B* (MIM# 606882; GenBank L25591), which maps on chromosome 13q14.3, has been cloned and found to encode a copper-transporting P-type ATPase (Bull *et al.*, 1993; Tanzi *et al.*, 1993; Yamaguchi *et al.*, 1993). Approximately 300 disease-causing mutations have been defined to date, a large part of which in populations of Mediterranean origin (http://uofa-medical-genetics.org/Wilson/index.html). Missense mutations are largely prevalent, while splice-site mutations are rarer. Among them, only a minority are detected in splicing consensus sequences. Further, only a few splicing mutations have been studied at the RNA level (Shimizu *et al.*, 1995; Shah *et al.*, 1997; Loudianos *et al.*, 2002; Deguti *et al.*, 2004). In this study, we report the characterization of the RNA transcript of three consensus splice-site mutations identified in WD patients by DNA analysis of *ATP7B* gene.

Materials and Methods

Patients

The study was carried out in six unrelated WD families of Italian origin coming predominantly from the region of Campania. The diagnosis of WD was based on low ceruloplasmin and copper serum concentrations, increased urinary copper excretion, and high liver copper concentration (Table 1).

DNA analysis

Genomic DNA was extracted from peripheral lymphocytes by standard methods. Mutation detection was performed by single strand conformation polymorphism (SSCP) analysis of the 21 amplified exons of the *ATP7B* gene (Lepori *et al.*, 2007). Sequencing was performed using dGTP technology and the ABI 3100 analyzer (Applied Biosystems–Perkin-Elmer, Norwalk, CT) according to the manufacturer's recommendations. The sequence software package (version 4.2; GeneCodes, Ann Arbor, MI) was used for sequence analysis.

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Table 1. Available Clinical and Laboratory Data of Patients

	Patient 1	*Patient 2*	*Patient 3*	*Patient 4*	*Patient 5*	*Patient 6*	*Patient 7*	*Patient 8*
Sex	M	F	F	M	M	M	M	F
Year of birth		1989	2000	2000	1993	1995	2003	1966
Clinical	1968							
Age at onset	17	6, 7/12	2, 5/12	6	6, 2/12	4	1	26
Age at diagnosis	17	6, 7/12	2, 5/12	6	6, 2/12	4	1	26
Ataxia	−	−	−	−	−	−	−	−
Tremor	−	−	−	−	−	−	−	−
Dysarthria	+	−	−	−	−	−	−	−
Dyslalia	−	−	−	−	−	−	−	−
Dystonia	+	−	−	−	−	−	−	−
Hepatomegaly	−	+	+	−	+	+	−	−
Asymptomatic		+	+	+	+	+	+	+
Kayser-Fleischer (KF) rings	+	−	−	−	−	−	−	−
Laboratory								
Ceruloplasmin (mg/dL)	4	2	2	4	5	6	11	3
Aspartate aminotransferase (AST) U/L	23	101	49	120	114	159	55	16
Alanine aminotransferase (ALT) U/L	26	223	50	170	148	424	57	17
Serum copper (γ/dL)	40	40	6	17	40	40	13	6
Urine Cu basal (γ/24 h)	835	119	22	97	262	214	ND	1470
Urine Cu after Penicillamine (γ/24 h)	n/a	ND	ND	902	ND	ND	ND	ND
Liver biopsy	ND						ND	n/a
Steatosis		+	+	+	+	+		
Liver cirrhosis		−	−	−	−	−		
Chronic hepatitis		−	−	−	−	−		
Inflammation		−	−	−	+	+		
Fibrosis		−	−	+	+	−		
Cu content (γ/g dry tissue)		1041	n/a	380	1056	1048		
Genotype	51 + 4 A → T/p.T8 58A	51 + 4 A → T/p.T1 220M	51 + 4 A → T/p.T1 220M	2121 + 3 A → G/p.G591 D	2447 + 5 G → A Homozygote	2447 + 5 G → A Homozygote	2447 + 5 G → A Homozygote	C72X/2447 + 5 G → A

Normal values: Cp, 25–62 mg/dL; AST, 5–40 U/L; ALT, 5–35 U/L; serum copper, 50–150 γ/dL; urine copper basal, <50 γ/24 h; liver copper, <250 γ/g dry tissue.
ND, not done; n/a, not available. Plus sign (+) indicates that the condition is present; minus sign (−) indicates that the condition is absent.

Table 2. RT-PCR Primers

Primer name	*Sequence 5′–3′*	*CDNA bases-exon*	*Direction*
WD-1	TGCGCAGCTCACCTGCCC	−517/−500 5′UTR	Forward
WD-2	GAAATCCTGTCCTCAATGGAC	242–222 Ex 2	Reverse
WD-3	TTCCCGGACCCCTGTTTGCT	−162/−143 5′UTR	Forward
WD-4	TCCCAGGCAGGGGTAGGCAAAG	89–68 Ex 2	Reverse
WD-5	TCCACGGGATATTATCAAAA	1842–1861 Ex 5	Forward
WD-6	CTGGCTAAACTCATGTCT	2374–2391 Ex 9	Reverse
WD-7	ATTGGCTTTCATGCTTCCC	1873–1891 Ex 6	Forward
WD-8	GACACAGGTCAGCCAACATG	2168–2187 Ex 8	Reverse
WD-9	ACCGATTACAATCCATACC	2832–2814 Ex 12	Reverse
WD-10	CAACGCTCATCACTTGGACCA	1911–1931 Ex 6	Forward
WD-11	GGTAGCTTTAATGGGCACA	2667–2649 Ex 11	Reverse

For the evaluation of the novel mutations we used the Human Splicing Finder (HSF) (http://www.umd.be/HSF/).

RNA analysis

To assess the pathogenicity of the detected splicing mutations, we carried out RNA analysis from peripheral lymphocytes. Total RNA was extracted from peripheral lymphoblasts and from normal controls (lymphoblasts and liver). RNA extraction was performed using the Ultraspec™A Reagent (Biotecx, Houston, TX) according to manufacturer recommendations. RT-PCR was carried out with the GeneAmpRNA PCR Core kit (Perkin Elmer, Branchburg, NJ) according to manufacturer instructions. First round RT-PCR was performed in 100 μL volumes on 300 ng of total RNA. A second round of PCR was carried out using 2 μL of the first PCR reaction product as template and appropriate nested primers (Table 2). In all experiments PCR-grade water was used as a DNA-free negative control to exclude any contamination during the PCR process. These controls contained all the necessary components for PCR except template DNA.

The amplified products were separated on 2% agarose gel, and the fragments were cut and purified using QIA Quick columns (Qiagen, Hilden, Germany) gel extraction kits, according to the manufacturer's protocol. Sequencing was performed as described above.

Results and Discussion

DNA analysis

Using the SSCP followed by direct sequencing, we identified three consensus splice mutations in our sample. The c.51 + 4 A → T, occurring in the consensus sequence of the donor splice site of intron 1, was detected in the homozygous state in one Italian family and in compound heterozygosity in two more Italian families, in one associated with the p.T1220M (c.3659 C → T) mutation and in the second with the p.T858A mutation (Lepori *et al.*, 2007). All families harboring these mutations came from the same region (Campania).

The 2121 + 3 A → G mutation, localized in the consensus sequence of the donor splice site of intron 7, was detected in the compound heterozygous state with the p.G591D (c.1772 G → A) mutation in a WD patient from the Apulia region.

The mutation c.2447 + 5 G → A, localized in the consensus sequence of the donor splice site of intron 9, was identified in the homozygous state in a family with three affected siblings and in compound heterozygosity with C72X (c.276 T → A) mutation (Lepori *et al.*, 2007) in another family, both of which from the Campania region.

In all families splice-site mutations segregated with the disease.

A screening of 100 normal chromosomes from 50 healthy people from Campania and Apulia failed to detect these consensus splice-site mutations.

Using the HSF (http://www.umd.be/HSF/) program, we evaluated the novel mutations.

The mutation c.2447 + 5 G → A resulted in the creation of two exonic splicing enhancer (ESE) motifs (SC35 and 9G8), while a Sironi splicing silencer motif (motif 2) (Sironi *et al.*, 2004) site was destroyed. The existing donor splice site was also weakened by 12.31%.

The mutation c.2121 + 3 A → G in intron 7 resulted in the creation of one ESE motif (SRp55), while three predicted putative exonic splicing silencer (PESS) octamer silencer sites were broken. Additionally, a Sironi Motif1 silencer site was created, and hnRNPA1, which is a modulator of splice-site selection, had its site weakened by 3.91% (Pozzoli and Sironi, 2005). The existing donor splice site was also weakened by 1.47%.

The mutation c.51 + 4A > G located in intron 1 resulted in the destruction of two predicted PESS octamer silencer sites (Zhang and Chasin, 2004). Additionally, a Sironi Motif1 silencer site was created, while a Sironi Motif2 silencer site showed a gain of function by 26.26% and hnRNPA1 had its site weakened by 4.11%. The existing donor splice site was weakened by 9.55%.

RNA analysis

RNA studies were performed on total RNA extracted from peripheral lymphocytes, the only easily accessible cells, as liver biopsies were not available. Illegitimate transcription (Chelly *et al.*, 1989) results in the presence of transcripts in these cells, albeit often in low amounts, thus allowing RNA studies by RT-PCR method. Using an RT-PCR method and appropriate pairs of primers, we characterized the RNA products for each detected mutation. The presence of contamination was excluded in all experiments; no RT-PCR products were observed in the negative control (data not shown).

c.51 + 4 T → A. Characterization of the effect of the c.51 + 4 T → A substitution on mRNA was carried out in a homozygote and in two compound heterozygotes for this mutation and the p.T1220M (c.3659 C → T) mutation, both of the heterozygous subjects were of Italian origin. Gel

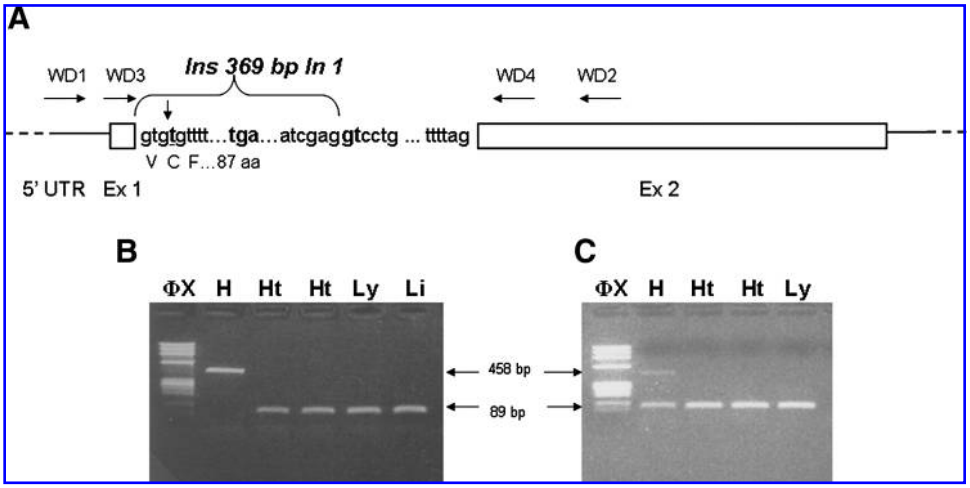

FIG. 1. RT-PCR analysis of the 51 + 4 AT substitution. (**A**) Genomic structure of the analyzed region of exon 1. Primers used for RNA studies are WD1, WD3, WD4, and WD2. Arrow indicates the intronic position of the T → A substitution. In bold is the gt cryptic donor splice site at the 370 nt position and the STOP codon at the 87 aa position. (**B**) Gel electrophoresis of nested PCR fragments obtained using WD1 and WD2 for the first PCR and WD3 and WD4 for the nested PCR from one homozygote (H), two heterozygote (Ht) patients, and normal controls from lymphocytes (Ly) and liver tissue (Li). RT-PCR products from normal controls and heterozygotes show only the normal 89 bp band. RT-PCR products from the homozygote show only an abnormal 458 bp band resulting from the insertion of a 369 bp intronic sequence. (**C**) Gel electrophoresis of the nested PCR fragments (using primers WD3 and WD2 from the first round PCR and WD3 and WD4 for the nested PCR) from the same subjects and a normal control from lymphocytes. RT-PCR products from normal controls and heterozygotes show only the normal 89 bp band. RT-PCR products from the homozygote show in addition to the normal 89 bp band also the abnormal 458 bp spliced product.

electrophoresis of RT-PCR products amplified using for the first-round PCR primers WD1 and WD2 and for the nested PCR WD3 sense primer and WD4 antisense primer (mapping in the 5′ untranslated region [UTR] and in exon 2, respectively) showed an abnormal band of 458 bp in the homozygous sample and an 89 bp normal band in both heterozygotes for the c.51 + 4 A → T and normal controls. (Table 2 and Fig. 1A, B). However, when first round PCR was performed using the WD3 and WD2 primers, and the nested PCR using WD3 and WD4 primers, the RT-PCR product from the normal controls and heterozygotes showed the same electrophoretic pattern as reported above (the 89 bp band), while the RT-PCR product from the homozygote, in addition to the abnormal 458 bp band, also showed the normal 89 bp band (Table 2 and Fig. 1A, C).

Sequence analysis of the abnormal PCR product revealed the presence of a 369 bp insertion from intron 1 resulting from the use of a cryptic GT donor splice site at the nucleotide position 370 of intron 1. The predicted amino acid sequence contained 20 amino acids coded by exon 1 followed by 88 amino acids and a STOP codon coded by the inserted intron sequence, and hence the protein is most likely not functional (Fig. 1A). Based on these data, we speculate that in the case of the homozygous sample, this discrepancy can be explained by the fact that nested PCR was carried out on a first-round PCR product obtained using a sense primer spanning positions from −517 to −499 in the 5′ UTR region of the *ATP7B* gene. In this way, only RNA transcripts initiating upstream of this region could be amplified (Loudianos *et al.*, 1999). In our case, these transcripts contained only the abnormal product. The second nested PCR was carried out using a first PCR product obtained using a sense primer spanning nt positions from 1 to 20 and probably amplifying additional RNA transcripts such as those previously described as initiating at positions −335, −284, −256, and −204 (Nanji *et al.*, 1997; Oh *et al.*, 1999) and containing both normal and abnormal products. The normal transcript is present in a larger amount compared to the abnormal one, most likely because short transcripts are preferentially amplified at the expense of the longer transcript resulting from the insertion of a 369 bp intronic sequence. Alternatively, it could be the effect of nonsense-mediated decay because the intron retention results in a STOP codon (Dietz, 1997). Only normal PCR products were present in the samples heterozygous for c.51 + 4 T → A even though the splice-site mutation occurs on the same haplotype in both the homozygous and heterozygous individuals. The same results were observed in a previous study (Deguti *et al.*, 2004), inducing the authors to consider the c.51 + 4 T → A substitution "potential splicing mutation not resulting in an alternatively spliced product by RT-PCR analysis." Analog observations were carried out during RNA analysis of another mutation, c.2731-2 A → G, occurring in the invariant AG acceptor splice site of exon 12 and detected in both homozygosity and heterozygosity (Shah *et al.*, 1997). In this case, RNA studies detected only the abnormal splicing product in the homozygous sample. These data could be due to the fact that an *in trans* competition for transcription factors occurs and the wild-type sequence is greatly preferred to the mutant sequence. Alternatively, in heterozygous individuals, the chromosome with the splice-site mutation may harbor additional polymorphisms that reduce the efficiency of the cryptic splice-site sequences so that intron retention is not favored (Shah *et al.*, 1997). No detailed clinical documentation was available from the homozygote for c.51 + 4 A → T. The only information obtained is that disease presentation was at the age of 41 years with hemolytic anemia due to liver failure. He underwent orthotopic liver transplantation. Pathologic findings of the resected patient's liver tissues and increased liver copper concentrations in combination with biochemical tests established the diagnosis of WD. Patient 1, a compound hetero-

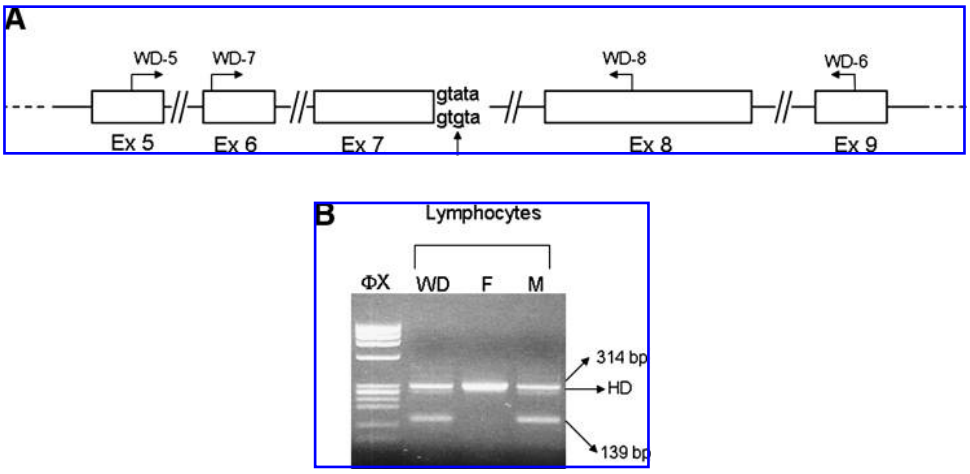

FIG. 2. RT-PCR analysis of the $2121+3\ A \rightarrow G$. (**A**) Schematic representation of the analyzed genomic region. An arrow shows the position of the mutation. Primers are WD5 and WD6 in the first round and WD7 and WD8 in the nested PCR. (**B**) Gel electrophoresis of nested PCR products, in analyzed members of the family. Sample from the proband (WD) and mother (M) presents both the 314 bp full-length and the 139 bp spliced of exon 7 band. Both are heterozygotes for the $2121+3\ A \rightarrow G$ mutation. Father (F) only presents the full-length band. HD, heteroduplex.

zygote for $c.51+4\ A \rightarrow T$ and p.T858A mutation, presented neurological manifestation at the age of 17 years (Table 1). Two sibling compound heterozygotes for $c.51+4\ A \rightarrow T$ and p. T1220M mutations, asymptomatic, were diagnosed after coincidental findings of elevated aminotransferase activity was detected in patient 2 and by DNA family screening in patient 3 at the ages of 6, 7/12 years and 2, 5/12 years, respectively (Table 1).

The $51+4\ A \rightarrow T$ was already detected in two different populations (Deguti *et al.*, 2004; Margarit *et al.*, 2005). In one case the analyzed RNA did not show abnormal bands (Deguti *et al.*, 2004). In the second case the mutation was found in heterozygosity in a WD patient of Spanish origin. Because it was also found in 13% of chromosomes in a normal population, it was considered a normal variant. According to recent information obtained (E. Margarit, personal communication) $51+4\ A \rightarrow T$ mutation was found only on WD chromosome and not in the normal population. The detection in normal chromosomes reported was the result of misinterpretation of SSCP patterns obtained during the screening of normal controls (E. Margarit, personal communication). In our cases, $51+4\ A \rightarrow T$ was detected only on WD chromosomes in patients diagnosed on the basis of clinical and biochemical data to be affected by WD; it segregates with the disease within the analyzed families and was absent in the control population.

c.2121 + 3 A→ G. RNA analysis for $2121+3\ A \rightarrow G$ was carried out in the proband and both parents. Agarose gel electrophoresis of the nested PCR products using the sense primer localized on exon 5 and the antisense primer localized

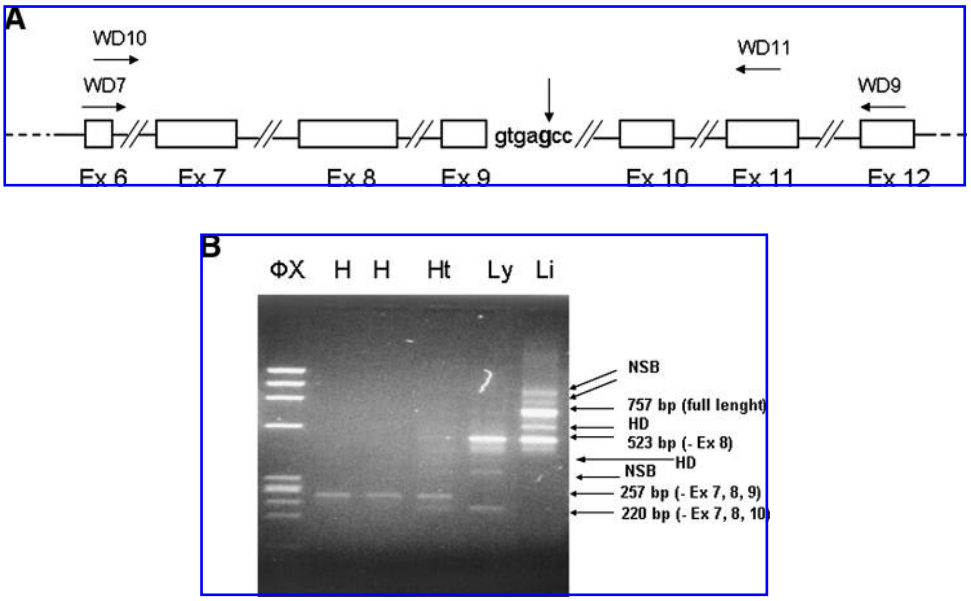

FIG. 3. RT-PCR analysis of the $2447+5\ G \rightarrow A$ mutation. (**A**) Schematic representation of the analyzed genomic region. An arrow shows the position of the mutation. Primers used are WD7 and WD9 in the first round PCR and WD10 and WD11 in the nested PCR. (**B**) Gel electrophoresis of nested PCR products from two homozygotes (H), one heterozygote (Ht), and the normal control from lymphocytes (Ly) and from liver tissue (Li). Both normal controls present two bands: a 757 bp band (not well visible in sample 4) corresponding to the full-length product and a 523 bp band corresponding to a transcript lacking exon 8. Control from lymphocytes also shows a 220 bp band that corresponds to a skipped transcript of exons 7, 8, and 10, in addition to the 757 and 523 bp bands. RT-PCR products from two homozygotes show only a 257 bp band that corresponds to transcript that skips exons 7, 8, and 9. RT-PCR products from heterozygote show both the 220 and 257 bp bands. NSB, nonspecific band; HD, heteroduplex.

on exon 8 showed a full-length major product in all family members and a minor product in the proband and the mother: both carriers of the 2121 + 3 A → G mutation (Table 2 and Fig. 2A, B). Sequence analysis of the minor product revealed skipping of exon 7, which creates a frameshift at codon 664, resulting in a truncated, nonfunctional protein.

The patient with 2121 + 3 A → G/p.G591D mutations is an asymptomatic WD patient in whom low ceruloplasmin and serum copper levels, increased urine copper, and liver biopsy and increased liver copper content established the clinical diagnosis of WD.

c.2447 + 5 G → A. RNA analysis for the c.2447 + 5 G → A mutation was carried out in two homozygotes from one family as well as in a compound heterozygote c.2447 + 5 G → A/p.C72×(c.276 T → A) from another family. Agarose gel electrophoresis of the nested PCR products obtained using the WD-10 sense primer localized on exon 6, and the WD-11 antisense primer mapping on exon 11 showed a major product of 757 bp in both normal liver cells and in a small amount of normal lymphocytes (Table 2 and Fig. 3A). Sequence analysis revealed that the 757 bp band corresponds to the full-length fragment. A second transcript of 523 bp was detected only in normal controls and in a very small amount (barely visible) in the lymphocytes of heterozygotes for c.2447 + 5 G → A mutation that by sequencing was found to correspond to a transcript with an in frame deletion of exon 8. A third transcript of 257 bp was present only in both the homozygotes and compound heterozygotes for the mutation and resulted, by sequence analysis, to correspond to a transcript with the in frame deletion of exons 7, 8, and 9 (Fig. 3B). Another PCR product of 220 bp, which was present only in both the heterozygotes for c.2447 + 5 G → A and normal lymphocytes, corresponds to a transcript with the deletion of exons 7, 8, and 10. Similar data have already been described in previous reports, suggesting that alternative splicing of the *ATP7B* gene is present and differentially regulated in normal tissues (Petrukhin *et al.*, 1994; Thomas *et al.*, 1995; Loudianos *et al.*, 2002). The presence of a consistent amount of normal transcript in the normal liver as compared with the lymphocytes (Fig. 2B) suggests that besides the presence of the two abnormal splicing products, for the normal controls, in the liver there is a different regulatory mechanism that permits the production of sufficient amounts of normal transcripts, thus maintaining the liver copper balance (Loudianos *et al.*, 2002).

The abnormal transcript of 257 bp corresponding to a transcript with the in frame deletion of exons 7, 8, and 9 RNAs was present only in both the homozygotes and compound heterozygote RNAs but not in normal controls. The existence of this additional alternative transcript produced by the c.2447 + 5 G → A substitution may result in an imbalance in the relevant protein isoforms normally present and probably in alterations of cell biochemistry resulting in the disease phenotype (Pagani and Baralle, 2004). These data suggest that the c.2447 + 5 G → A substitution results in the prevalent production of an exon 7, 8, and 9–lacking mRNA producing a protein lacking Tm1–Tm4 regions that is predicted to be functionless (Fig. 3B). Clinical data were available from three homozygotes siblings of one analyzed family and the compound heterozygote patient from the second family. Two subjects were diagnosed as WD patients at the age of 6 and 4 years, respectively, after coincidental findings of elevated aminotransferase activity (Table 1). WD diagnosis was carried out in their 1-year-old sibling using DNA sequence-based family screening. Similarly, the compound heterozygote patient from the second family was diagnosed at the age of 26 years after coincidental finding of elevated aminotransferase activity.

Because of the limited number of clinical observations reported and the presence of compound heterozygosity in the analyzed cases, these data do not allow genotype–phenotype considerations.

The results of this study are obtained from illegitimate transcriptional products of peripheral lymphocytes rather than on liver RNA. Nevertheless, they suggest that mutations residing in the consensus sequence of *ATP7B* gene splice sites are disease causing probably by interfering with the production of the normal protein. Documentation of pathogenic mutations is important for the accurate diagnosis of WD, and it will be pertinent to screen for the presence of these novel mutations in a larger population group from southern Italy.

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Disclosure Statement

There is no conflict of interest, real or perceived.

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