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Molecular analysis of Wilson disease in Taiwan: identification of one novel mutation and evidence of haplotype-mutation association

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Abstract Wilson disease (WND) is caused by a deficiency of the copper-transporting enzyme, P-type ATPase (ATP7B). Twelve different mutations have previously been identified in Taiwan Chinese with Wilson disease. We, herein, report another 4 missense mutations, 1 of which is novel. We did haplotype analysis of Taiwanese WND chromosomes, using three well characterized short tandem repeat markers (haplotype was assigned in the order of D13S314-D13S301-D13S316). Association correlation was found between the mutations and their respective haplotypes. Haplotype-deduced pedigree analysis was shown to be helpful in the mutation analysis of WND chromosomes and in the molecular assessment of both pre-symptomatic WND patients and carriers. Given the complexity and heterogeneity of the mutation spectrum of ATP7B, we suggest that haplotype analysis should be performed before full-scale mutation analysis.

Key words Wilson disease (WND) · ATP7B · Mutation analysis · Haplotype analysis · Short tandem repeat (STR) markers · Taiwanese

Introduction

Wilson disease (WND; McKusick 277900) is inherited in a recessive mode, with a disease prevalence rate of between 1 in 35,000 and 1 in 100,000 live births (Danks 1995). It is a disorder of copper transport that is characterized by reduced incorporation of copper into ceruloplasmin and by decreased biliary excretion. This leads to copper accumulation in the liver and, consequently, to progressive liver damage. The clinical features of WND are attributable to the toxic accumulation of copper in the liver, and in other tissues, such as kidney, brain, and cornea.

WND is caused by a defect in a copper transporting P-type ATPase (EC 3.6.1.36), which is coded by the *ATP7B* gene, which is closely related to *ATP7A*, the Menkes disease (McKusick 309400) gene. *ATP7B* consists of 21 exons and encodes a polypeptide of 1465 amino acids (Bull et al. 1993; Chelly and Monaco 1993; Mercer et al. 1993; Vulpe et al. 1993). Earlier genetic linkage studies showed that the WND locus segregated with the red cell enzyme esterase D on chromosome 13 (Frydman et al. 1985). Subsequent linkage analysis refined the disease locus to a genomic region flanked proximally by the DNA marker, D13S31, and distally by D13S59 (Bowcock et al. 1987; Stewart et al. 1993).

We had previously identified 12 different mutations in 70% Taiwanese WND chromosomes analyzed (Tsai et al. 1998). We, herein, report the finding of another 4 missense mutations, 1 of which is novel. With the majority of WND mutant alleles being identified in Taiwan, we investigated whether those alleles were associated with specific haplotypes. Haplotype analysis, using three short tandem repeat (STR) markers, D13S314, D13S301, and D13S316, has been shown to be a useful indicator of WND mutation (Thomas et al. 1995; Nanji et al. 1997; Chuang et al. 1996). With the use of these three STR markers, haplotypes of all WND chromosomes were established. Haplotype-mutation association was observed in most WND chromosomes analyzed. The established association was shown to be informative in carrier assessment and pre-symptomatic diagnosis of WND patients' family members. It is also helpful for mutation analysis of newly identified WND patients.

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Subjects and methods

Subjects

Peripheral blood was collected from 77 normal individuals from 20 randomly selected families, and from 31 WND patients, as well as their relatives, from 27 unrelated families. Mutation analysis had previously been performed in 25 of these 31 WND patients (Tsai et al. 1998), while 6 patients were newly referred to this study. Genomic DNA was prepared from the peripheral blood using a DNA Extractor WB kit (Wako, Tokyo, Japan). The diagnosis of WND was established by clinical symptoms, the presence of Kaiser-Fleischer rings in the cornea, and biochemical tests (low ceruloplasmin and low serum copper concentrations, and high urinary and high hepatic copper content).

Mutation analysis of the *ATP7B* gene

Exons 1–21 of *ATP7B* were polymerase chain reaction (PCR) amplified and were subjected to mutation analysis by single-strand conformation polymorphism (SSCP), using a GenePhor DNA Electrophoresis System (Pharmacia, Uppsala, Sweden) following the protocol described by Tsai et al. (1998). 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, Hilden, Germany). Direct sequencing was performed using a Taq DyeDeoxy Terminator sequencing kit (PE Applied Biosystems, Foster City, CA, USA) with an ABI Prism 377 DNA sequencer (PE Applied Biosystems).

STR polymorphism analysis

To analyze the haplotypes of the normal population and the WND patients, along with their relatives, three STR markers (D13S314, D13S301, and D13S316) flanking the WND locus were used. These markers contain either di- or tetranucleotide repeats and had been reported in previous studies (Petrukhin et al. 1993; Thomas et al. 1993; Thomas et al. 1994). Nucleotide sequences of amplification primers for D13S314 and D13S316 were described previously by Thomas et al. (1994), while those for D13S301 were described by Petrukhin et al. (1993). One of each primer set was 5′ end-labeled with fluorescent dye. Three different fluorescent dyes were used, as follows: D13S314 with tetrachloro-6-carboxyfluorescein (TET, green), D13S301 with 5-carboxyfluorescein (FAM, blue), and D13S316 with 2′,7′-dimethyloxy-4′,5′-dichloro-6-carboxyfluorescein (HEX, yellow).

The PCR reaction was carried out in a total volume of 25 μl containing genomic DNA; 2–6 pmole of each primer; 1X Taq polymerase buffer (1.5 mM $MgCl_2$); and 0.25 units of AmpliTaq DNA polymerase (Perkin Elmer, Foster City, CA, USA). PCR amplification was performed in a programmable thermal cycler GeneAmp PCR System 2400 (Perkin Elmer). Cycling conditions for D13S314 were set as follows: one cycle at 94°C for 5 min, 35 cycles of 94°C for 15 s, 52°C for 20 s, and 72°C for 30 s, and one final cycle of extension at 72°C for 40 min. Cycling conditions for D13S301 and D13S316 were the same as those for D13S314 except that the annealing temperature was set at 47°C. Appropriate amounts of PCR products (0.75 μl) were mixed with 1.75 μl of premixed solution (formamide:loading buffer [blue dextran, 50 mg/ml; ethylenediaminetetraacetic acid (EDTA), 25 mM]:standard = 5:1:1). Either Genescan-350 TAMRA (6-carboxy-tetramethylrhodamine, red) or Genescan-500 TAMRA (PE Applied Biosystems) was used as the reference molecular size standard. Electrophoretic analysis was performed with the use of a 6% denaturing polyacrylamide gel and with an ABI Prism 377 DNA Sequencer. The data were analyzed with the software, GeneScan Analysis 2.1 (PE Applied Biosystems).

Results

Mutation analysis of the *ATP7B* gene

Besides the 12 mutations previously reported by Tsai et al. (1998), we identified another 4 missense mutations (A874V, V1216M, E1173K, and D1279G) in Taiwanese WND chromosomes; the A874V mutation was recently reported in Japanese and Korean WND chromosomes (Yamaguchi et al. 1998; Kim et al. 1998), and V1216M and E1173K were identified in Mediterranean WND chromosomes (Loudianos et al. 1998, 1999). However, D1279G is a novel mutation with nonconservative amino acid substitution. Glutamic acid 1173 is located near the ATP loop region, and replacement by the basic lysine residue may affect the binding of ATP. Aspartic acid 1279 is located between the hinge region and the seventh transmembrane domain of ATPase, and replacement by the glycine residue may affect the folding of the polypeptide.

Haplotype association of WND chromosomes

The allele size definition and allele distribution for STR markers of D13S314, D13S301, and D13S316 among normal and WND chromosomes are listed in Table 1. A total of 11, 14, and 10 alleles were identified for each marker, respectively. Table 2 lists all the identified WND chromosomes' haplotypes with their respective mutations. Among these identified WND mutations, we observed haplotype-mutation association among most mutations. The G943D mutation, which was reported only in Taiwanese WND patients (Tsai et al. 1998), was found exclusively associated with the 11-1.5-5.5 haplotype (n = 7). R778L, the most frequently found WND mutation in Taiwanese, was associated with either 8-4-4 (n = 4) or 8-4-5.5 (n = 4). Mutation P992L was associated with either 8.5-6.5-2 (n = 2) or 8.5-6.5-5.5 (n = 3). R778Q, the other mutation besides R778L found in amino acid position 778, was exclusively associated with 7.5-0.5-5.5 (n = 3). Q1142H, reported only in

Table 1. Allele-size definition and distribution of STR markers in Taiwanese WND families

Marker	Band size (bp)	Allele	No. of chromosomes	
			Normal	WND
D13S314[a]	154	5.5	1	0
	153	6	7	1
	151	7	32	9
	150	7.5	3	3
	149	8	10	10
	148	8.5	6	5
	147	9	1	0
	143	10.5	6	1
	141	11	46	25
	138	11.5	3	0
	136	12.5	1	0
D13S301[b]	158	−2	1	0
	157	−1.5	3	1
	155	−0.5	3	2
	153	0.5	25	13
	152	1	2	0
	151	1.5	10	11
	150	2	2	0
	149	2.5	4	0
	148	3	11	2
	146	4	44	18
	144	5	8	1
	142	6	1	0
	141	6.5	0	6
	131	11.5	2	0
D13S316[a]	152	2	20	7
	150	3	3	2
	149	3.5	1	0
	148	4	4	5
	147	4.5	3	0
	145	5.5	77	37
	144	6	2	0
	143	6.5	1	3
	142	7	2	0
	138	9	3	0

STR, Short tandem repeat; WND, Wilson disease
[a] Amplification primers used for D13S314 and D13S316 previously described (Thomas et al. 1994)
[b] Amplification primers used for D13S301 previously described (Petrukhin et al. 1993)

Taiwanese (Tsai et al. 1998), was found associated with either 7-4-2 ($n = 3$) or 7-4-5.5 ($n = 1$). The 11-0.5-5.5 haplotype, the most frequently found haplotype among WND chromosomes, was associated with mutations IVS4-1G → C ($n = 3$), A874V, 2304insC, and other unidentified WND alleles ($n = 4$).

Haplotype and pedigree analysis in WND families

The results of haplotype and pedigree analysis for eight WND families are shown in Fig. 1. Although some of the mutations have not yet been identified, we were able to deduce these WND chromosomes' haplotypes. In the process of pedigree analysis of WND families, we found that the haplotype data were useful in assessing the status of both carriers and pre-symptomatic patients who had a sibling diagnosed with Wilson disease and had yet, themselves, to develop clinical symptoms. As shown in family F (Fig. 1),

Table 2. Haplotype/mutation association in Taiwanese WND patients

Haplotype	Mutation	WND chromosomes	Normal chromosomes
6-(-1.5)-5.5	NI	1	0
7-4-2	Q1142H	3	1
7-4-3	NI	1	1
7-4-5.5	C490X	1	12
	Q1142H	1	
	W1153C	1	
7-5-5.5	NI	1	2
7-6.5-2	N1270S	1	0
7.5-0.5-5.5	R778Q	3	1
8-4-4	R778L	4	3
	IVS17-2A → T	1	
8-4-5.5	R778L	4	2
8.5-6.5-2	P992L	2	0
8.5-6.5-5.5	P992L	3	0
10.5-3-5.5	NI	1	1
11-(-0.5)-3	E1173K	1	0
11-(-0.5)-5.5	NI	1	1
11-0.5-5.5	A874V	1	11
	IVS4-1G → C	3	
	2304insC	1	
	NI	4	
11-0.5-6.5	NI	1	0
11-1.5-2	NI	1	0
11-1.5-5.5	G943D	7	5
	NI	2	
11-1.5-6.5	D1279G	1	0
11-3-6.5	NI	1	0
11-4-5.5	523insA	1	7
	V1216M	1	

NI, Mutation not identified

the propositus was newly diagnosed with Wilson disease. The haplotype data indicated she was homozygous, with a haplotype of 11-1.5-5.5, which was associated with mutation G943D. Direct sequencing analysis of the G943D mutation confirmed that she was homozygous for the G943D mutation (data not shown). In family G, the propositus was shown to carry the 11-1.5-5.5 haplotype. Direct sequencing showed that he inherited the G943D mutation from his father (data not shown). His sister, at the time of the haplotype analysis, had not shown clinical symptoms of Wilson disease. Although the other mutation had not been identified, haplotype data indicated that his sister shared the same haplotype, and she was subsequently diagnosed as a pre-symptomatic WND patient (Fig. 1). In family H, the propositus was a newly recruited WND patient. He was shown to carry haplotypes of 11-0.5-5.5 and 7-4-2. To identify WND mutations, these two haplotype-associated mutations were screened first. In a very short time, we identified that he was a compound heterozygote for the Q1142H and IVS4-1G → C mutations.

Discussion

To date, 16 different mutations have been identified in Taiwanese WND chromosomes. Because each non-identified WND allele was checked for known mutations

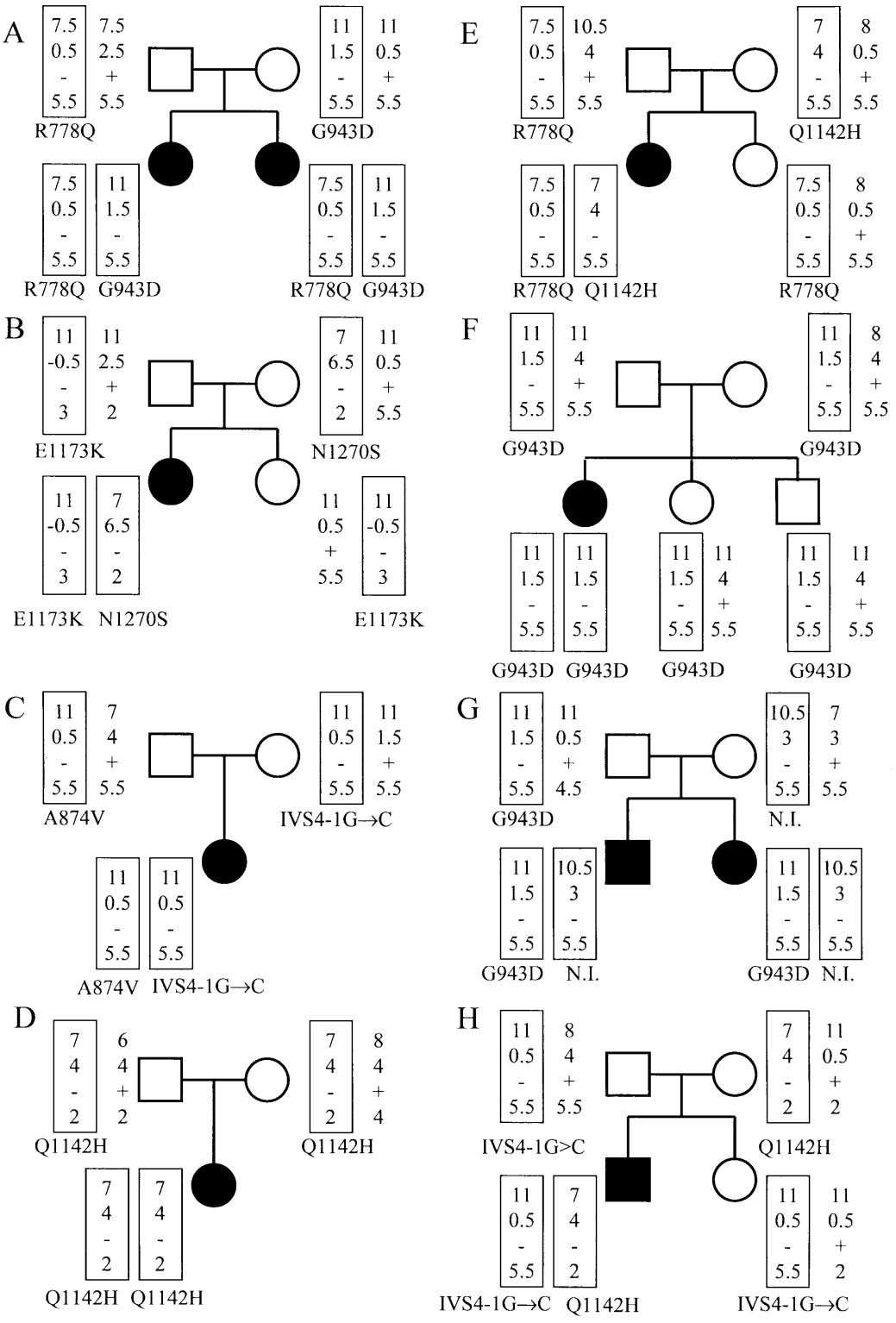

Fig. 1. Haplotypes, identified mutations, and pedigrees of eight Wilson disease (*WND*) families (*A* to *H*). *Numbers* indicate alleles of each short tandem repeat (STR) marker and are defined in *Table 1*. *Blackened boxes and circles* indicate the affected WND probands. The haplotype is arranged from the *top to bottom* in the order of D13S314-D13S301-ATP7B-D13S316. *Normal chromosomes* and *WND chromosomes* are labeled as "+" and "−", respectively. Haplotypes carrying WND mutations are *boxed*. *N.I.* represents WND mutations not yet identified

with the same haplotype, we can estimate that there are probably 11 more unidentified mutations existing. Because of the high heterogeneity of the Wilson disease mutation spectrum in Taiwanese, haplotype analysis was suggested to be the initial step before full-scale mutation analysis was performed in newly recruited WND patients. The haplotype data of WND chromosomes was compared with data in a previous study performed by Chuang et al. (1996). In their study, only R778L and R778Q were identified, and were reported to be associated with haplotypes 8-4-4 and 8-1-6, respectively. While the R778L-associated haplotypes were the same in both studies, the R778Q-associated haplotypes (7.5-0.5-5.5 in ours and 8-1-6 in Chuang's) showed a minor discrepancy. This was probably due to a lack of consensus in

allele definition by the different laboratories. We also noted that haplotypes 11-2-6 and 11-1-6 were the most frequent in Chuang's study. These two haplotypes would correspond to haplotypes 11-0.5-5.5 and 11-1.5-5.5, respectively, in this study.

Some WND mutations, e.g., R778L, P992L, A874V, 2304insC, and N1270S, were identified in both Taiwanese and Japanese (Chuang et al. 1996; Nanji et al. 1997; Tsai et al. 1998; Yamaguchi et al. 1998). It would be interesting to determine whether these common mutations also shared the same haplotypes in these two ethnic populations. However, we found this task difficult, even in Japanese WND chromosomes themselves. For example, while R778L was found to be associated with haplotypes: 5-5-6, 7-5-4, 7-5-5, 7-5-5.5, and 7-5-7 in one study of Japanese WND patients (Nanji et al. 1997), the same mutation was found to be associated with a haplotype of either 8.5-6-5.5 or 8.5-6-7 in an other study (Yamaguchi et al. 1998). This discrepancy was probably derived from either different amplification primers used or different allele definitions used by the different laboratories. Recently, R778L and A874V were identified in Korean WND chromosomes, with R778L being the most frequent WND allele (37.5%) in Koreans (Kim et al. 1998). The WND chromosomes identified in these three different ethnic populations in Northeast Asia area implied that Taiwanese, Japanese, and Koreans may have originated from the same ancestor. Haplotype study of these shared mutations in these three populations should be able to prove this hypothesis. A consensus on allele definition, however, is necessary for haplotype comparison among different ethnic populations. Because we do not yet have such a consensus, we listed our allele-size definition in Table 1. On the other hand, the presence of other population-specific mutations in these three ethnic groups suggests that there still is heterogeneity in WND chromosomes within each population (Nanji et al. 1997; Tsai et al. 1998; Yamaguchi et al. 1998; Kim et al. 1998).

Because non-identified WND mutations still account for about 20%–30% of the mutations in Taiwanese WND patients, it is still difficult to make a direct correlation between haplotypes and their respective WND mutations. We believe that, with more WND mutations being identified, the haplotype-mutation association will become more significant, and it will also assist mutation identification for newly diagnosed WND patients.

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References

Bowcock AM, Farrer LA, Cavalli-Sforza LL, Hebert JM, Kidd KK, Frydman M, Bonne-Tamir B (1987) Mapping the Wilson disease locus to a cluster of linked polymorphic markers on chromosome 13. Am J Hum Genet 41:27–35

Bull PC, Thomas GR, Rommens JM, Forbes JR, Cox DW (1993) The Wilson disease gene is a putative copper transporting P-type ATPase similar to the Menkes gene. Nat Genet 5:327–335

Chelly J, Monaco AP (1993) Cloning the Wilson disease gene. Nat Genet 5:317–318

Chuang LM, Wu HP, Jang MH, Wang TR, Sue WC, Lin BJ, Cox DW, Tai TY (1996) High frequency of two mutations in codon 778 in exon 8 of the *ATP7B* gene in Taiwanese families with Wilson disease. J Med Genet 33:521–523

Danks DM (1995) Disorders of copper transport. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds). The metabolic and molecular bases of inherited disease, 7th ed. McGraw-Hill, New York, pp 2211–2235

Frydman M, Bonne-Tamir B, Farrer LA, Conneally PM, Magazanik A, Ashbel S, Goldwitch Z (1985) Assignment of the gene for Wilson disease to chromosome 13: linkage to the esterase D locus. Proc Natl Acad Sci USA 82:1819–1821

Kim EK, Yoo OJ, Song KY, Yoo HW, Choi SY, Cho SW, Hahn SH (1998) Identification of three novel mutations and a high frequency of the Arg778Leu mutation in Korean patients with Wilson disease. Hum Mutat 11:275–278

Loudianos G, Dessi V, Lovicu M, Angius A, Nurchi A, Sturniolo GC, Marcellini M, Zancan L, Bragetti P, Akar N, Yagci R, Vegnente A, Cao A, Pirastu M (1998) Further delineation of the molecular pathology of Wilson disease in the Mediterranean population. Hum Mutat 12:89–94

Loudianos G, Dessi V, Lovicu M, Angius A, Altuntas B, Giacchino R, Marazzi M, Marcellini M, Sartorelli MR, Sturniolo GC, Kocak N, Yuce A, Akar N, Pirastu M, Cao A (1999) Mutation analysis in patients of Mediterranean descent with Wilson disease: identification of 19 novel mutations. J Med Genet 36:833–836

Mercer JF, Livingston J, Hall B, Paynter JA, Begy C, Chandrasekharappa S, Lockhart P, Grimes A, Bhave M, Siemieniak D (1993) Isolation of a partial candidate gene for Menkes disease by positional cloning. Nat Genet 3:20–25

Nanji MS, Nguyen VTT, Kawasoe JH, Inui K, Endo F, Nakajima T, Anezaki T, Cox TW (1997) Haplotype and mutation analysis in Japanese patients with Wilson disease. Am J Hum Genet 60:1423–1429

Petrukhin K, Fischer SG, Pirastu M, Tanzi RE, Chernov I, Devoto M, Brzustowicz LM, Cayanis E, Vitale E, Russo JJ, Matseoane D, Boukhgalter B, Wasco W, Figus AL, Loudianos J, Cao A, Sternlieb I, Evgrafov O, Parano E, Pavone L, Warburton D, Ott J, Penchaszadeh GK, Scheinberg IH, Gilliam TC (1993) Mapping, cloning and genetic characterization of the region containing the Wilson disease gene. Nat Genet 5:338–343

Stewart EA, White A, Tomfohrde J, Osborne-Lawrence S, Prestridge L, Bonne-Tamir B, Scheinberg IH, St George-Hyslop P, Giagheddu M, Kim JW, Seo JK, Lo WH, Ivanova-Smolenskaya IA, Limborska SA, Cavalli-Sforza LL, Farrer LA, Bowcock AM (1993) Polymorphic microsatellites and Wilson disease (WD). Am J Hum Genet 53:864–873

Thomas GR, Roberts EA, Rosales TO, Moroz SP, Lambert MA, Wong LT, Cox DW (1993) Allelic association and linkage studies in Wilson disease. Hum Mol Genet 2:1401–1405

Thomas GR, Bull PC, Roberts EA, Walshe JM, Cox DW (1994) Haplotype studies in Wilson disease. Am J Hum Genet 54: 71–78

Thomas GR, Roberts EA, Walshe JM, Cox DW (1995) Haplotypes and mutations in Wilson disease. Am J Hum Genet 56:1315–1319

Tsai CH, Tsai FJ, Wu JY, Chang JG, Lee CC, Lin SP, Yang CF, Jong YJ, Lo MC (1998) Mutation analysis of Wilson disease in Taiwan and description of six new mutations. Hum Mutat 12:370–376

Vulpe C, Levinson B, Whitney S, Packman S, Gitschier J (1993) Isolation of a candidate gene for Menkes disease and evidence that it encodes a copper-transporting ATPase. Nat Genet 3: 7–13

Yamaguchi A, Matsuura A, Arashima S, Kikuchi Y, Kikuchi K (1998) Mutations of *ATP7B* gene in Wilson disease in Japan: identification of nine mutations and lack of clear founder effect in a Japanese population. Hum Mutat Suppl 1:S320–322