

• CLINICAL RESEARCH •

Correlation of ATP7B genotype with phenotype in Chinese patients with Wilson disease

Xiao-Qing Liu, Ya-Fen Zhang, Tze-Tze Liu, Kwang-Jen Hsiao, Jian-Ming Zhang, Xue-Fan Gu, Ke-Rong Bao, Li-Hua Yu, Mei-Xian Wang

Xiao-Qing Liu, Jian-Ming Zhang, Ke-Rong Bao, Li-Hua Yu, Mei-Xian Wang, Department of Neurology, Xinhua Hospital, Shanghai Second Medical University, Shanghai 200092, China

Ya-Fen Zhang, Xue-Fan Gu, Shanghai Institute for Pediatric Research, Shanghai 200092, China

Tze-Tze Liu, Kwang-Jen Hsiao, Genome Research Center and Institute of Genetics, National Yang Ming University, and Department of Medical Research and Education, Taipei Veterans General Hospital, Taipei 11216, Taiwan, China

Supported by the National Ministry of Education Teacher Education Plan in China 2000 NO.1424

Correspondence to: Dr. Xiao-Qing Liu, Department of Neurology, Xinhua Hospital, Shanghai Second Medical University, 1665 Kongjiang Rd, Shanghai 200092, China. xqliu@public8.sta.net.cn

Telephone: +86-21-65790000 **Fax:** +86-21-65791316

Received: 2003-06-30 **Accepted:** 2003-07-30

Abstract

AIM: To determine the mutational characterization of P-type ATP7B gene and to explore the correlation of ATP7B genotype to phenotype in Chinese patients with Wilson disease (WD).

METHODS: Seventy-five patients with WD from 72 no-kinship families, 44 males and 31 females, were enrolled in this study. The age of onset ranged from 4 to 39 years, ≤ 18 years in 72 patients. Some exons of ATP7B gene mutations were analyzed in patients with WD by using biochemical methods, polymerase chain reaction-single strand configuration polymorphism (PCR-SSCP) and DNA sequence analysis. A total of 778 coding regions were identified with restriction enzyme Msp I. The activity of Cu-ATPase was assessed by measuring inorganic phosphorus.

RESULTS: Sixty-six of 75 patients (88%) had with hepatic manifestations, 39 of them had only hepatic manifestations, 27 patients had hepatic and neurological manifestations or other symptoms at the same time (16 patients had associated neurological manifestation, 3 patients had osteopathy, 8 patients had other symptoms). Eight of the 75 patients (10.7%) had only neurological symptoms, one patient (5 years old) had no symptom. Twelve changing patterns were detected in ATP7B gene by DNA sequencing, including seven mutations (R778L, C656X, G943D, V1140A, V1106I V1216M and 1384del17), six polymorphisms (IVS4-5t/c, A2495G, C2310G, IVS18+6c/t and IVS20+5a/g). R778L occurred in 49/66 patients (74%) with hepatic manifestations, homozygosity of R778L in 16 patients, heterozygosity of R778L in 33 patients. V1106I mutation of ATP7B gene occurred in 2 patients with delaying onset of clinical symptoms. Cu-ATPase activity of three patients with known mutations (R778L/V1106I/A2495G, R778L/V1216M and R778L/R778L) were determined, and the activity of Cu-ATPase was decreased by 44.55%, 88.23% and 69.49% respectively.

CONCLUSION: 1384del17bp is a novel mutation found in

WD patients. R778L is the most common mutation of ATP7B gene. There is a correlation between R778L and hepatic manifestations in WD patient.

Liu XQ, Zhang YF, Liu TT, Hsiao KJ, Zhang JM, Gu XF, Bao KR, Yu LH, Wang MX. Correlation of ATP7B genotype with phenotype in Chinese patients with Wilson disease. *World J Gastroenterol* 2004; 10(4): 590-593

<http://www.wjgnet.com/1007-9327/10/590.asp>

INTRODUCTION

Wilson disease (WD), or hepatolenticular degeneration, is an autosomal recessive disorder of copper metabolism caused by ATP7B gene mutation. The clinical manifestations of copper accumulation produced by enzyme deficiency usually presented after birth, but rarely occurred before 5 years old. Patients with WD most often present with either progressive liver degeneration or neurologic symptoms, or both. Three major clinical patterns of live disease in WD are hepatic cirrhosis, chronic active hepatitis and fulminant hepatic failure. Neurological manifestations are bradykinesia, rigidity, tremor and dyskinesia, *etc.* Copper-transporting P-type ATP7B gene has been identified as a defective gene in WD. The WD locus (ATP7B, OMIM#277900), consisting of 21 exons, has been isolated by using YAC mapping on 13q14.3^[1]. Mutation screening in WD patients has led to the detection of at least 200 disease-specific mutations (HGMD). The published data suggest that some mutations appear to be population specific, while others are common to many populations. Arg778Leu has been identified as the most common mutation in the Asian population, accounting for 28-44% of WD chromosomes. The codon H1069Q mutation has been reported to have a high allele frequency in the eastern and northern European populations. This study was to investigate the mutational characterization of P-type ATP7B gene and to explore the correlation between genotype and phenotype in Chinese patients with WD.

MATERIALS AND METHODS

Subject

Seventy-five patients with WD from 72 no-kinship families, 44 males and 31 females, were enrolled in this study. All WD patients were from the Neurological Clinic in Xinhua Hospital and Shanghai Children's Medical Center, Shanghai Second medical University. The age at onset of symptoms was 4 to 39 years, ≤ 18 years in 72 patients. The diagnosis of WD was confirmed by serum ceruloplasmin (or copper oxides activity), urinary copper of 24 h, liver and/or brain imaging features (CT or MRI).

Polymerase chain reaction (PCR)

DNA was isolated from peripheral blood. Some exons (3, 5, 7, 8, 10, 12, 15, 16, 17, 18 and 19) were amplified by PCR with the primers previously reported^[2]. Reactions were carried out in 50 μ l [H_2O 33 μ l, buffer (10 \times) 5 μ l, dNPT (10 mmol/L)

4 µl, primer (12.5 µmol/L) 4 µl, Taq DNA polymerase 2 units]. Amplification was performed 35 cycles, 94°C for 40 s, each cycle was at 58–65°C for 1 min, at 72°C for 1 min, degeneration at 94°C for 5 min and a final extension at 72°C for 10 min.

Single strand configuration polymorphism (PCR-SSCP) and sequencing

Five µl of PCR product and equivalence denaturing solution were degenerated at 95°C for 10 min, then they were placed directly on ice. Six µl was added into a gel, using the auto-electrophoresis system (Pharmacia Biotech). Exons exhibiting an irregular shift by SSCP were subjected to direct sequencing for mutation identification. Before direct sequencing, PCR products were purified from agarose gel, using the Quik kit. Direct sequencing was performed using the ABI PRISM™ 377 DNA sequencer (PE Applied Biosystems).

Restriction-enzyme analysis

The CGG to CTG transition at exon 8 splice acceptor site was recognized by Msp I restriction-endonuclease. In normal exon 8, PCR product (296 bp) was amplified into two products of 256 bp and 40 bp. R778L would not be recognized, and PCR product (296 bp) was not digested.

Cu-ATPase activity

Ten ml peripheral venous blood was put in a heparinization bottle, lymphocytes were collected, and then split and membrane was collected^[3]. Cu-ATPase was assessed by a kit made in Nanjing Jiancheng Biotechnology Company, and protein was quantified by UV/spectrophotometer (PE).

RESULTS

Clinical features

Sixty-six of 75 patients (88%) had hepatic manifestations, 39 of them had only hepatic manifestations (Table 1), and 27 patients of them had hepatic manifestations and other symptoms at the same time. Among the 27 patients who had both hepatic and other symptoms, 16 patients were associated with neurological manifestations, 3 patients with musculoskeleton symptoms, 8 patients with other symptoms (hemolytic anemia

or renal lesions). Eight of the 75 patients had only neurological symptoms, one patient had no symptom identified by family screening and microsatellite DNA analysis^[4].

Table 1 Clinical manifestations of WD patients

Clinical manifestation	Patient No. (%)
Cirrhosis, chronic active hepatitis	66(88)
Enlargement	25(33)
Rigidity, tremor, ataxia, dyskinesia, dysarthria,	24(32)
Behavioral disturbances, psychosis	4(5)
K-F rings	49(65)
Hemolysis, petechia	6(8)
Hematuria, proteinuria	6(8)
Amenorrhea, menoxenia, hairiness	5(7)
Degenerative joint disease	3(4)

Analysis of mutated genes

Twelve changing patterns were detected in ATP7B gene by DNA sequencing, including five missense mutations (R778L, G943D, C656X, V1140A, V1106I and V1216M), one deletion (1384del17) (Figures 1 and 2) and six polymorphisms (IVS4-5t/c, 2495A>G, C2310G, IVS18+6c/t and IVS20+5a/g) (Table 2). With restriction-enzyme analysis and sequencing in exon 8, R778L and L/L770 were identified in 54/75 WD patients, 16 patients showed homozygous, and 38 patients heterozygous.

Correlation between genotype and phenotype

In 66 patients with hepatic manifestations, 49 patients (74%) carried R778L (arginine to leucine) mutation and L/L770 polymorphism (2310 site C to G). V1106I mutation occurred in two WD patients with later onset of illness and had presenting neurological manifestations when they were 39 and 48 years old respectively.

Cu-ATPase activity

Cu-ATPase activity was determined in 3 patients with known mutations (R778L/V1106I, R778L/V1216M and R778L/R778L), activity of cu-ATPase was decreased by 44.55%, 88.23% and 69.49% respectively, compared with normal value.

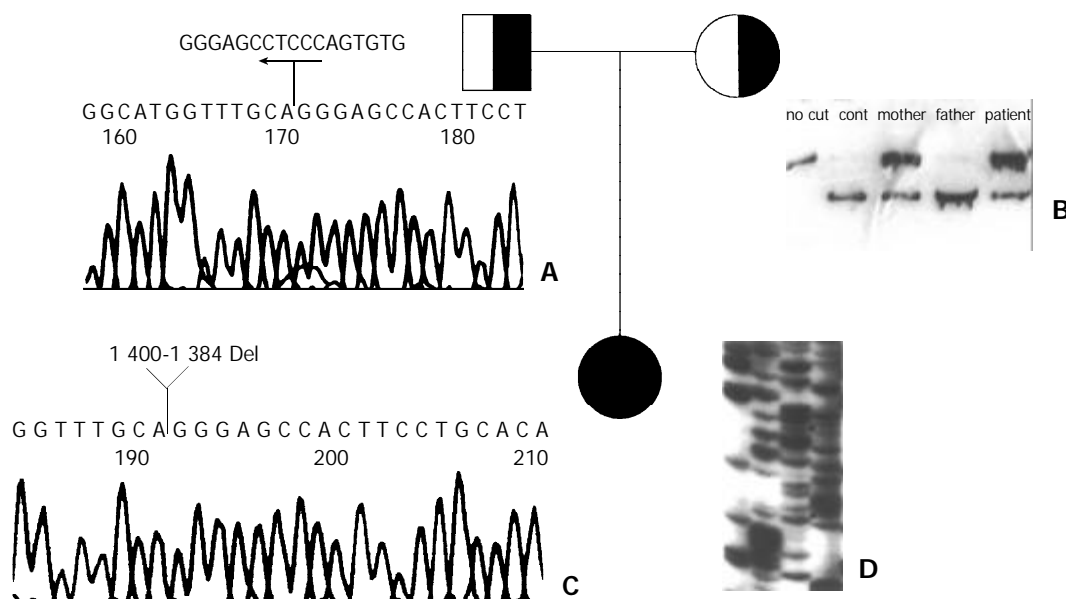


Figure 1 Family analysis of patient 038. A: Patients father carrying 1384Del17bp (ATP7B genes position 1 384-1 400 bp deletion). B: Msp I digested PCR product of ATP7B gene exon 8 of patients mother shows that she was R778L carrier. C and D: Patients genotypes are R778L/1384Del17 bp, 1384Del17 bp from his father and R778L from his mother.

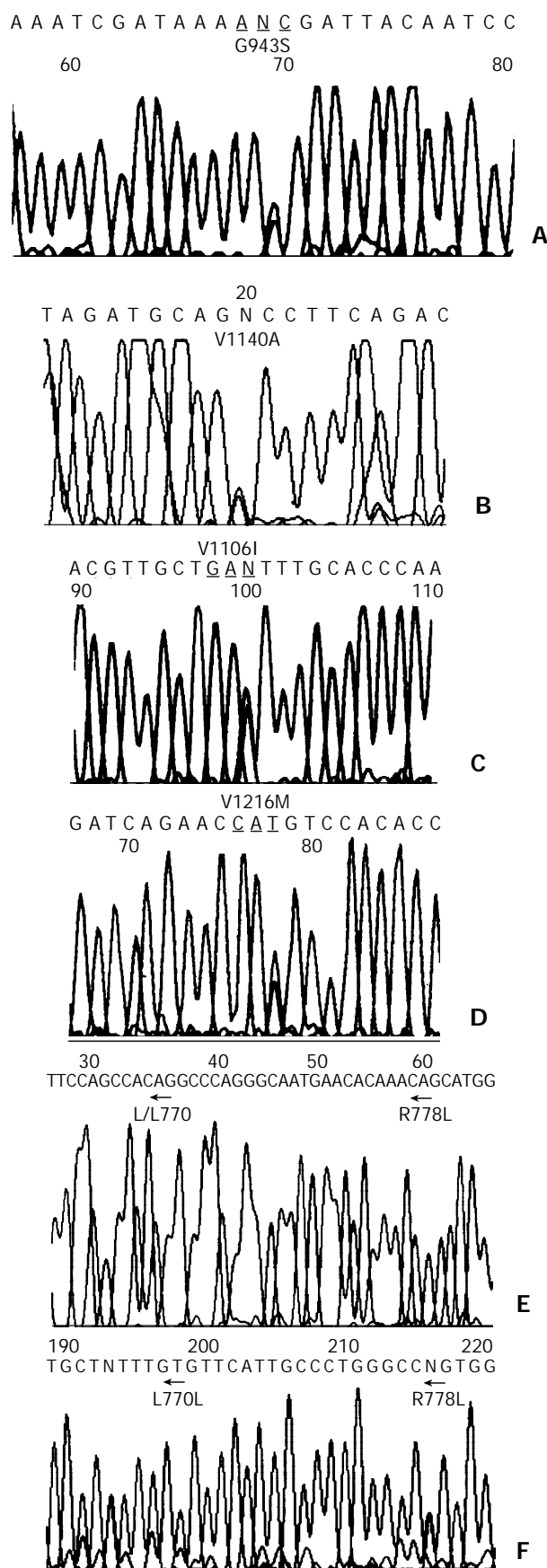


Figure 2 Nucleic acid sequence of PCR products of WD gene. A: Gly943Asp(G943D) heterozygous for a G-A mutation at position 2828, B: Val1140Ala (V1140A) heterozygous for a T-C mutation at position 3419, C: Val1106Ile (V1106I) heterozygous for a G-A mutation at position 3316, D: Val1216Met (V1216M) heterozygous for a G-A mutation at position 3646. E. and F: Arg778 Leu (R778L) homozygous and heterozygous for a G-T mutation at position 2333.

Table 2 Genotypes of ATP7B in WD patients

Exon	Mutation	Amino acid	Codon
3	1384del17	His stop	1 384 delete 17 bp
7	C656X	Cys→X	1971 C splline
8	R778L	Arg→Leu (CGG→CTG)	2333 G→T
12	G943D	Gly→Asp (GGT→GAT)	2828 G→A
15	V1106I	Val→Ile (GTC→ATC)	3316 G→A
16	V1140A	Val→Ala (GTC→GCC)	3419 T→C
17	V1216M	Val→Met (GTG→ATG)	3646 G→A
<i>polymorphism</i>			
4	IVS4-5T/C		1708-5 t→c
8	L/L770		2310 C→G
10	2495A>G		2495 A→G
18	IVS18+6C/T		3903+6 c→t
20	IVS20+5A/G		4124+5 a→g
21	IVS21+3		4398+3 t→g

DISCUSSION

Wilson disease is an autosomal recessive disorder in copper transport. Disturbance of copper metabolism leads to accumulation of copper resulting in tissue damage, principally in the liver and brain. The disorder manifests as progressive liver degeneration, which is one of the important causes of chronic liver disease in childhood, although it is rare. Without early intervention, WD has a higher mortality and morbidity. ATP7B gene for WD is distributed worldwide, and has been demonstrated in almost all races. Epidemiological survey indicated that the prevalence of the disease was 1/30 000 live births, with a corresponding gene frequency of 0.3-0.7%, and a heterozygote carrier frequency of 1%^[5]. The prevalence of the disease is high in China.

There was a correlation between the age of onset and initial manifestations. Initial clinical feature was diverse. The age of onset ranged from 6 years to 15 years old in most patients with WD. In this group of patients, 72 patients were ≤ 18 years old, accounting for 96%, and 61 patients (81%) were ≤ 12 years old. In a large series of patients, the initial clinical manifestations were found to be 42% in liver, 34% in neurological system, 10% in psychiatric system, 12% in hematological system, 1% in kidney. Less commonly, patients had skeletal or other symptoms^[5]. In our patients, the initial clinical manifestations were found to 52% in liver (Cirrhosis, chronic active hepatitis), 21% in neurological system, 33% in saliva enlargement of spleen, 8% in kidney. Skeletal symptoms occurred in 3 patients (4%). The subjects in this study were most pediatric patients, it might be responsible for higher percentage of liver impairments in this study, compared with that in other reports.

To date, at least 200 different mutations have been identified in WD chromosomes. Some mutations appear to be population specific. H1069Q has been identified as the most common mutation in the eastern and northern European populations^[6-10]. Caca *et al* reported that an allele frequency of H1069Q was 63% in 82 WD patients, homozygous in 32 patients (39%), heterozygous in 39 patients (48%)^[11]. The H1069Q mutation was found in 27/42 WD patients (64.3%) from 39 Hungarian families, homozygous in 9, heterozygous in 18^[12]. The R778L has been reported to have a high allele frequency in the Oriental populations, such as Japanese, Korean, and Chinese. The allele frequency of R778L was 37.9% in Korean patients with WD^[13,14], 27% in Japanese WD^[15-19] and 28-44% in Chinese^[20-30]. In this study all the 75 WD patients were Han people. The R778L mutation was identified in 68/144 alleles with an allele frequency of 45.6%, in 54 WD patients (72%), homozygous in 16 patients, heterozygous in 38 patients. The average age of the patients was 9.5 years in homozygous, 10.7 years in heterozygous, there was no significant difference

between two groups. In our patients, we did not detect the H1069Q mutation, the most common WD mutation found in the European population. Our data showed that patients had clinical manifestations at preschool age or school age, liver impairment was the most common clinical manifestation, and allele frequency of the R778L was higher in patients with liver impairment. It is suggested that there might be a correlation between R778L mutation and liver impairment at initial evaluation.

The V1106I mutation was identified in two WD patients with late onset of phenotypes. One patient had neurological manifestations (tremor, and dysarthria) at the age of 39, progressing slowly. She was diagnosed as WD based on the decreased activity of Cu-ATPase and serum ceruloplasmin, Kayser-Fleischer rings, and increased urinary copper excretion. Treatment with oral D- penicillamine initiated at 9 months after diagnosis was made, showed clinical improvement. The genotype of the patient (014) was V1140A/V1106I. The other patient (026) having a past history of chronic hepatitis had psychiatric symptoms at the age of 48 years. Tremor gradually appeared and became progressively worse. After oral D- penicillamine clinical symptoms were remitted. Her genotype was R778L/V1106I. The other mutation did not detected by sequencing in exons 1 to 21 in these two patients. The results of family analysis of patient (038) with 1384De117bp/R778L genotype demonstrated that 1384De117bp was originated paternally and R778L maternally. R778L/V1216M (033) was found in one patient. G to A mutation at position 3646 occurred in exon 17 of ATP7B. The results demonstrated that the activity of Cu-ATPase was significantly decreased in lymphocytic membrane by 44.55% in patients with R778L/V1106I genotype, 88.23% in patients with R778L/V1216M genotype, 69.49% in patients with R778L/R778L (034) genotype. It can be presumed that the decreased degree of Cu-ATPase is correlated with the severity of clinical manifestations and genotypes.

REFERENCES

- Bull PC**, Barwell JA, Hannah HT, Pautler SE, Higgins MJ, Lal M, Cox DW. Isolation of new probes in the region of the Wilson disease locus, 13q14.2-q14.3. *Cytogenet Cell Genet* 1993; **64**: 12-17
- Thomas GR**, Forbes JR, Roberts EA, Walshe JM, Cox DW. The Wilson disease gene: spectrum of mutations and their consequences. *Nat Genet* 1995; **9**: 210-217
- Shah AB**, Chernov I, Zhang HT, Ross BM, Das K, Lutsenko S, Parano E, Pavone L, Evgrafov O, Ivanova-Smolenskaya IA, Annerén G, Westermarck K, Urrutia FH, Penchaszadeh GK, Sternlieb I, Scheinberg IH, Gilliam TC, Petrukhin K. Identification and Analysis of Mutations in the Wilson Disease Gene (ATP7B): Population Frequencies, Genotype-phenotype Correlation, and Functional Analyses. *Am J Hum Genet* 1997; **61**:317-328
- Liu XQ**, Zhang YF, Gu XF, Bao KR. Polymorphism analysis of ATP7B related microsatellite DNA haplotype in Wilson disease. *Lab Medicine* 2002; (Suppl): S50-51
- Gollan JL**, Gollan TJ. Wilson disease in 1998: genetic, diagnostic and therapeutic aspects. *J Hepatol* 1998; **28**: 28-36
- Czlonkowska A**, Rodo M, Gajda J, Ploos van Amstel HK, Juyn J, Houwen RHJ. Very high frequency of the His1069Gln mutation in Polish Wilson disease patients. *J Neurol* 1997; **244**: 591-599
- Ivanova-Smolenskaya IA**, Ovchinnikov IV, Karabanov AV, Deineko NL, Poleshchuk VV, Markova ED, Illarioshkin SN. The His1069Gln mutation in the ATP7B gene in Russian patients with Wilson disease. *J Med Genet* 1999; **36**: 174
- Tamacka B**, Gromadzka G, Rodo M, Mierzejewski P, Czlonkowska A. Frequency of His1069Gln and Gly1267Lys mutations in Polish Wilson's Disease population. *Eur J Neurol* 2000; **7**: 495-498
- 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. Mutation analysis in patients of Mediterranean descent with Wilson disease: identification of 19 novel mutations. *J Med Genet* 1999; **36**: 833-836
- Palsson R**, Jonasson JG, Kristjansson M, Bodvarsson A, Goldin RD, Cox DW, Olafsson S. Genotype-phenotype interactions in Wilson's disease: insight from an Icelandic mutation. *Eur J Gastroenterol Hepatol* 2001; **13**: 433-436
- Caca K**, Ferenci P, Kühn HJ, Polli C, Willgerodt H, Kunath B, Hermann W, Mössner J, Berr F. High prevalence of the H1069Q mutation in East German patients with Wilson disease: rapid detection of mutations by limited sequencing and phenotype-genotype analysis. *J Hepatol* 2001; **35**: 575-581
- Fireisz G**, Lakatos PL, Szalay F, Polli C, Glant TT, Ferenci P. Common mutations of ATP7B in Wilson disease patients from Hungary. *Am J Med Genet* 2002; **108**: 23-28
- Yoo HW**. Identification of novel mutations and the three most common mutations in the human ATP7B gene of Korean patients with Wilson disease. *Genet Med* 2002; **4**: 43S-48S
- Kim EK**, Yoo OJ, Song KY, Yoo HW, Choi SY, Cho SW, Hahn SH. Identification of three novel mutations and a high frequency of the Arg778Leu mutation in Korean patients with Wilson disease. *Hum Mutat* 1998; **11**: 275-278
- Okada T**, Shiono Y, Hayashi H, Satoh H, Sawada T, Suzuki A, Takeda Y, Yano M, Michitaka K, Onji M, Mabuchi H. Mutational analysis of ATP7B and genotype-phenotype correlation in Japanese with Wilson's disease. *Hum Mutat* 2000; **15**: 454-462
- Shimizu N**, Nakazono H, Takeshita Y, Ikeda C, Fujii H, Watanabe A, Yamaguchi Y, Hemmi H, Shimatake H, Aoki T. Molecular analysis and diagnosis in Japanese patient with Wilson's disease. *Pediatr Int* 1999; **41**: 409-413
- Kusuda Y**, Hamaguchi K, Mori T, Shin R, Seike M, Sakata T. Novel mutations of the ATP7B gene in Japanese patients with Wilson disease. *J Hum Genet* 2000; **45**: 86-91
- Takeshita Y**, Shimizu N, Yamaguchi Y, Nakazono H, Saitou M, Fujikawa Y, Aoki T. Two families with Wilson disease in which siblings showed different phenotypes. *J Hum Genet* 2002; **47**: 543-547
- Nanji MS**, Nguyen VTT, Kawasoe JH, Inui K, Endo F, Nakajima T, Anezaki T, Cox DW. Haplotype and mutation analysis in Japanese patients with Wilson disease. *Am J Hum Genet* 1997; **60**: 1423-1429
- Lee CC**, Wu JY, Tsai FJ, Kodama H, Abe T, Yang CF, Tsai CH. Molecular analysis of Wilson disease in Taiwan: identification of one novel mutation and evidence of haplotype-mutation association. *J Hum Genet* 2000; **45**: 275-279
- Chuang LM**, Wu HP, Jang MH, Wang TR, Sue WC, Lin BJ, Cox DW, Tai TY. High frequency of two mutations in codon 778 in exon 8 of the ATP7B gene in Taiwanese families with Wilson disease. *J Med Gene* 1996; **33**: 521-523
- Fan Y**, Yu L, Jiang Y, Xu Y, Yang R, Han Y, Cui Y, Ren M, Zhao S. Identification of a mutation hotspot in exon 8 of Wilson disease gene by cycle sequencing. *Chin Med J* 2000; **113**: 172-174
- Yang RM**, Fan YX, Yu L, Cai YL, Shi SL, Wang XP, Han YZ, Ren MS, Zhao SY. Identification of a novel mutation in Wilson disease gene. *Zhonghua Yixue Zazhi* 1997; **77**: 344-347
- Wu ZY**, Wang N, Lin MT, Fang L, Murong SX, Yu L. Mutation analysis and the correlation between genotype and phenotype of Arg778Leu mutation in chinese patients with Wilson disease. *Arch Neurol* 2001; **58**: 971-976
- Wang Y**, Wu ZY, Murong ZX, Lin MT, Fang L. Hot point mutations of Wilson disease gene in Chinese with DNA sequencing. *Zhonghua Shenjingke Zazhi* 1998; **31**: 20-24
- Xu P**, Liang X, Jankovic J, Le WD. Identification of a high frequency of mutation at exon 8 of the ATP7B gene in a Chinese population with Wilson disease by fluorescent PCR. *Arch Neurol* 2001; **58**:1879-1882
- Liu XQ**, Zhang JM, Gu XF, Wu J, Zhang YF, Xue HP, Xu XP, Bao KR, Chen RG. A study on the relationship between Arg778Leu mutation of ATP7B gene and clinical phenotype in Wilson disease. *Zhonghua Erke Zazhi* 1999; **37**: 359-361
- Liu XQ**, Zhang YF, Liu TT, Gu XF, Bao KR. Clinical application of D13S301 maker in the diagnosis of children with Wilson disease. *Linchuang Erke Zazhi* 2002; **20**: 614-616
- Wu Z**, Wang N, Murong S, Lin M. Identification and analysis of mutations of the Wilson disease gene in Chinese population. *Chin Med J* 2000; **113**: 40-43
- Tsai CH**, Tsai FJ, Wu JY, Chang JG, Lee CC, Lin SP, Yang CF, Jong YJ, Lo MC. Mutation analysis of Wilson disease in Taiwan and description of six new mutations. *Hum Mutat* 1998; **12**: 370-376

Edited by Wang XL