Analysis of Exon 8 of ATP7B Gene in Thai Patients with Wilson Disease

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Objective: *Determine the frequency of mutations in exon 8 of ATP7B gene.*
Material and Method: *The exon 8 of ATP7B gene in twenty 20 unrelated Thai patients with Wilson disease (WD) was analyzed.*
Results: *Three heterozygous mutations were identified in four patients. The Arg778Leu (G2333T) and 2299insC mutations have been previously reported. The authors also identified a novel missense mutation, Thr766Arg (C2297G). Despite the Arg778Leu mutation being common in East Asian populations, its frequency in Thais was only 5% in the presented patients.*
Conclusion: *Sequencing of the exon 8 of the ATP7B gene is insufficient for the diagnostic service testing in Thais.*

Keywords: *Wilson disease, Exon 8, ATP7B gene, Arg778Leu*

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Wilson disease (WD) is an autosomal recessive disorder of copper excretion resulting in an accumulation and toxicity of copper frequently affecting the brain and liver[1]. Unlike many other genetic diseases, effective treatment for WD is available. Copper chelating agents, such as D-penicillamine, trientine, and ammonium tetrathiomolybdate, promote copper excretion and zinc helps in reducing intestinal copper absorption. Thus family members of patients, especially first-degree relatives, are necessarily screened for WD in order to receive early treatment. Patients with neurological disease are often not difficult to diagnose since they almost invariably develop Kayser-Fleischer ring and low level of serum ceruloplasmin. However, diagnoses in some patients with hepatic disease are difficult since biochemical indicators can be normal or similar to the value of heterozygous carriers[1].

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WD is caused by mutations of a copper transporting P-type ATP synthase (*ATP7B*) gene in chromosome 13q14.3[2]. There are over 300 mutations located throughout 21 exons of the *ATP7B* gene of which mutations are often compound heterozygous. However, a few common mutations occur in a high frequency as hot spots in specific populations. Arg 778Leu mutation in exon 8 has occurred at 12-39% in the East Asian populations including Chinese, Japanese, and Korean[3-5]. Other mutations in exon 8, such as 2304delC and C2250G mutations are also relatively common in Chinese suggesting that exon 8 may be a hot spot for identification of WD mutations in East Asians[6]. His1069Gln mutation is a common mutation in the eastern and northern European populations, which accounts for up to 49%[7,8]. Some mutations appear to be common in specific countries, such as C813A mutation in India and 3402delC mutation in Brazil[9,10].

According to the data of common Arg778Leu mutation in East Asian populations, the authors conducted a study on exon 8 of the *ATP7B* gene in Thai WD patients. If mutations in exon 8 are a highly frequent cause of WD in the presented population, analysis of

only exon 8 in combination with bio-chemical tests may be practical for service testing of WD in Thailand.

Material and Method

Patients

The authors reviewed clinical data from the medical records of patients with WD who were treated at the Department of Medicine, Ramathibodi Hospital between January 1994 and October 2006. Thirty-five unrelated patients were included. The inclusion criteria were clinical manifestations compatible with WD plus one or more positive laboratory results including low serum ceruloplasmin (< 20 mg/dl), high copper in liver tissue (> 250 μg/g dry weight), or high urine copper excretion (> 100 μg/day). Fifteen patients were male and 20 patients were female. Age at onset ranged from 1.5 to 72 years. All patients with neurological disease had age onset of less than 40 years old (ranging from 11 to 39 years) whereas one-third of patients with hepatic disease had age of onset of over 40 years old. Twenty out of 35 patients had available DNA samples for the present study. There was no known consanguinity between parents of each patient.

Exon 8 analysis

Genomic DNA samples were isolated from peripheral blood samples by standard techniques. PCR-based screening of the exon 8 of the *ATP7B* gene was conducted as previously described[2]. Automated direct sequencing of both forward and reverse strands of the PCR products was performed using a Bigdye Terminator sequencing kit (Applied Biosystems) and an ABI 3100 DNA sequencer. All sequence data were compared to the normal gene sequence (NCBI reference mRNA sequence; NM_00053) using Bioedit Sequence Alignment Editor program[11].

A PCR-RFLP was designed to screen for the new C2297G mutation, in which a restriction site for the endonuclease *Hae*III recognized the presence of the mutant nucleotide and three other restriction sites including nucleotide position 2153, 2207 and 2332. In the presence of the mutant nucleotide at position 2297, the 296-bp product is cleaved into five fragments of 53, 54, 92, 32 and 65 bp while the wild-type PCR products is cleaved into four fragments of 53, 54, 124, and 65 bp (Fig. 1b).

Four of the human ATP7B protein was analyzed for comparison of amino acids of the transmembrane domain by using Prodom database. They were automatically generated from the SWISS-PROT and TrEMBL sequence databases[12].

Results

The authors identified three heterozygous mutations in four unrelated patients, of which two mutations were previously described and one was a novel missense mutation (Table 1). All identified mutations were heterozygous suggesting that these patients had compound heterozygous mutations. There were G to T at the nucleotide position 2333 (G2333T, Arg788Leu), C insertion at the position 2299 (2299insC) and a novel C to G at the nucleotide position 2297 (C2297G, Thr766Arg) (Fig. 1a). The new Thr766Arg was identified in a 22-year-old patient who developed Parkinsonism, marked postural tremor and Kayser-Fleischer ring for 6 months. It was absent in 100 normal control samples. Amino acid position 766 is in a transmembrane domain 4 (Tm4) of the ATP7B. Sequence of amino acids of the Tm4 was compared to transmembrane domain of ATP7 protein of other species showing that it is very highly conserved between different species (Fig. 1c).

The common Arg788Leu was identified in two unrelated patients with neurological disease. The 2299insC mutation was identified in monozygotic twins both of whom had combined neurological and

Table 1. Summary of clinical and molecular features of patients with identified mutations of *ATP7B* gene

Patient ID	Sex	Age at onset (years)	Duration (years)	Clinical manifestations	Mutations	Type of mutations
4*	M	20	2	Parkinsonism, cirrhosis	2299insC	Frameshift
5*	M	22	3/12	Parkinsonism, cirrhosis	2299insC	Frameshift
6	M	11	8	Postural tremor	G2333T	Missense
11	M	22	6/12	Parkinsonism	C2297G**	Missense
20	F	13	8/12	Ataxia, parkinsonism	G2333T	Missense

* Patient 4 and 5 are monozygotic twin

** C2297G mutation is a novel mutation, which is firstly described in this study

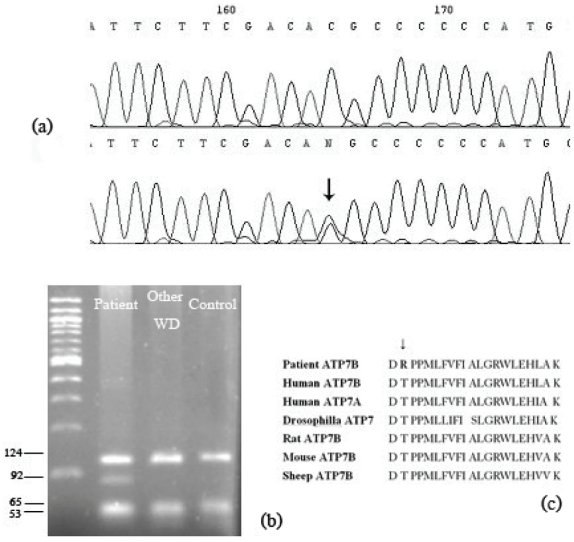

Fig. 1 Analysis of the C2297G mutation. (a) Electropherogram shows the heterozygous C to G transitions (arrows on lower panel) at position 2297 of the ATP7B gene. (b) Agarose gel electrophoresis containing products following digestion of the original 296-bp product with HaeIII. In the present of the mutant nucleotide, the 296-bp product is cleaved into 5 fragments of 53, 54, 92, 32 and 65 bp while the wild-type PCR products is cleaved into 4 fragments of 53, 54, 124 and 65 bp (The 53, 54 and 65 bp bands are unable to be visualized separately). (c) Table demonstrating amino acid position 766 (arrow) and part of Tm4 highly conserved among various species

hepatic diseases, Parkinsonism, and cirrhosis. The younger brother, who developed symptoms three months before treatment started, had a better outcome. The older brother had onset two years earlier than his brother. He became bedridden from Parkinsonism although his liver function tests were back to normal.

Discussion

ATP7B gene encodes a copper transporting P-type ATPase and it is most richly expressed in the liver[1]. Other tissues that express ATP7B include brain, kidney, heart, lung, mammary gland, and placenta[1]. Almost two-thirds of all WD mutations are missense mutations[2]. The distribution of these mutations over *ATP7B* gene are mostly clustered between exons 6 and 8, which encode a region just upstream of the first transmembrane domain (Tm), and between exons 21 and 22, a region encoding the last Tm domain[2]. Although, most WD mutations are often identified in single families, some mutations are common, and account for a large number of WD patients. Common mutations are often regional-specific, such as His1069Gln mutations in Europeans and North Americans, and Thr778Leu mutations in East Asians[13]. Mutations in exon 8, especially mutations at codon 778, are widely observed to be most common in East Asian populations including Chinese, Japanese and Korean[3,5,6,13-16].

The authors have identified three different heterozygous mutations in exon 8 of *ATP7B* gene in five WD patients of Thai origin in which a novel C2297G mutation is described. It is a missense mutation, which causes an amino acid substitution from threonine to arginine (Thr766Arg). It is in the Tm4 domain of the ATP7B, which is functionally important and highly evolutionarily conserved between different species. Several mutations have been described in Tm4, such as Asp765Gly, Pro768His, Met769Ile and the common Arg778Leu, which were predicted to disrupt Tm4[14-16]. The mutation was also absent in 100 ethnic-matched control samples. These evidences support a pathogenic role of the novel Thr766Arg mutation.

The Arg778Leu and 2299ins-C mutations were previously reported. The Arg778Leu has been reported as the most common mutation of the WD gene in East Asian populations[3-5]. However, the authors identified Arg778Leu mutation in only 5% of the studied chromosomes. Thai WD patients appear to have the lowest prevalence of Arg778Leu mutation compared to previous reports from other East Asian countries. The third mutation, 2299ins-C mutation, was a frameshift mutation, which is also one of the common mutations in various ethnic groups[2,4]. All identified mutations in exon 8 accounts for 10% of the studied WD chromosomes. Therefore, analysis of Arg778Leu or exon 8 sequencing is not sufficient for routine genetic testing in the presented population. Other approaches, such as those using polymorphic dinucleotide markers or intragenic single nucleotide polymorphism markers as genetic testing may be helpful to identify pre-symptomatic siblings of the affected individuals[4,9].

In summary, the authors identified a novel Thr766Arg mutation in a Thai WD patient. Overall mutations of exon 8 of the *ATP7B* gene in Thais appear to be less prevalent compared to other East Asians. Further direct sequencing of the whole *ATP7B* gene will be helpful to identify other more common WD mutations in order to plan for an appropriate genetic testing in the Thai population.

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Potential conflict of interest

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References

1. Ala A, Walker AP, Ashkan K, Dooley JS, Schilsky ML. Wilson's disease. Lancet 2007; 369: 397-408.
2. Thomas GR, Forbes JR, Roberts EA, Walshe JM, Cox DW. The Wilson disease gene: spectrum of mutations and their consequences. Nat Genet 1995; 9: 210-7.
3. Chuang LM, Wu HP, Jang MH, Wang TR, Sue WC, Lin BJ, et al. High frequency of two mutations in codon 778 in exon 8 of the ATP7B gene in Taiwanese families with Wilson disease. J Med Genet 1996; 33: 521-3.
4. Nanji MS, Nguyen VT, Kawasoe JH, Inui K, Endo F, Nakajima T, et al. Haplotype and mutation analysis in Japanese patients with Wilson disease. Am J Hum Genet 1997; 60: 1423-9.
5. Park S, Park JY, Kim GH, Choi JH, Kim KM, Kim JB, et al. Identification of novel ATP7B gene mutations and their functional roles in Korean patients with Wilson disease. Hum Mutat 2007; 28: 1108-13.
6. Xu P, Liang X, Jankovic J, Le W. 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: 1879-82.
7. Maier-Dobersberger T, Ferenci P, Polli C, Balac P, Dienes HP, Kaserer K, et al. Detection of the His1069Gln mutation in Wilson disease by rapid polymerase chain reaction. Ann Intern Med 1997; 127: 21-6.
8. Todorov T, Savov A, Jelev H, Panteleeva E, Konstantinova D, Krustev Z, et al. Spectrum of mutations in the Wilson disease gene (ATP7B) in the Bulgarian population. Clin Genet 2005; 68: 474-6.
9. Gupta A, Aikath D, Neogi R, Datta S, Basu K, Maity B, et al. Molecular pathogenesis of Wilson disease: haplotype analysis, detection of prevalent mutations and genotype-phenotype correlation in Indian patients. Hum Genet 2005; 118: 49-57.
10. Deguti MM, Genschel J, Cancado EL, Barbosa ER, Bochow B, Mucenic M, et al. Wilson disease: novel mutations in the ATP7B gene and clinical correlation in Brazilian patients. Hum Mutat 2004; 23: 398. DOI: 10.1002/humu.9227. PubMed PMID: 15024742.
11. Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser 1999; 41: 95-8.
12. Corpet F, Servant F, Gouzy J, Kahn D. ProDom and ProDom-CG: tools for protein domain analysis and whole genome comparisons. Nucleic Acids Res 2000; 28: 267-9.
13. Ferenci P. Regional distribution of mutations of the ATP7B gene in patients with Wilson disease: impact on genetic testing. Hum Genet 2006; 120: 151-9.
14. Gu YH, Kodama H, Du SL, Gu QJ, Sun HJ, Ushijima H. Mutation spectrum and polymorphisms in ATP7B identified on direct sequencing of all exons in Chinese Han and Hui ethnic patients with Wilson's disease. Clin Genet 2003; 64: 479-84.
15. 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-8S.
16. Okada T, Shiono Y, Hayashi H, Satoh H, Sawada T, Suzuki A, et al. Mutational analysis of ATP7B and genotype-phenotype correlation in Japanese with Wilson's disease. Hum Mutat 2000; 15: 454-62.

การวิเคราะห์ Exon 8 ในจีน ATP7B ในผู้ป่วยโรค Wilson disease ชาวไทย

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ผู้ป่วยโรค Wilson disease ส่วนใหญ่มีความผิดปกติในจีน ATP7B ในลักษณะจีนด้อย การศึกษาที่มีมาก่อนหน้านี้ในคนเอเชียตะวันออก ได้แก่ จีน, ญี่ปุ่น และเกาหลี แสดงให้เห็นว่าอาจมากถึง 50% ของผู้ป่วย Wilson disease ในชนชาติดังกล่าวมีความผิดปกติในส่วนของ Exon 8 ของจีน ATP7B ผู้นิพนธ์จึงได้วิเคราะห์ Exon 8 ในจีน ATP7B ในผู้ป่วยโรค Wilson disease ชาวไทย เพื่อหาความถี่ของลักษณะผิดปกติในยีน ATP7B ในตำแหน่งดังกล่าว ผู้นิพนธ์ค้นพบความผิดปกติ 3 แบบในผู้ป่วย 4 ราย จากผู้ป่วยทั้งหมด 20 รายดังนี้ พบ ความผิดปกติ Arg778Leu (G2333T) และ 2299insC mutations ซึ่งเคยมีรายงานมาก่อนในประเทศอื่น โดยที่ความผิดปกติ Arg778Leu เป็นความผิดปกติที่พบบ่อยที่สุดในชาวเอเชียตะวันออก แต่จากงานวิจัยนี้ พบว่าความผิดปกติชนิดนี้พบเพียง 5% ของผู้ป่วยชาวไทย นอกจากนี้คณะผู้นิพนธ์ได้ค้นพบลักษณะความผิดปกติ ชนิดใหม่ที่ไม่เคยมีรายงานมาก่อน คือ Thr766Arg (C2297G mutation)

โดยสรุป ผู้นิพนธ์รายงานความผิดปกติใน Exon 8 ในจีน ATP7B ในผู้ป่วย Wilson disease ชาวไทยพบว่าความถี่ที่พบความผิดปกติในบริเวณดังกล่าวต่ำกว่าที่รายงานในประเทศแถบเอเชียตะวันออกอื่น ๆ มาก ไม่เพียงพอที่จะใช้เป็นการทดสอบเพื่อการวินิจฉัยในงานบริการได้