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Development of a high-resolution melting method for the screening of Wilson disease-related *ATP7B* gene mutations

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ABSTRACT

Background: Wilson disease is an autosomal recessive inherited disorder of copper metabolism. The condition is characterized by excessive deposition of copper in many organs and tissues. The major physiologic aberration is excessive absorption of copper from the small intestine and impaired biliary copper excretion. The genetic defect is located at copper-transporting adenosine triphosphatase (ATPase) gene (*ATP7B*).
Methods: A high-resolution melting analysis (HRM) was designed to characterize the *ATP7B* hotspot mutations. Genomic DNA was extracted from peripheral blood samples from 14 patients and 50 normal controls. The 21 exons of *ATP7B* were screened by HRM analysis. Our methodology was confirmed by direct DNA sequencing.
Results: We have confirmed the 10 different hotspot mutations and 7 polymorphisms in the *ATP7B* gene, and also identified 1 newly-identified sequence variant (p.A476T) and 1 novel SNP (p.L776L) in 50 normal Taiwanese individuals. We estimate that the carrier frequency of WD in the Taiwanese population as probably 0.03.
Conclusions: HRM analysis is accepted as a rapid, accurate and low-cost method to screen *ATP7B* gene mutations.

1. Introduction

Wilson disease (WD) is an autosomal recessive inherited disorder of copper metabolism resulting in pathological accumulation of copper in many organs and tissues. The characterizations of the disease are the presence of liver disease, neurologic symptoms and Kayser–Fleischer ring. The WD is characterized by excess hepatic copper accumulation and impaired biliary copper excretion. The consequence of copper accumulation is the development of severe neurologic and hepatic disease [1]. In 1993, the *ATP7B* gene was cloned and found to encode a copper-transporting P-type ATPase required for biliary copper excretion. Although the characterization of the molecular genetic basis of this disease has provided insight into the mechanisms of copper homeostasis, clinical studies of specific patients have not been useful in elucidating the mechanism of hepatic copper metabolism [2]. Wilson disease is lethal if left untreated. Incidence is estimated to be 1:30,000 in most populations [3].

The diagnosis of WD is determined by the signs and symptoms, in conjunction with laboratory testing that indicates impaired hepatic copper metabolism. However, these standard tests may give false-positive results or false-negative results. Failure to diagnose a WD patient can result in lost opportunities for prophylactic therapy, whereas a false-positive diagnosis may lead to an inappropriate administration of potentially toxic drugs to those patients. Therefore, molecular diagnosis can be a good way to overcome all such limitations when all siblings and first-degree relatives of affected patients are screened. To date, over 480 mutations of the *ATP7B* gene have been reported in WD [4] over one half of these occur rarely in any given population. Most patients are compound heterozygotes, possessing alleles with two different mutations. Generally, mutations can be detected in 90% of patients. Most of them (60%) are homozygous or compound heterozygous for *ATP7B* mutations (two abnormal copies), 30% have only one abnormal copy, and 10% have no detectable mutation. The study of genotype-phenotype correlation in WD is difficult because of allelic heterogeneity [5,6]. Clinical and

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Table 1
The known mutations were used to assess the sensitivity of the HRM method for mutation scanning.

Mutation	Sequence	Exon	Allele
p.C490X	TGC→TGA	3	1
c.1708-1G>C	agA→acA	5	2
c.1708-5T>G	ttg→tgg	5	1
p.R778L	CGG→CTG	8	10
p.R778Q	CGG→CAG	8	4
p.A874V	GCG→GTG	11	2
p.P992L	CCC→CTC	13	5
p.S986F	TCC→TTC	13	1
p.Q1142H	CAG→CAC	16	1
p.T1178A	ACA→GCA	16	1

biochemical testing, which included serum concentrations of ceruloplasmin, increased excretion of urinary copper, and presence of the Kayser–Fleischer ring, usually establish a diagnosis of WD [7]. However, it is difficult to interpret in some patients because there is a diverse clinical and biochemical phenotype for WD. Molecular testing for *ATP7B* mutations has greatly enhanced the ability to diagnose WD in affected patients and also in their siblings when the diagnosis is difficult to establish by clinical and biochemical testing. Direct sequencing of *ATP7B* for disease-specific mutations is now the standard for molecular diagnosis [8]. Of the existing wide range of mutation detection methodologies, sequencing is the favored one because of its ability to identify the specific DNA sequence change. This method for large-scale detection of mutations is expensive, time consuming and labor intensive if the entire *ATP7B* gene needs to be screened. In contrast, HRM analysis is less expensive and more efficient (96 or 384 wells at the same time). Also, the PCR amplification products obtained from HRM analysis could be directly used for direct sequencing without any pretreatment. Compared with direct sequencing method, the HRM method is more feasible and economically beneficial in mutational scanning.

To date, multiple methodologies have been made available by researchers for screening *ATP7B* gene mutation which present a number of advantages and disadvantages and all have their advocates. Most of the diagnostic tools to detect *ATP7B* gene mutation are costly, complex, time consuming and require extensive sample preparation. Such methods include PCR-SSCP, direct sequencing, real-time amplification refractory mutation system (ARMS), conformation-sensitive gel electrophoresis, multiplex PCR, DHPLC, BI-PASA, reverse dot-blot, and SYBR green intercalator method based on the ARMS [9–18].

HRM method is rapidly becoming the most important mutation scanning methodology. It is a closed-tube method, which indicates that PCR amplification and subsequent analysis are sequentially performed in the well. This makes it more convenient than other scanning methodologies. This study aimed to assess the value of the HRM analysis using real-time polymerase chain reaction (PCR) (LightCycler® 480; Roche Applied Science) for scanning *ATP7B* gene mutations.

2. Patients and methods

2.1. Patients

All DNA study samples were obtained from Kaohsiung Medical University Hospital and China Medical University Hospital. A total of 14 positive samples with a diagnosis of Wilson disease with known genotype [9] and 50 unaffected individuals from the general population were analyzed in this study. Genomic DNA samples were extracted from peripheral whole blood using NucleoSpin® Blood Kit (Macherey-Nagel, GmbH & Co. KG) according to the manufacturer's instructions. This study was approved by the Institute Review Board (IRB) of Kaohsiung Medical University Hospital.

2.2. Assay design and PCR conditions

Good amplicon design was essential to obtain robust and reproducible HRM analysis. The difference between wild type and heterozygote curves became smaller and more difficult to differentiate when the product length increased [19]. Besides, extra care was needed to design PCR reactions to avoid primer dimers and non-specific amplification in HRM analysis. We designed the primer sets on the *ATP7B* DNA sequences (GenBank accession number NM_00053). In this study, 26 pairs of primers for HRM analysis were newly selected using Primer3 software (Supplementary data). Appropriate PCR fragments were named as L1–L52 as shown in Supplementary data. For exon 2, five sets of primers were used to amplify the exon in two overlapping segments.

2.3. The HRM technique

PCR reactions were carried out in duplicate in 10 μl final volume using the LightCycler ® 480 High-Resolution Melting Master (Reference 04909631001, Roche Diagnostics) 1× buffer—containing Taq polymerase, nucleotides and the dye ResoLight—and 20 ng DNA. The primers and $MgCl_2$ were used at a concentration of 0.25 μmol/l and 2.5 mmol/l, respectively, for detecting the *ATP7B* gene mutations. The HRM assays were conducted using the LightCycler® 480 Instrument (Roche Diagnostics) provided with LightCycler® 480 Gene Scanning Software Ver. 1.5 (Roche Diagnostics).

The PCR program required SYBR Green I filter (533 nm), and it consisted of an initial denaturation activation step at 95 °C for 10 min, followed by a 45-cycle program (denaturation at 95 °C for 15 s, annealing at 58 °C or 60 °C (Supplementary data) 15 s and elongation at 72 °C for 15 s with the reading of the fluorescence; acquisition mode: single). The melting program included three steps: denaturation at 95 °C for 1 min, renaturation at 40 °C for 1 min and subsequent melting that consists of a continuous fluorescent reading of fluorescence from 60 to 98 °C at the rate of 25 acquisitions per °C. The shapes of the difference-plot curves of the duplicate of each DNA sample must be reproducible both in shape and peak height.

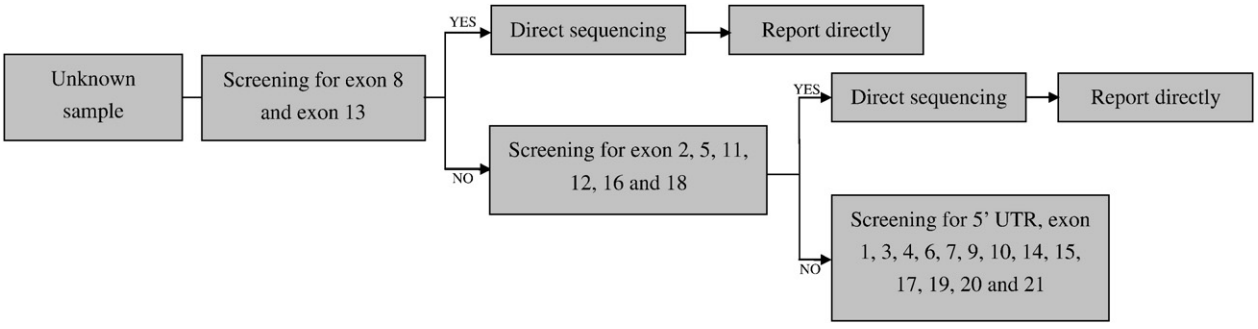

Fig. 1. The proposed ATP7B mutation detection strategy in patients with Wilson disease.