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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.

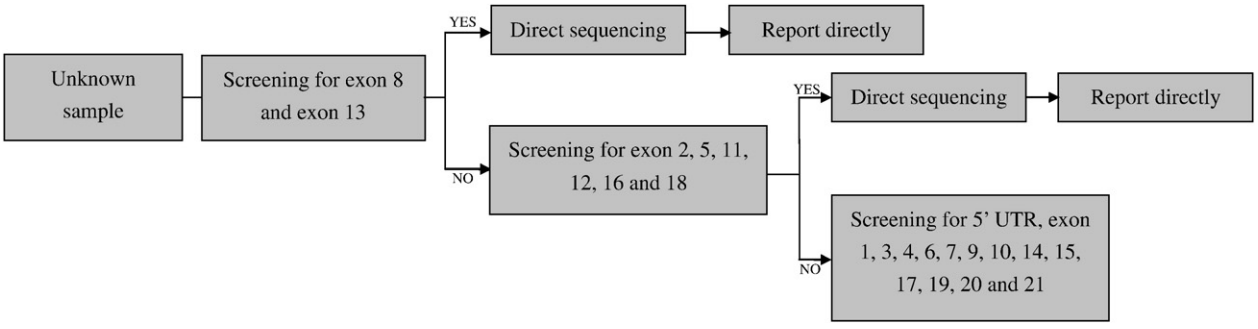


Fig. 1. The proposed ATP7B mutation detection strategy in patients with Wilson disease.

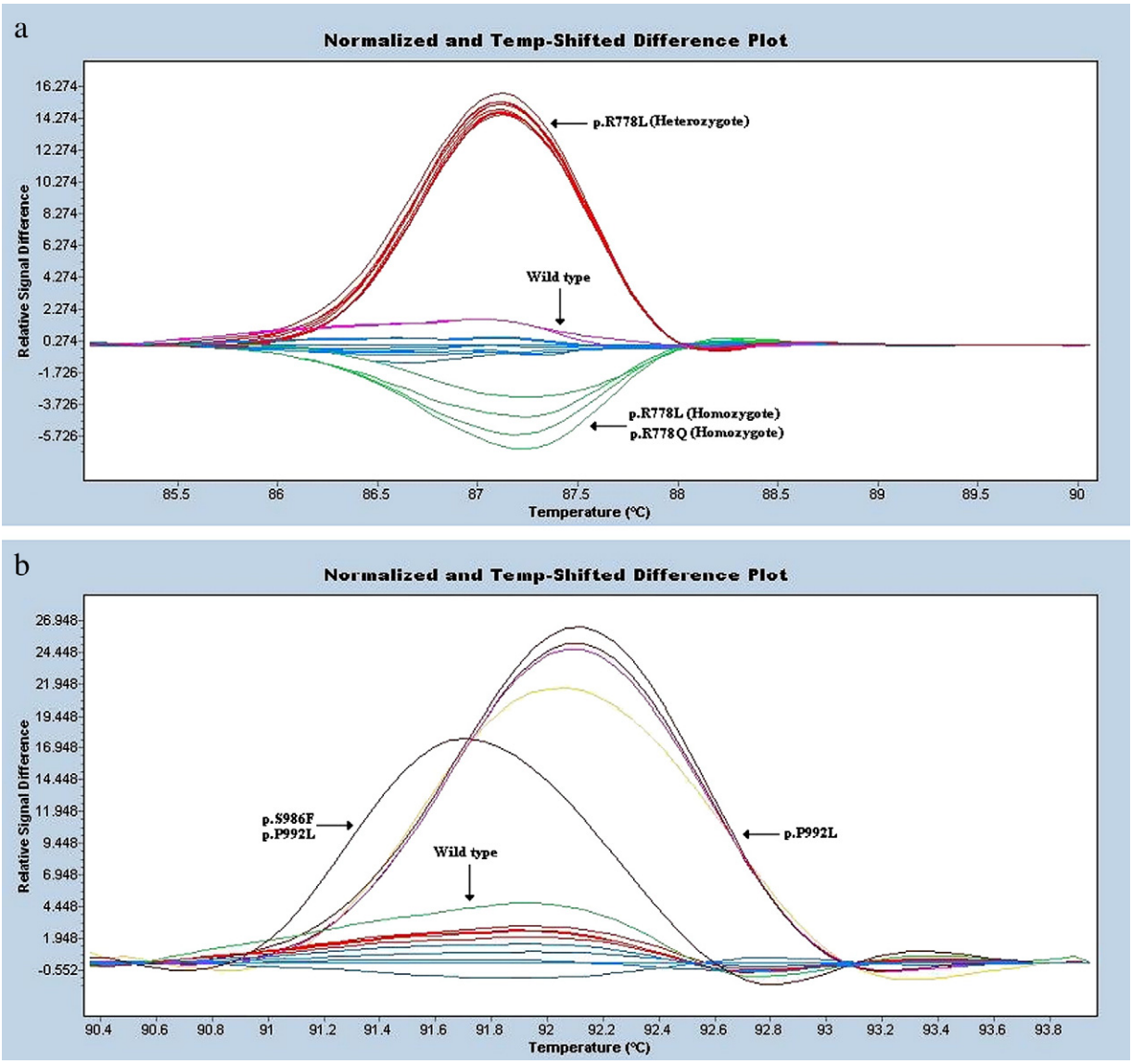


Fig. 2. Screening of the ATP7B mutation in step 1. Normalized plots, and normalized and temperature-shifted difference plots for the mutation screening of ATP7B gene of 14 patients. (a) represents heterozygous mutation of p.R778L as well as homozygous mutation of p.R778L and p.R778Q can be distinguished from wild type. (b) represents a novel mutation, p.S986F.

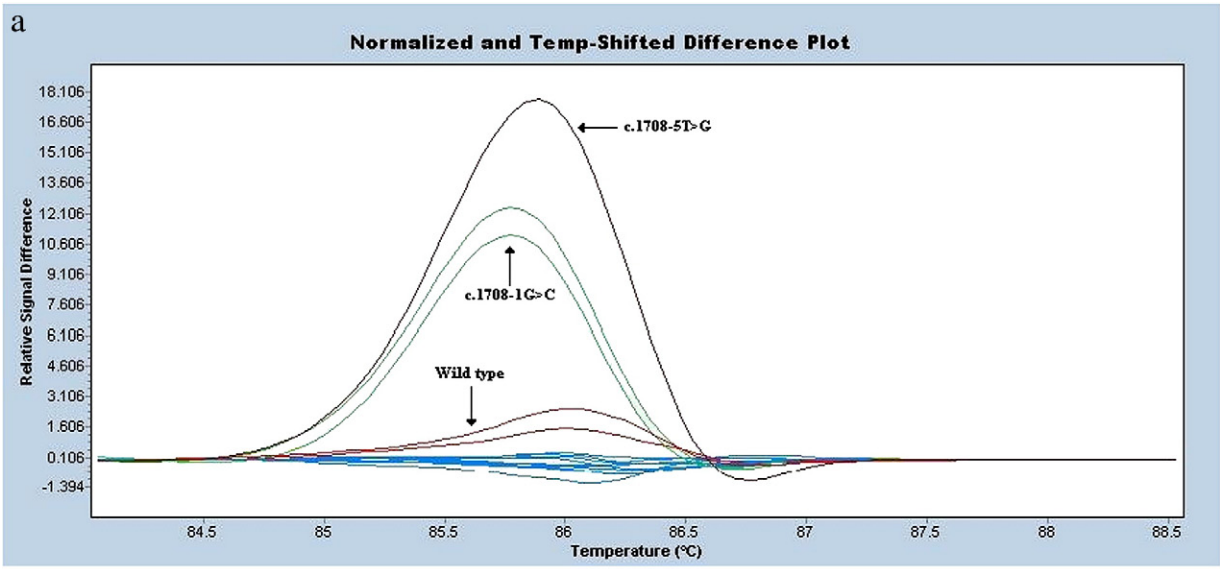
2.4. Gene scanning

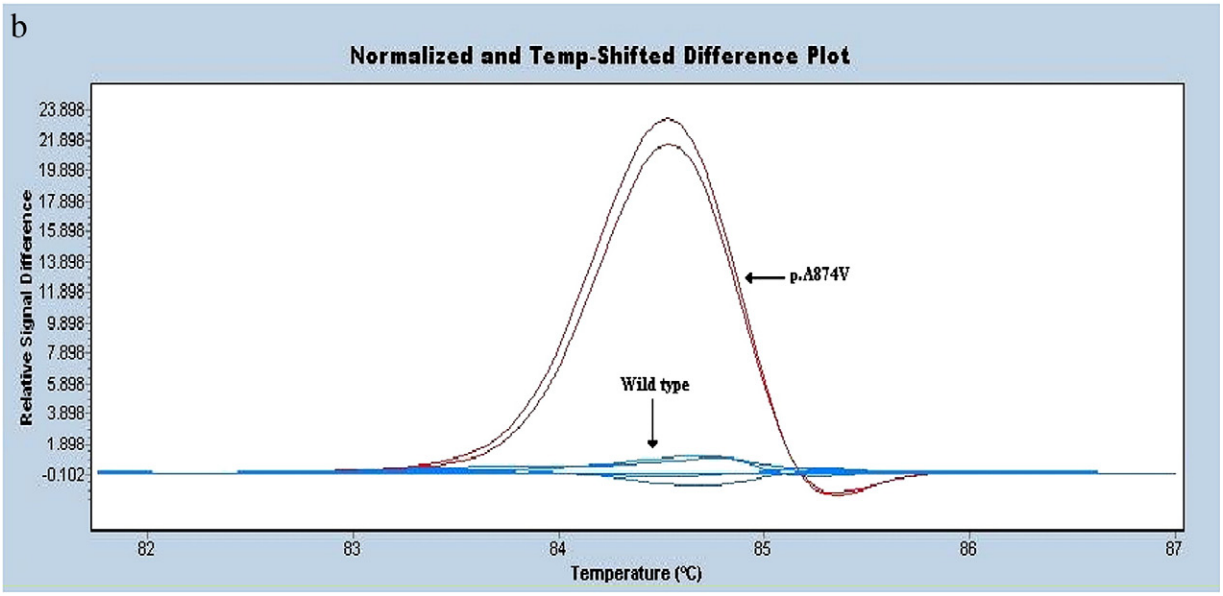
The melting curve analysis performed by the Gene Scanning Software is comprised of three steps: normalization of melting curves which involves equaling to 100% of the initial fluorescence and to 0% of the fluorescence remnant after DNA dissociation: shifting of the temperature axis of the normalized melting curves to the point where the entire double-stranded DNA is completely denatured: and finally, analyzing the difference plot analyzes the differences in melting curve shape by subtracting the curves from wild-type and mutation DNA by means of the difference plot which helps in clustering the samples into groups. In order to evaluate the discriminating power of each mutation in relation to the wild-type control DNA, the resolution which is defined as the difference of the relative fluorescence signals between the maximum of the peaks of the mutation and the wild-type DNA in the difference plot [20].

According to Laurie et al. [21], a mutation is clearly differentiated from wild-type DNA when the absolute value of resolution is greater than 5. However, the shape of the curve is also an important consideration and may indicate the presence of a variant even if the resolution is smaller than 5. If the shape of the melting curve is not similar with each other, we will confirm it by direct DNA sequencing to prevent any false-negative result. Furthermore, analysis of the melting curves with the high sensitivity setting of 0.5 was carried out by Gene Scanning software. (The default sensitivity setting of Gene Scanning software is 0.3).

2.5. Direct DNA sequencing

To confirm the results of the HRM analysis, a sequencing analysis was also performed in all samples was also performed in all samples. After HRM analysis, the samples were purified using PCR-M^{TM} clean up system (VIOGEN, Sunnyvale CA , USA). The PCR products generated after HRM were sequenced directly. The sequence reaction was performed in a final volume of 10 μl including 1 μl of the purified PCR product, 2.5 μmol/l of one of the PCR primers, 2 μl of ABI PRISM terminator cycle sequencing kit v3.1 (Applied Biosystems) and 2 μl 5× sequence buffer. The sequencing program started from 96 °C for 1 min and then followed by 25-cycle PCR program (denaturation 96 °C 10 s; annealing 50 °C 5 s and elongation 60 °C 4 min). The sequence detection was performed in the ABI Prism 310 Genetic Analyzer (Applied Biosystems) according to standard protocols.


a
Normalized and Temp-Shifted Difference Plot
Relative Signal Difference
18.106
16.606
15.106
13.606
12.106
10.606
9.106
7.606
6.106
4.606
3.106
1.606
0.106
-1.394
c.1708-5T>G
c.1708-1G>C
Wild type
84.5
85
85.5
86
86.5
87
87.5
88
88.5
Temperature (°C)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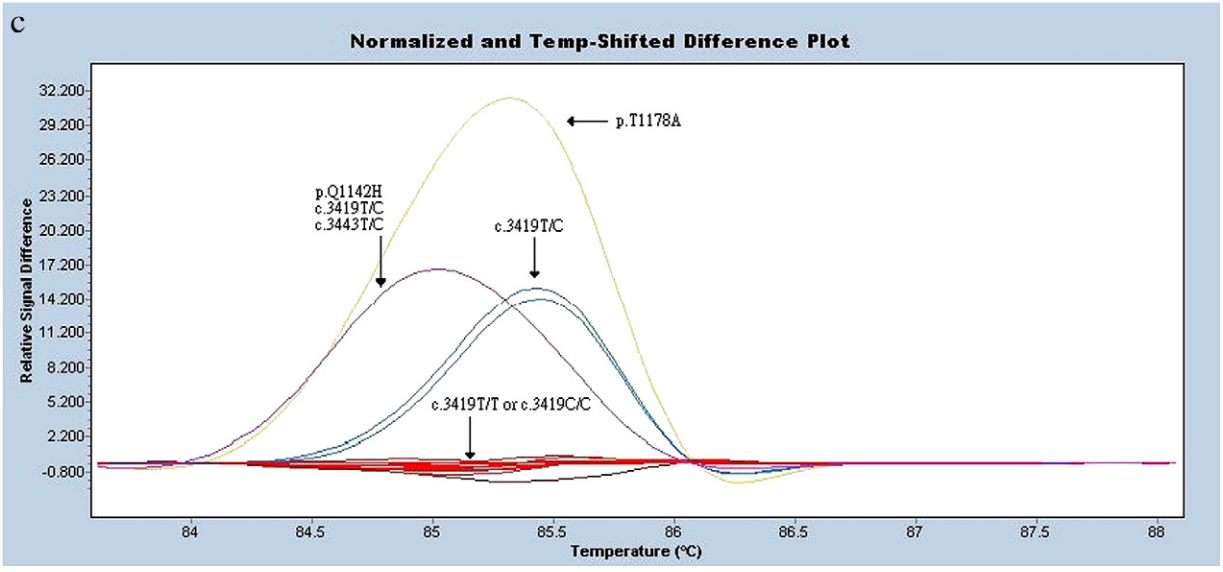
Normalized and Temp-Shifted Difference Plot
Relative Signal Difference
23.898
21.898
19.898
17.898
15.898
13.898
11.898
9.898
7.898
5.898
3.898
1.898
-0.102
p.A874V
Wild type
82
83
84
85
86
87
Temperature (°C)


c
Normalized and Temp-Shifted Difference Plot
Relative Signal Difference
32.200
29.200
26.200
23.200
20.200
17.200
14.200
11.200
8.200
5.200
2.200
-0.800
p.T1178A
p.Q1142H
c.3419T/C
c.3443T/C
c.3419T/C
c.3419T/T or c.3419C/C
84
84.5
85
85.5
86
86.5
87
87.5
88
Temperature (°C)

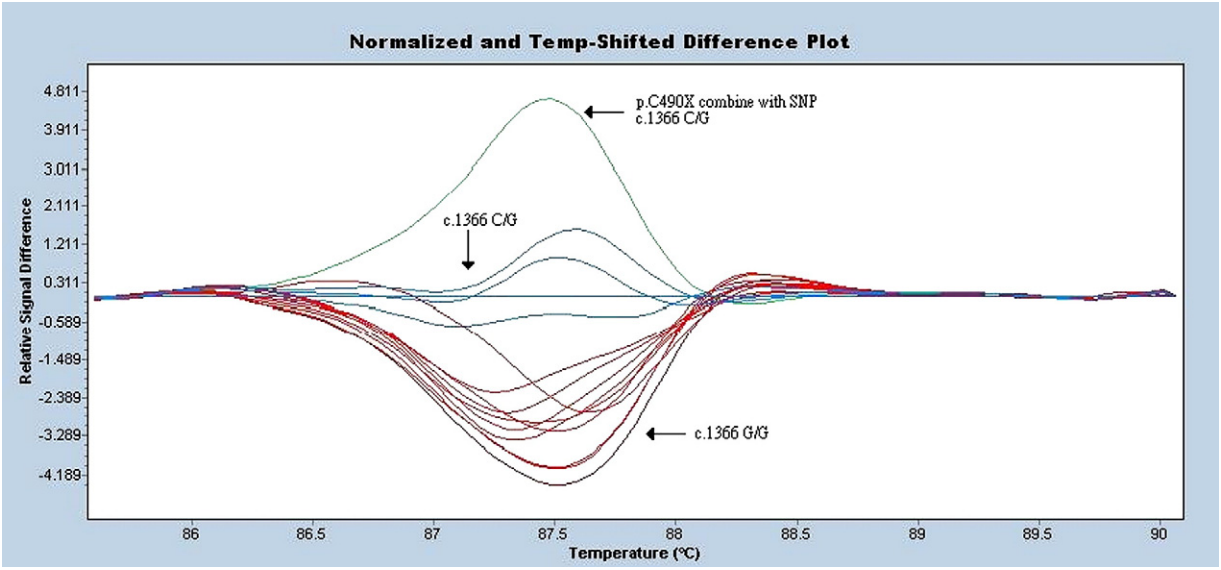


Fig. 4. Screening of the ATP7B mutation in step 3. Normalized plots, and normalized and temperature-shifted difference plots for the mutation screening of ATP7B gene of 14 patients. This figure represents the melting profile of the ATP7B mutation, c.1366C>G and p.C490X. The c.1366C>G and p.C490X are clearly distinguished in the normalized and normalized temperature-shifted difference plots.

3. Results

3.1. Optimization of HRM curve analysis

Complete *AT7PB* gene mutational screening required investigation of 21 exons. Fifty-two of primers were designed for amplification and amplicon melting of these exons. We evaluated 26 PCR amplicons (165–381 bp) with an average of 262 bp to cover the 21 exons using the 96-well LightCycler system. The melting curve data showed the difference in normalized temperature-shifted data between the mutation samples and normal samples. In this study, we easily and accurately extended application of HRM analysis to genotyping of *ATP7B* gene variants. A total of 10 known mutations (p.C490X, c.1708-1G>C, c.1708-5T>G, p.R778L, p.R778Q, p.A874V, p.P992L, p.S986F, p.Q1142H, and p.T1178A) were used to assess the sensitivity of the HRM method for mutation scanning (Table 1).

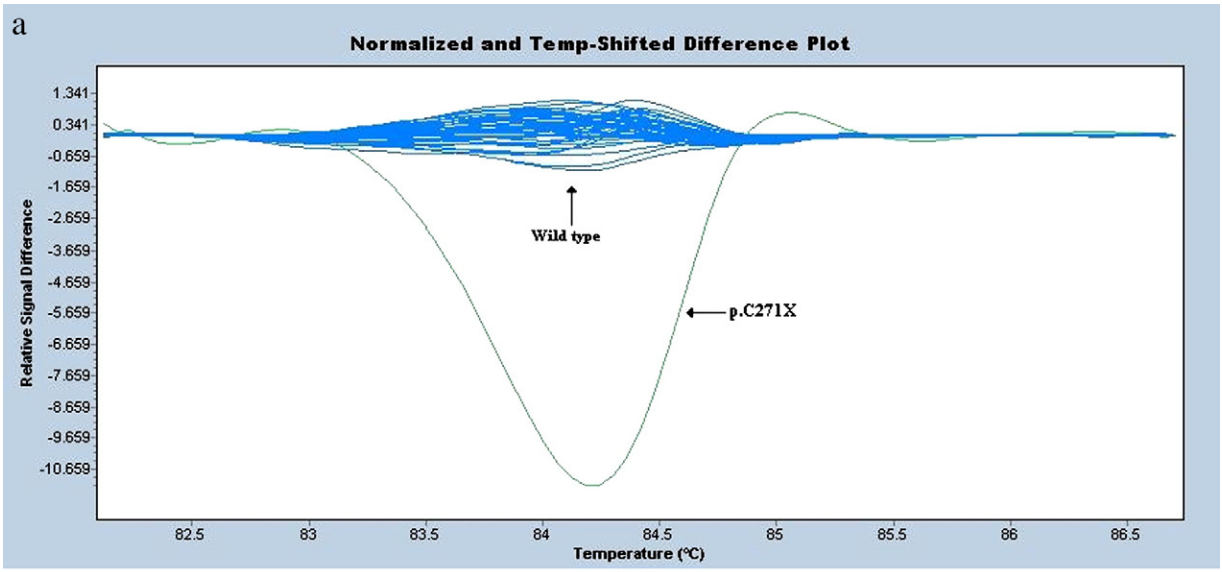
Because each population has different hotspot mutations, we proposed an *ATP7B* mutation detection strategy in patients with Wilson disease (Fig. 1). At the beginning, we screened for exon 8 and exon 13 in order to detect p.R778L and p.P992L. Based on our previous report the frequencies of p.R778L and p.P992L among Taiwanese patients are 43.1% and 14.5%, respectively [9,10]. If the clinical sample is detected by HRM as the p.R778L and p.P992L, then the results can be quickly reported to the clinicians. In this step, heterozygous mutation of p.R778L as well as homozygous mutation of p.R778L and p.R778Q can be distinguished from wild type (Fig. 2a). Interestingly, we identified a novel mutation, p.S986F (Fig. 2b). At the second step, we analyzed the mutation in the exons 2, 5, 11, 12, 16 and 18 for the cases without mutations in exons 8 and 13. The melting profiles of the *ATP7B* mutation, c.1708-1G>C, c.1708-5T>G, p.A874V, p.Q1142H, p.T1178A, are shown in Fig. 3a–c. In addition, a mutation, c.1708-5T>G reported in the population of Japan, Hong Kong and China (Fig. 3a), was first identified in a Taiwanese population [22–24]. We found a case that harbors a reported mutation, p.Q1142H. Meanwhile, this case also harbors two SNPs, c.3419T>C and c.3443T>C. These SNPs were confirmed by direct sequencing of PCR products. The melting curve will be changed if SNP occurs according to our previous publication [25]. Indeed, the melting curve shifts to the left because of the interference of SNPs (Fig. 3c).

Finally, we screened for the 5′ UTR, exons 1, 3, 4, 6, 7, 9, 10, 14, 15, 17, 19, 20 and 21. Fig. 4 represents the normalized and temperature-shifted difference plots, the melting profile of the *ATP7B* mutation, p.C490X. It is a case of heterozygous mutation of p.C490X combined with a non-disease variant point mutation c.1366C>G. It can also differentiate point mutation c.1366C>G from wild type in this primer set. All of the results were confirmed by direct DNA sequencing. We analyzed these samples using our strategy step by step: the results showed that 19 alleles with *ATP7B* mutations could be detected at the first step, 8 alleles with *ATP7B* mutations could be detected at the second step, and only one allele with *ATP7B* mutation need to be analyzed at the third step. Thus, employing this strategy the *ATP7B* mutation can be quickly identified in the large-scale clinical samples. About 67.86% of *ATP7B* mutation alleles can be detected at the first step and can achieve 96.43% at the second step. However, if an abnormal melting curve is noted, the precise mutation can be confirmed with direct DNA sequencing.

3.2. Application of HRM analysis for ATP7B mutation analysis in 50 normal individuals

We recruited 50 healthy Taiwanese subjects from the Kaohsiung Medical University Hospital. Then, we screened *ATP7B* mutation according to our strategy as previously described. Interestingly, we revealed three mutations among 50 normal individuals, including p.C271X, p.P992L, and p.D1296N. The melting profile of the *ATP7B* mutation, p.C271X, p.P992L, and p.D1296N is shown in the Fig. 5a–c. The c.3903+6T>C and p.D1296N can be clearly distinguishable from the wild type. The results show that mutation p.P992L was found in Taiwanese populations: other mutations include p.C271X, and p.D1296N which have not been identified in Taiwanese and Chinese-Han populations before. In this section, we identified 9 SNPs by HRM analysis and confirmed these by direct DNA sequencing: p.S406A,

Fig. 3. Screening of the ATP7B mutation in step 2. Normalized plots, and normalized and temperature-shifted difference plots for the mutation screening of ATP7B gene of 14 patients. (a) represents the melting profile of the ATP7B mutation, c.1708-1G>C and c.1708-5T>G. (b) represents the melting profile of the ATP7B mutation, p.A874V. (c) represents the melting profile of the ATP7B mutation, p.T1178A, p.Q1142H, c.3419T>C and c.3443T>C. The c.1708-1G>C, c.1708-5T>G p.T1178A, p.Q1142H, c.3419T>C and c.3443T>C are clearly distinguished in the normalized and normalized temperature-shifted difference plots.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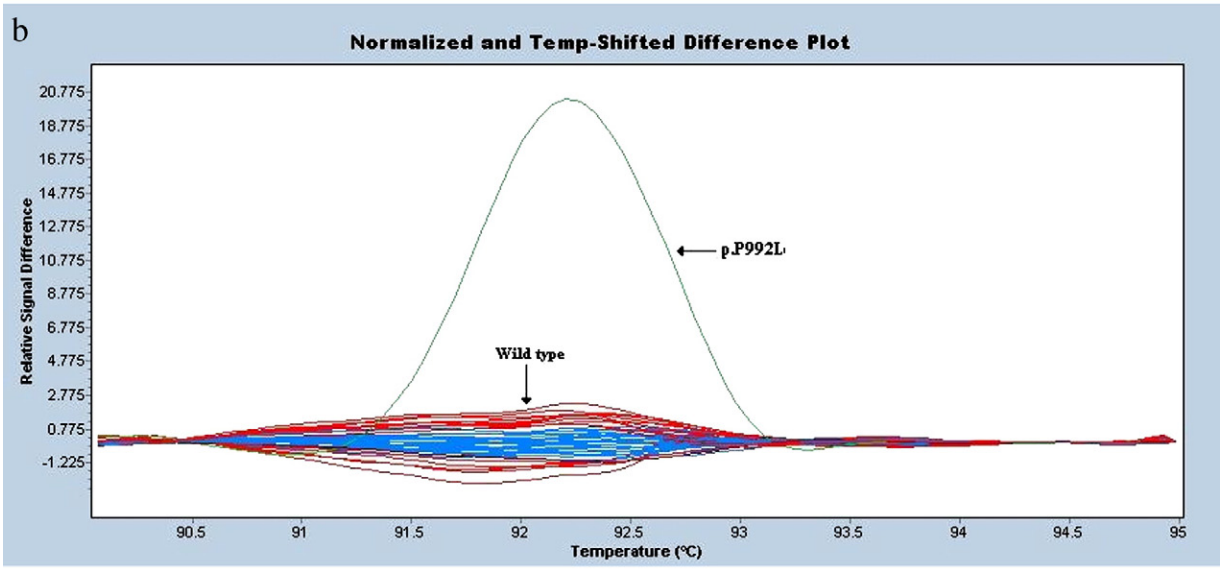
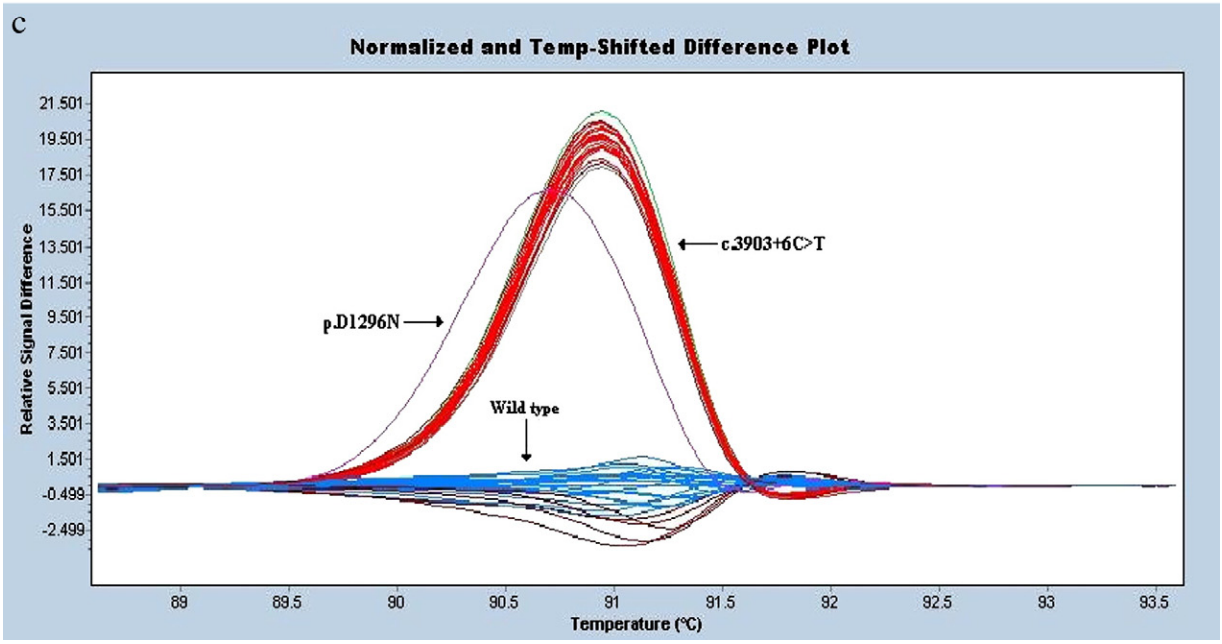
Normalized and Temp-Shifted Difference Plot
Relative Signal Difference
1.341
0.341
-0.659
-1.659
-2.659
-3.659
-4.659
-5.659
-6.659
-7.659
-8.659
-9.659
-10.659
Wild type
p.C271X
82.5
83
83.5
84
84.5
85
85.5
86
86.5
Temperature (°C)


b
Normalized and Temp-Shifted Difference Plot
Relative Signal Difference
20.775
18.775
16.775
14.775
12.775
10.775
8.775
6.775
4.775
2.775
0.775
-1.225
p.P992L
Wild type
90.5
91
91.5
92
92.5
93
93.5
94
94.5
95
Temperature (°C)


c
Normalized and Temp-Shifted Difference Plot
Relative Signal Difference
21.501
19.501
17.501
15.501
13.501
11.501
9.501
7.501
5.501
3.501
1.501
-0.499
-2.499
c.3903+6C>T
p.D1296N
Wild type
89
89.5
90
90.5
91
91.5
92
92.5
93
93.5
Temperature (°C)

p.L456V, p.A476T, p.L776L, p.K832R, p.I929V, p.K952R, p.A1140V and c.3903+6T>C. One newly-identified sequence variant, p.A476T and one novel SNP, p.L776L were identified in Taiwanese populations. Intriguingly, three SNPs including p.K832R, p.I929V, and c.3903+6T>C have not been previously reported in Taiwanese populations, but in Chinese-Han populations [11,26]. The frequencies of these nucleotide changes in 50 healthy Taiwanese subjects are shown in Table 2. Three mutant alleles were identified by screening 100 alleles for the presence of p.C271X, p.P992L and p.D1296N mutations. Therefore, we calculated that the carrier frequency is probably 0.03 (3/100) in Taiwanese population. Larger scale of samples may be necessary to confirm the carrier frequency in future study because our sample size is quite small in this study.

Table 2
Polymorphisms in *ATP7B* identified in 50 normal individuals using HRM analysis.

Polymorphism	Sequence	Exon	Allele
p.S406A	TCT→GCT	2	7
p.L456V	GTG→CTG	3	25
p.A476T	GCA→ACA	3	1
p.L776L	CTG→CTA	8	1
p.K832R	AAG→AGG	10	27
p.I929V	ATC→GTC	12	1
p.K952R	AAA→AGA	12	26
p.A1140V	GCC→GTC	16	25
c.3903+6T>C	AGAgtga	18	26

3.3. Application of HRM analysis for ATP7B mutation analysis in one family

The diagnosis of WD is suspected in one family with unknown *ATP7B* mutation. These clinical samples were obtained from parents and 2 proband. Those clinical samples were analyzed by HRM analysis and confirmed by direct DNA sequencing.

Cases 1 and 2 were siblings. Case 1 was compound heterozygous mutation (p.I1148T/p.R778L), and; Case 2 was a carrier of the heterozygous mutation, p.R778L. Their father and mother were both carriers of the heterozygous mutation, p.I1148T and p.R778L respectively (Fig. 6a,b). The HRM was able to detect the mutations precisely. The results were confirmed by direct DNA sequencing.

4. Discussion

HRM analysis represents the next generation of mutation scanning technology and offers considerable time and cost savings over the previously described methods [19]. In the presence of a saturating double-stranded DNA-binding dye, amplicons are slowly heated to full denaturation while the fluorescence is monitored [27]. Amplicons heterozygous for a sequence variant yield altered melting curves in comparison to normal control samples. Compared with those methods, HRM costs less and works more efficiently (96 or 384 wells at one time). Recently, we have used this method to detect the mutations of the *HBB* gene in Chinese and *JAK2* V617F [25,28]. Zhao X et al. [29] first reported the scanning of the *ATP7B* gene mutation in Chinese populations by HRM analysis. However, the authors designed only 2 primer sets to detect *ATP7B* gene mutations. In this study, we designed 52 PCR primers encompassing the 21 exons of the *ATP7B* gene.

Up-to-date Wilson disease has over 480 distinct mutations reported that include missense and nonsense mutations, deletions and insertions [4]. Molecular diagnosis with 21 exons sequencing analysis is time-consuming and expensive. Then, we surveyed the high prevalence mutations of the *ATP7B* gene in East Asian countries including China, Hong Kong, Japan, Korea and Taiwan. Therefore, we proposed *ATP7B* mutation detection strategy in patients with Wilson disease in this study (Fig. 1).

Wan L et al. [9] reported that WD chromosomes in the Taiwanese population are predominantly located at exons 8, 11, 12, 13, 16, 17, and 18 and the authors recommend screening these 7 exons first by PCR-RFLP and SSCP on those individuals in Taiwan who have a higher risk in having WD, before whole gene and promoter sequencing analysis. Some mutations are associated with a severe impairment of copper transportation resulting in severe liver disease very early in life; other mutations appear to be less severe with disease appearance in mid-adulthood. Recently, Davies LP et al. [30] carried out a mutation analysis on 300 WD patients of various origins and new mutations not previously reported were identified: European white (p.L217X, c.918_931, c.1073delG, c.3082_3085delAAGAinsCG, p.V536A, p.S657R, p.A971V, p.T974M, p.Q1004P, p.D1164N, p.E1173G, p.I1230V, p.M1359I, and c.2355+4A>G), Sephardic Jewish (p.Q286X), Filipino (p.G1149A), Lebanese (p.R1228T), Japanese (p.D1267V) and Taiwanese (p.A1328T). Mutation p.H1069Q is highly prevalent in Caucasians with 26–70% of all alleles [1], whereas p.R778L accounts for up to 44% frequencies in East Asia [9,24,26,32–33]. Wan et al. [9] found ten different mutations among 29 WD patients; among them four were novel (Ala11186Pro, Thr1178Ala, Ala1193Pro, and Pro1273Gln) by PCR-RFLP combined single-stranded conformation polymorphism (SSCP) analysis. The Arg778Leu mutation exhibited the highest allelic frequency (43.1%) in a Taiwanese population. According to their study, they recommend to screen codon 778 first and then perform SSCP on exons 8, 11, 12, 13, 16, 17 and 18 followed by sequencing analysis.

In a Chinese population, Wu et al. [26] identified 18 mutations (7 novel) and 11 polymorphisms (3 novel). They revealed that Arg778Leu and Thr935Met mutations are hotspots in the Chinese population. Meanwhile, Liu et al. [31] identified 1384del17bp as a novel mutation in WD patients and R778L is the most common mutation of the *ATP7B* gene. There is a correlation between R778L and hepatic manifestations in WD patients and the R778L has been reported to have a high allele frequency in Oriental populations such as Japanese, Korean, and Chinese. The allele frequency of R778L was 37.9% in Korean patients with WD [32], 27% in Japanese WD [22,33] and 37.7% in Chinese [26].

Meanwhile, Nicastro et al. [34] recommended a 2-step approach to detect *ATP7B* gene mutations using SSCP and direct sequencing in Italian children presenting with WD. Their results showed that 91.3% of studied WD alleles can be identified using 2-step approach. In addition, Mak CM and Lam [35] also proposed a five-exon screening approach to East Asian populations. Exons 8, 12, 13, 16, and 18 should be screened for Northern Chinese, Koreans and Taiwanese, and exons 5, 8, 12, 13, and 16 for Hong Kong Chinese and Japanese populations. This five-exon approach can cover about 70% of the mutations in the East Asian populations. Accordingly, we surveyed the high prevalent mutations of the *ATP7B* gene in East Asian countries including China, Hong Kong, Japan, Korea and Taiwan. Therefore, we proposed an *ATP7B* mutation detection strategy in patients with WD in the Taiwanese population (Fig. 1) using an HRM analysis. About 67.86% of *ATP7B* mutation alleles can be detected at the first step and can achieve 96.43% at the second step. Compared with Mak and Lam [35] proposed algorithm, our approach encompasses 8 exons and we can cover over 90% of the mutations in the Taiwanese population. In this study, we identified one newly-identified sequence variant (p.A476T) and 1 novel SNP (p.L776L) in 50 normal Taiwanese individuals.

Fig. 5. Application of HRM analysis for ATP7B mutation analysis in 50 normal individuals. Normalized plots, and normalized and temperature-shifted difference plots for the mutation screening of ATP7B gene of 50 normal individuals. (a) represents the melting profile of the ATP7B mutation, p.C271X. (b) represents the melting profile of the ATP7B mutation, p.P992L. (c) represents the melting profile of the ATP7B mutation, p.D1296N and c.3903+6C>T. The p.C271X, p.P992L, p.D1296N and c.3903+6C>T are clearly distinguished in the normalized and normalized temperature-shifted difference plots.

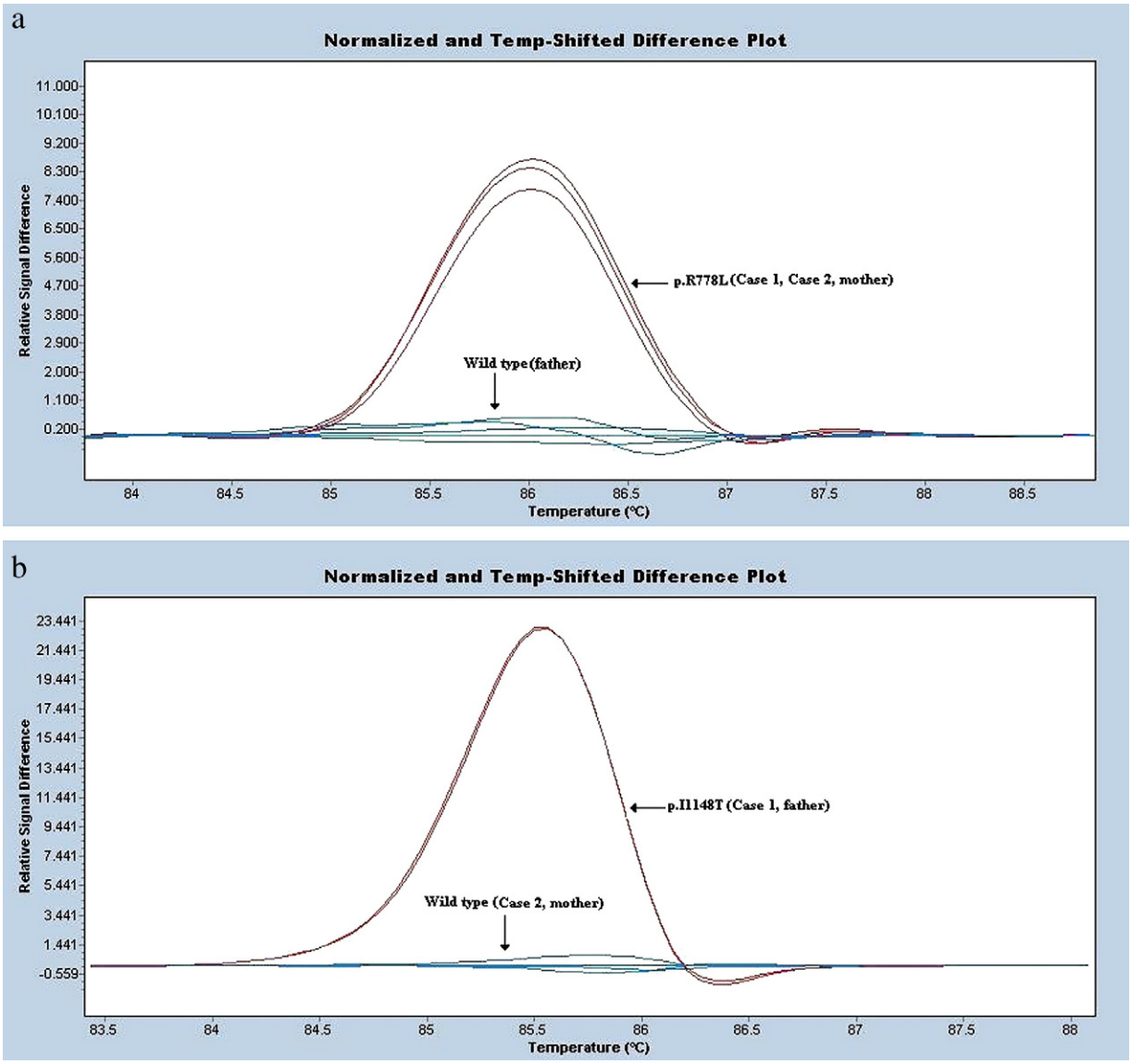


Fig. 6. Application of HRM analysis for ATP7B mutation analysis in one suspected family. Case 1 was compound heterozygous mutation (p.I1148T/p.R778L), however; Case 2 was a carrier of the heterozygous mutation, p.R778L. Their father and mother were both carriers of the heterozygous mutation, p.I1148T and p.R778L respectively.

In this study, we have demonstrated a rapid and sensitive method for mutation scanning of the *ATP7B* gene using melting analysis. This simple, high-performance, accurate, high-resolution, cost and time-saving technique can be used as a sensitive and specific tool for the detection of variations in DNA. With this highly sensitive and specific diagnostic tool, a researcher could manage a large quantity of clinical samples in the laboratory. Our results demonstrated that HRM analysis represents a good tool to detect mutations in the *ATP7B* gene.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.cca.2010.04.030.

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