Abstract. The present study aimed to analyze gene mutations in patients with β-ureidopropinoase deficiency and establish a rapid detection method for β-ureidopropinoase (UPB1) pathogenic variations by high resolution melting (HRM) analysis. DNA samples with known UPB1 mutations in three patients with β-ureidopropinoase deficiency were utilized to establish a rapid detection method for UPB1 pathogenic variations by HRM analysis. Further rapid screening was performed on two patients diagnosed with β-ureidopropinoase deficiency and 50 healthy control individuals. The results showed that all known UPB1 gene mutations can be analyzed by a specially designed HRM assay. Each mutation has specific HRM profiles which could be used in rapid screening. The HRM method could correctly identify all genetic mutations in two children with β-ureidopropinoase deficiency. In addition, the HRM assay also recognized four unknown mutations. To conclude, the results support future studies of applying HRM analysis as a diagnostic approach for β-ureidopropinoase deficiency and a rapid screening method for UPB1 mutation carriers.

Introduction

β-ureidopropinoase deficiency (Online Mendelian Inheritance in Man no. 613161) is a rare autosomal recessive disease which is caused by mutations in the β-ureidopropinoase (UPB1) gene. The clinical phenotypes of reported cases are complicated. Several patients presented with severe neurological involvement or mild psychomotor development delay, while other patients do not show any symptoms (1). Most patients are diagnosed before the age of two years, while numerous patients who do not present with any symptoms may not have been diagnosed (1). Since the disease lacks clinical specificity, auxiliary examination plays an important role in diagnosis. Gas chromatography-mass spectrometry (GC-MS) is widely used to diagnose this disease, however, analysis of gene mutation is the main tool used to confirm diagnosis (2,3).

High-resolution melting analysis (HRM), a novel technology for mutation, polymorphism and epigenetic alteration detection in double-stranded DNAs, has been one of the most widely used molecular diagnostic techniques in clinical and research settings (4). The basic principle of HRM is that DNA-saturated fluorescent dye is added into the PCR reaction system (5). The PCR amplicon is denatured by heating in a certain temperature range (6). Specialized instruments detect the degenerated double-stranded DNA fluorescent signal and a melting curve is plotted (7). The combination of fluorescent dye with PCR products is monitored in real-time during the heating process (5). The PCR amplicon is denatured by heating in a certain temperature range (6). Specialized instruments detect the degenerated double-stranded DNA fluorescent signal and a melting curve is plotted (7). The combination of fluorescent dye with PCR products is monitored in real-time during the heating process (5). The single-nucleotide mismatch leads to double-stranded DNA unwinding in the heating process (8). Fluorescent dyes are released from DNA molecules that are locally opened up, and the presence of mutations can be determined from the fluorescence intensity and time curves according to different melting temperature and shape of the melting curve, which allows wild-type and heterozygous mutation types to be effectively distinguished (9).

Considering the advantages of HRM in gene screening and detection, HRM in combination with other molecular diagnostic techniques can expand the application scope and improve the efficiency of HRM. For example, by using competitive multiplex PCR and HRM, copy number variations can
be detected accurately (10). Recently, the rapid development of instruments, DNA dyes and analysis software significantly enhanced the sensitivity, specificity and accuracy of HRM (11). This provides a fast, efficient and economic molecular diagnostic platform for molecular diagnosis of inherited disease, molecular profiling and target therapy of cancer, identification of pathogens and individualized medicine (12-15). The present study evaluated the potential application of HRM analysis for identifying gene mutations in patients and carriers with β-ureidopropinoase deficiency.

Materials and methods

Clinical details. The methods used in the present study were performed in accordance with approved guidelines by the Committee for the Ethical Review of Research Involving Human Subjects at Tianjin Children's Hospital. A total of 50 healthy individuals were recruited as controls in this study from October 2018 to August 2019 in Tianjin Children's Hospital (Tianjin, China), including 27 males and 23 females, with a mean age of 2 years (range, 6 months to 12 years old). Physical examination and GC-MS results of these controls were normal. The controls had no relationship with any of these five patients. Peripheral blood specimens were collected from all subjects after informed consent was obtained from their parents.

During the screening of suspected children by urine GC/MS, those identified with abnormal increase in pyrimidine degradation metabolites, including N-carbamyl-β-alanine (β-UP), N-carbamyl-β-aminoisobutyric acid (β-UIB), uracil, thymine, dihydouracil and dihydrothymine, were diagnosed as β-ureidopropinoase deficiency. Five patients were recruited to the present study. The first three families have been previously reported (16). Case 1, a 2-month-old male, was hospitalized with jaundice for nearly 2 months and progressive aggravation with occasional clay-like tool. The male was diagnosed with β-ureidopropinoase deficiency by GC-MS (16).

Case 2, a 3-day-old male, was admitted due to temporary convulsion. His father also suffered from β-ureidopropinoase deficiency. Elevated pyrimidines and metabolites in urine were detected by GC-MS. The male was diagnosed with β-ureidopropinoase deficiency by GC-MS (16).

Case 3, a 2-year-old male, was admitted because of language delay. The male was able to sit by himself in 7 months, climb in 10 months and walk until 14 months. The male was unable to speak until 2 years and 4 months. The results of GC-MS urine screening showed that the male has β-ureidopropinoase deficiency (16).

Case 4, an 8-month-old male, entered with a one-day history of dizziness, vomiting and stroking twice. The boy was delivered with a birth weight of 3.65 kg. No obvious abnormalities were detected in neurological examinations. The result of GC-MS urine screening showed the boy has β-ureidopropinoase deficiency.

Case 5, a 20-month-old boy, was admitted due to motor retardation. No obvious abnormalities were detected in examinations. β-ureidopropinoase deficiency was initially identified by GC-MS performed in the clinical setting.

Genomic DNA extraction. A total of 1 ml peripheral blood was collected with EDTA anticoagulant. Genomic DNA was isolated using a Blood Genomic DNA Mini kit (CoWin Biosciences) according to the manufacturer's instructions. DNA quality and concentration were assessed using a NanoDrop™ 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Inc.).

PCR primer selection and amplification. Primers for PCR amplification of UPBI genes with a size range of 271-490 bp were selected (16). The entire UPBI coding region were amplified by PCR using these primers. Primers were synthesized by Genewiz, Inc. PCR reactions were performed in a total reaction volume of 25 µl (16). The amplicons were detected using 1.5% agarose gel electrophoresis and visualized by ChemiDoc XRIS+ Imaging system (Bio-Rad Laboratories, Inc.). The DNA products were sent to Genewiz, Inc. for Sanger sequencing. Sequences from amplified UPBI were compared to known sequences using Chromas Lite and Gene Runner (Frank Buquicchio and Michael Spruyt; version 6.5.51x64).

HRM primer selection. Primers for HRM amplification of UPBI genes with a size range of 77-249 bp were designed (Table I). Primers were synthesized by Suzhou Jinweizhi Biotechnology Co., Ltd. The primers for HRM are shorter than those used for PCR.

HRM reaction. HRM was performed using a Forget-Me-Not EvaGreen qPCR Master Mix kit (Biotium, Inc.; cat. no. 31042-1). The total volume of the reaction was 20 µl, including 10 µl premix (2X), 1 µl forward and 1 µl reverse primers (10 µM) and 25 ng genomic DNA template. Amplification was performed using a LightCycler 480 High Throughput Real-Time Fluorescence Quantitative PCR system (Roche Diagnostics GmbH; 96-wells). The following thermocycling conditions were used for the PCR: Initial denaturation at 95°C for 5 min; 48 cycles of denaturation at 95°C for 5 sec, annealing at 60°C for 10 sec and extension at 72°C for 20 sec with fluorescence reading and single point acquisition mode. The subsequent melting analysis process of PCR amplification products included three steps: Denaturation at 95°C for 1 min; renaturation at 40°C for 1 min followed by continuous fluorescence reading mode at 65-95°C. The rise rate was 0.02°C/sec, and data acquisition was 25 times/°C (once every 0.04°C rise in temperature). The melting curve was analyzed using LightCycler 480 Gene Scanning software (version 1.5; Roche Diagnostics GmbH). Each sample was performed in duplicate. All fragments showing HRM aberrant patterns in controls (cases 1, 2 and 3) and the two families (cases 4 and 5) were amplified by PCR analysis. All PCR products were purified and sequenced by Genewiz, Inc. Subsequently, the PCR products were sent to Genewiz, Inc. for Sanger sequencing.

Results

HRM development. DNA samples of the first three families (cases 1, 2 and 3) were detected using whole exome sequencing. These families with known mutations were used to determine proper parameters and normalized HRM profile (Table II). PCR amplifications of exon fragments 1, 7, 9 and 10 of the UPBI gene followed by HRM analysis were performed. All selected mutations were confirmed by Sanger sequencing.
**Table I.** *UPB1* gene primers of high resolution melting curve analysis.

| Name    | Direction | Sequence (5'-3') | Length of amplification (bp) |
|---------|-----------|------------------|-----------------------------|
| EX1-F   | Forward   | GTGCCGCCGACACAAGCACTG | 165                         |
| EX1-R   | Reverse   | CATTAGCTTAGCCTCTTGG |                            |
| EX2-F   | Forward   | AAGTGGAGCAGACTGCATCA | 246                         |
| EX2-R   | Reverse   | ATGGTCAAAGAGTCTGCAC |                            |
| EX3-F   | Forward   | CCATATGCATTAGGATGAGT | 201                         |
| EX3-R   | Reverse   | AGGCAGCAGAAAGCAC    |                            |
| EX4-F   | Forward   | TGCCCTTTCTCTATTATAG | 140                         |
| EX4-R   | Reverse   | CCAGAGGCACCGTTAATG  |                            |
| EX5-F   | Forward   | ATAAAGTGGAACCTCGCAA | 216                         |
| EX5-R   | Reverse   | ATCTTTAGGTTGCTCAC   |                            |
| EX6-F   | Forward   | GAGTCTAAGGAAATCTTGAAGG | 249                      |
| EX6-R   | Reverse   | CAGACCCCAACACTGA    |                            |
| EX7-F   | Forward   | GCTGAGCATCCACTGATTC | 228                         |
| EX7-R   | Reverse   | CATGTTGTCAGAGGGAAC  |                            |
| EX8-F   | Forward   | TAACTGTTCCTGCTCAC   | 235                         |
| EX8-R   | Reverse   | GAAGCAGACAGAAAGGG   |                            |
| EX9A-F  | Forward   | AGCCCAACGTGCATCTAC  | 77                          |
| EX9A-R  | Reverse   | AAATGACCAAAGTCTTG   |                            |
| EX9B-F  | Forward   | AAGCTCACAGATGTTTTCTT | 226                      |
| EX9B-R  | Reverse   | AGAGGACGACCGCAACACAG |                      |
| EX10-F  | Forward   | CGAGCCTTCCTGTACGTTTC | 246                      |
| EX10-R  | Reverse   | CAGAGGTGTCTCTCACC    |                            |

*UPB1*, β-ureidopropinase; 9A, the front part of exon 9; 9B, the latter part of exon 9.

**Table II.** Mutations of the *UPB1* gene verified in the five families.

| Case          | Polymorphism | Mutation    | Exon   |
|---------------|--------------|-------------|--------|
| Case 1        | c.91G>A      | Heterozygous| 1      |
|               | c.977G>A     | Homozygous  | 9      |
| Case 1's father | c.977G>A    | Heterozygous| 9      |
| Case 1's mother | c.91G>A     | Heterozygous| 1      |
|               | c.977G>A     | Heterozygous| 9      |
| Case 2        | c.851G>T     | Heterozygous| 7      |
|               | c.977G>A     | Homozygous  | 9      |
| Case 2's father | c.977G>A   | Homozygous  | 9      |
| Case 2's mother | c.851G>T    | Heterozygous| 7      |
| Case 3        | c.853G>A     | Heterozygous| 7      |
|               | c.917-1 G>A  | Heterozygous| Intron 8 |
| Case 3's father | c.853G>A   | Heterozygous| 7      |
| Case 3's mother | c.917-1 G>A | Heterozygous| Intron 8 |
| Case 4        | c.977 G>A    | Homozygous  | 9      |
| Case 4's father | c.977 G>A   | Heterozygous| 9      |
| Case 4's mother | c.977 G>A   | Heterozygous| 9      |
| Case 5        | c.91G>A      | Heterozygous| 1      |
|               | c.977 G>A    | Homozygous  | 9      |
| Case 5's father | c.977 G>A  | Heterozygous| 9      |
| Case 5's mother | c.91G>A     | Heterozygous| 1      |
|               | c.977 G>A    | Heterozygous| 9      |
Figure 1. High resolution melting analysis of the UPB1 gene in the three families (cases 1, 2 and 3). The (A) melting curves and (B) difference plot of exon 1. The (C) melting curves and (D) difference plot of exon 7. The (E) melting curves and (F) difference plot of exon 9A. The (G) melting curves and (H) difference plot of exon 9B. WT, wild-type; het, heterozygous; hom, homozygous; UPB1, β-ureidopropinoase.

Figure 2. High resolution melting analysis of the UPB1 gene in the two families (cases 4 and 5) and 50 controls. The (A) melting curves and (B) difference plot of exon 1. a, c.91G>A heterozygous mutation. The (C) melting curves and (D) difference plot of exon 9B in the two families. b, c.977G>A heterozygous mutation. c, c.977G>A homozygous mutation. WT, wild-type; het, heterozygous; hom, homozygous; UPB1, β-ureidopropinoase.
As shown in Fig. 1, each mutation had specific melting curve profiles which significantly differed from the wild-type. HRM also clearly divided heterozygote variants with homozygote variants in each sample. However, when the c.917-1G>A mutation was analyzed, the melting curve profile showed that there was no curve variation with wild-type controls. Hence, a primer checking was performed and it was found that although the primers uncovered the mutation site, the placement of primers were not appropriate enough for HRM splice-site variation analysis. A set of primers were then redesigned to differentiate this mutation. This data indicated that for pathogenic splice-site mutation HRM, the primer needs to extend into part of the intronic region until the splice donor/acceptor site is incorporated.

**HRM to identify UPB1 mutations in β-ureidopropinoase deficiency.** To evaluate the diagnostic utility of HRM to detect UPB1 mutations, two β-ureidopropinoase deficiency cases (cases 4 and 5) with unknown mutations were selected. The melting curves of different UPB1 mutations were used as positive controls, and the wild-type UPB1 gene was used as the normal control. The results are as follows. Case 4 carried a c.977G>A p.R326Q homozygous mutation from his parents. Case 5 carried a maternal c.91G>A p.G31S heterozygous mutation and a c.977G>A p.R326Q homozygous mutation from his parents (Fig. 2). Sanger sequencing was performed to verify the genotyping results (Fig. 3).

**Application to carrier screening.** To validate the use of HRM for mutation carrier scanning in β-ureidopropinoase deficiency, 50 randomly chosen healthy children samples were first tested by HRM. Among the 50 samples negative for β-ureidopropinoase deficiency, 46 samples were identified as wild-type and 4 samples were identified to have harbored mutations. All melting curves of 46 healthy children were consistent with those of normal melting profiles (Fig. 4). The four mutant samples carried c.977G>A, c.957G>A, c.1072-63C>A, c.1072-70G>A and c.1122G>A heterozygous mutations.

Following HRM analysis, all samples were genotyped by Sanger sequencing. For each of these mutation types and genotypes, HRM and Sanger sequencing gave consistent results (Fig. 5).

**Discussion**

β-ureidopropinoase deficiency is an autosomal recessive disease. The pathogenic gene, **UPB1**, is located at chromosome 22q11.2 and consists of 10 exons. The cDNA is 1,152 bases long and encodes 384 amino acids (117). β-ureidopropinoase is the ultimate reactive enzyme in the metabolic pathways of uracil and thymine (18). Defects in the activity of β-ureidopropinoase directly affects the degradation of β-UP and β-UIB, resulting in the abnormal increase of β-UP and β-UIB in body fluids (2,19). Concurrently, the uracil, thymine, 5,6-dihydrouracil (DHU) and 5,6-dihydrothymine (DHT) of its upstream metabolic substrates also accumulate to varying degrees (2). It eventually leads to a series of clinical symptoms mainly of nervous system abnormalities and dysplasia, known as β-ureidopropinoase deficiency (18).
Routine blood and biochemical tests showed no specific changes in patients with β-ureidopropinoase deficiency (2). The MRI results of some patients are abnormal without specificity (1). Therefore, β-ureidopropinoase deficiency cannot be diagnosed by routine examination. Nowadays, GC-MS metabolic screening of pyrimidine metabolism evaluation of the β-ureidopropinoase catalytic activity has been widely used (2). Although GC-MS is routinely used for the diagnosis of β-ureidopropinoase deficiency, genetic testing has become increasingly important, particularly in the identification of asymptomatic patients and healthy carriers. With the development of gene detection technology, the mutant sites of the \textit{UPB1} gene that causes β-ureidopropinoase deficiency have been gradually discovered. According to the ClinVar database, >50 mutations have been found in exons 1-10 and their introns of UPB1 (https://www.ncbi.nlm.nih.gov/clinvar/?term=UPB1%5Bgene%5D). Previously, we reported seven Chinese patients with five mutations in the \textit{UPB1} gene (11). Our study showed that the c.977G>A mutation was the most common mutation of \textit{UPB1} in the Northern Chinese population, which suggested that a significant percentage of individuals with Chinese ethnicity may have β-ureidopropinoase deficiency (20). Therefore, β-ureidopropinoase deficiency may be more prevalent than the current estimates, owing to a number of undiagnosed patients. Moreover, β-ureidopropinoase deficiency is associated with a variable clinical phenotype. Although only a low number of cases have been reported, patients have presented with such symptoms as seizures, delayed myelination, growth delay, microcephaly, mental retardation, brainstem hypoplasia, atrophy of cerebri and cortical dysplasia (16). Children with β-ureidopropinoase deficiency may be difficult to identify due to the variable clinical phenotypic presentation, the subtlety of symptoms or the costly and time-consuming nature of gene screening. To meet the demands of clinical application, the development of high throughput, cost-effective and rapid DNA assays offers the possibility of avoiding missed diagnosis in susceptible individuals. HRM allows for large-scale carrier testing screening, which can provide sufficient capacity for the detection of known mutations in the \textit{UPB1} gene, playing a major role in accurate diagnosis.

Since the introduction of HRM, its advantages and disadvantages have been the focus of a number of researches (4,21,22). HRM technology is widely used in mutation scanning due to its simplicity, accuracy, rapidity, low cost, short cycle time and high throughput (23). Because each DNA segment has its own unique sequence, similar to DNA fingerprinting, it has a unique melting curve shape during heat denaturation, which makes HRM analysis highly specific, stable and repeatable (8). In addition, compared with other mutation screening methods, HRM analysis is a real closed-tube method, which indicates that the amplification product could be directly analyzed by the melting curve without any other treatment, make screening more convenient (24). At present, mutation detection methods have been relatively established, such as TaqMan probe-based PCR and the molecular beacon method, which have higher requirements for probe design (25). Besides, molecular hybridization-based methods, such as dynamic allele-specific hybridization and oligonucleotide ligation assays, which also involve expensive sequence-specific probes, increases the cost of detection (18,19,26,27). Other PCR-combined molecular methods are also available, such as single-strand conformation polymorphism and denaturing gradient gel electrophoresis (28,29). These techniques have their own distinctiveness, but the experimental design is relatively complex. Compared with them, HRM does not require specific probes, is relatively simple in the experimental design and detection is not limited by mutation type. Furthermore, HRM is inexpensive, which do not require fluorescent probes, with a cost of approximately $0.6 per reaction, when compared to a cost of approximately $3 per reaction for Sanger.

Figure 4. High resolution melting of the \textit{UPB1} gene in 50 controls. The (A) melting curves and (B) difference plot of exon 9B in the 50 controls, including c.957G>A heterozygous mutation and c.977G>A heterozygous mutation. The (C) melting curves and (D) difference plot of exon 10, including c.1072-63C>A heterozygous mutation, c.1072-70G>A heterozygous mutation and c.1122G>A heterozygous mutation. WT, wild-type; \textit{UPB1}, β-ureidopropinoase.
Since the price of genetic testing is still generally high, HRM is a more convenient and low-cost rapid detection technology. However, HRM still has several limitations, such as low ability to identify complex gene fragments with multiple mutations (polymorphisms) or GC-rich templates, the analysis sensitivity is limited by the length of amplification products and the detection ability of large fragments is lower compared with small fragments (4,11). The requirements for the quality of nucleic acid samples, experimental apparatus and fluorescent

Figure 5. UPB1 gene mutations (indicated by the black arrows) in the 50 controls. Sample with heterozygous mutations in (A) c.977G>A, (B) c.957G>A, (C) c.1122G>A, (D) c.1072-65C>A and (E) c.1072-70G>A. UPB1, β-ureidopropinase.
As β-ureidopropinoase is a rare disease, it is difficult to collect a large number of pedigrees to comprehensively evaluate the specifications of HRM in detecting the mutation hotspots of the UPB1 gene. Therefore, 50 normal samples were screened to further confirm that HRM analysis can accurately distinguish heterozygote and wild-type mutations of the UPB1 gene. In the present study, HRM can not only verify the genotypes of 50 normal control cases according to the known melting curves of wild-type and mutant PCR products within 400 bp were 100% (32). Therefore, the length of each amplified fragment was set to <400 bp when designing primers. Moreover, in most studies, pathogenic mutations are more widely studied than benign polymorphic sites. The presence of polymorphic sites will affect the positive rate of HRM and increase sequencing workload (33).

Several researchers also use samples with polymorphic sites and wild-type samples as well as normal controls to distinguish polymorphic sites from pathogenic mutants (34). Through appropriate condition optimization and disadvantage avoidance, effectiveness in HRM analysis was achieved in the present study.

As β-ureidopropinoase deficiency is a rare disease, it is difficult to collect a large number of pedigrees to comprehensively evaluate the specifications of HRM in detecting the mutation hotspots of the UPB1 gene. Therefore, 50 normal samples were screened to further confirm that HRM analysis can accurately distinguish heterozygote and wild-type mutations of the UPB1 gene. In the present study, HRM can not only verify the genotypes of 50 normal control cases according to the known melting curves of wild-type and mutant UPB1 gene, but also find new unknown mutants. Of the 50 samples negative for β-ureidopropinoase deficiency, four mutant samples carried five mutations. One of the controls have two mutations, which were c.957G>A heterozygous mutation and c.1072-70G>A heterozygous mutation without any clinical phenotype. The c.957G>A is a synonymous mutation which has been reported as a benign/likely benign mutation in the ClinVar database (35). c.1072-70G>A is a deep intronic mutation, which is unlikely to cause disease. This screening result further indicated that the PCR-HRM method for the UPB1 gene established in the present study has high sensitivity and specificity. It can not only identify known mutations, but also identify other mutations in the PCR coverage area, which is suitable for high-throughput screening of the target population. Following HRM analysis, all samples were genotyped by Sanger sequencing. The results showed that HRM has the same accuracy as Sanger sequencing in detecting UPB1 mutations (Table III). However, several types of variants cannot be distinguished using the HRM method, such as A/T or G/C, where melting profiles may become indistinguishable (36). In the present study, the UPB1 mutation presented here did not harbor any A/T or G/C nucleotide change, which may not lead to erroneous results. There might be a need for further optimization of HRM parameters that are specific for UPB1 mutations. Although Sanger sequencing is still the gold standard to detect mutations in the clinical setting, for patients diagnosed with β-ureidopropinoase deficiency, HRM can be used to scan the entire UPB1 gene, and subsequently sequence suspicious fragments to determine the pathogenic mutation, which can decrease the sequencing workload.

A previous study revealed an association between mutations in the UPB1 gene and 5-fluorouracil (5-FU) toxicity in subjects of Chinese/Asian ethnicity (35). 5-FU is widely used for therapy of cancers, however, some patients may occur symptoms of severe 5-FU toxicity (36). The entire molecular mechanisms underlying 5-FU-related toxicity remains unclear. It was suggested that UPB1 variants may impair the 5-FU catabolic pathway, resulting in a delay of 5-FU clearance (37). Knowledge of this association might be useful to help prevent 5-FU toxicity complications by screening cancer patients for frequent UPB1 variants before prescribing 5-FU or a related drug. Since the HRM technique can be performed in a shorter period of time, it can be an ideal screening tool.

In summary, the present study established a PCR-HRM screening method for the pathogenic gene UPB1 of β-ureidopropinoase deficiency, which is suitable for rapid screening of large samples of clinically suspected β-ureidopropinoase deficiency. It may also be a suitable technique for evaluating the hotspot mutation regions of the UPB1 gene, mutation site-related toxicity prediction and population-based mutation analysis. The sensitive, simple and cost-effective advantages of the UPB1 HRM analysis makes it suitable for its use in future research.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Natural Science Foundation of Tianjin City (grant no. 16JQNJJC1900), the National Natural Science Foundation of China (grant no. 81771589), the Program of Tianjin Science and Technology Plan (grant no. 18ZXDBSY00170) and the Program of Tianjin Health Bureau (grant no. 2014KZ031).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

| Procedure | + | _ | Total |
|-----------|---|---|------|
| +         | 29 | 0  | 29   |
| _         | 0  | 101| 101  |
| Total     | 29 | 101| 130  |

Sensitivity, 100%; specificity, 100%; positive predictive value, 100%; negative predictive value, 100%.
Authors' contributions

XX contributed in conception and design of the study, literature search and drafting the manuscript. IZ contributed in conception and design of the study, manuscript editing and review. QZ, CW, XZ and WX contributed in clinical data acquisition, manuscript editing and review. YL and JS contributed in conception and design of the study, literature search, experimental studies, manuscript editing and review. All authors have accepted responsibility for the entire content of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Tianjin Children’s Hospital. Written informed consent was obtained from the parents/guardians of all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. van Kuilenburg AB, Dobritzsch D, Meijer J, Krumpel M, Selim LA, Rashed MS, Assmann B, Meinsma R, Lohkamp B, Ito T, et al: beta-ureidopropionase deficiency: Phenotype, genotype and protein structural consequences in 16 patients. Biochim Biophys Acta 1822: 1096-1108, 2012.

2. Kuhara T, Ohse M, Inoue Y and Shinka T: Five cases of beta-ureidopropionase deficiency detected by GC/MS analysis of urine metabolome. J Mass Spectrom 44: 214-221, 2009.

3. Nakajima Y, Meijer J, Dobritzsch D, Ito T, Meinsma R, Abel ing NG, Roelofsen J, Watanabe Y, Tashiro K, et al: Clinical, biochemical and molecular analysis of 13 Japanese patients with beta-ureidopropionase deficiency demonstrate high prevalence of the c.977G>A (p.R326Q) mutation [corrected]. J Inherit Metab Dis 37: 801-812, 2014.

4. Wittwer CT: High-resolution DNA melting analysis: Advancements and limitations. Hum Mutat 30: 857-869, 2009.

5. Wittwer CT, Reed GH, Gundry CN, Vandersteen JG and Pryor RJ: High-resolution genotyping by amplicon melting analysis using LCGreen. Clin Chem 49: 853-860, 2003.

6. Gundry CN, Vandersteen JG, Reed GH, Pryor RJ, Chen J and Wittwer CT: Amplicon melting analysis with labeled primers: A closed-tube method for differentiating homozygotes and heterozygotes. Clin Chem 49: 396-406, 2003.

7. Lyon E and Wittwer CT: LightCycler technology in molecular diagnostics. J Mol Diagn 11: 93-101, 2009.

8. Nguyen-Dumont T, Calvez-Kelm FL, Forey N, McKay-Chopin S, Garrattino S, Gioia-Patricia L, De Silva D, Weigel R, Sangrajrang S, Lesueur F, et al: Description and validation of high-throughput simultaneous genotyping and mutation scanning by high-resolution melting curve analysis. Hum Mutat 30: 884-890, 2009.

9. Montgomery JL, Sanford LN and Wittwer CT: High-resolution DNA melting analysis in clinical research and diagnostics. Expert Rev Mol Diagn 10: 219-240, 2010.

10. Zhou L, Palais RA, Paxton CN, Geiersbach KB and Wittwer CT: Copy number assessment by competitive PCR with limiting copy number assessment by competitive PCR with limiting DNA extraction from dried blood spots (DBS) for diagnostic P. J Clin Diagn Res 8: FC01-FC04, 2014.

11. Natoli ME, Rohrman BA, De Santiago C, van Zyl GU and Richards-Kortum RR: Paper-based detection of HIV-1 drug resistance using isothermal amplification and an oligonucleotide ligation assay. Anal Biochem 544: 64-71, 2018.

12. Zarhorakova D, Lelkova P, Gregor V, Magner M, Zeman J and Martasek P: MECP2 mutations in Czech patients with Rett syndrome and Rett-like phenotypes: Novel mutations, genotype-phenotype correlations and validation of high-resolution melting analysis for mutation scanning. J Hum Genet 61: 617-625, 2016.

13. Azzurra D, Aussó S, Rodriguez-Moranta F, Guardiola J, Sanjuan X, Lobaton T, Boadas J, Piqueras M, Monfort D, Guinó E, et al: New methylation biomarker panel for early diagnosis of dysplasia or cancer in high-risk inflammatory bowel disease patients. Inflamm Bowel Dis 24: 2555-2564, 2018.

14. Müller KE, Zampieri RA, Aoki JI, Muxel SM, Nerland AH and Floeter-Winter LM: Amino acid permease 3 (aap3) coding sequence as a target for Leishmania identification and diagnosis of leishmaniasis using high resolution melting analysis. Parasit Vectors 11: 421, 2018.

15. Rezaei F, Haelili M, Fooladi AL and Feizabadi MM: High-resolution melting Curve analysis for rapid detection of streptomycin and ethambutol resistance in Mycobacterium tuberculosis. J Biomed Sci (Bucure) 12: 319, 2017.

16. Yang Y, Cai C, Wang C, Sun B, Zhang X, Fan W, Hu W, Meng Y, Lin S, Zhang C, et al: Clinical and genetic analysis of 7 Chinese patients with beta-ureidopropionase deficiency. Medicine (Baltimore) 98: e14021, 2019.

17. van Kuilenburg AB, Meinsma R, Assmann B, Hoffmann GF, Voit T, Ribes A, Lorente I, Busch R, Mayatepek E, Abel ing NG, et al: Genetic analysis of the first 4 patients with beta-ureidopropionase deficiency. Nucleosides Nucleotides Nucleic Acids 25: 1093-1098, 2006.

18. van Kuilenburg AB, Meinsma R, Beke E, Assmann B, Ribes A, Lorente I, Busch R, Mayatepek E, Abel ing NG, van Cruchten A, et al: beta-Ureidopropionase deficiency: An inborn error of pyrimidine degradation associated with neurological abnormalities. Hum Mol Genet 13: 2793-2801, 2004.

19. van Kuilenburg AB, van Lente H, Ratmann GG, Assmann B, Hoffmann GF, Brautigam C and van Gennip AH: Confirmation of the enzyme defect in the first case of beta-ureidopropionase deficiency. Beta-alanine deficiency. Adv Exp Med Biol 486: 243-246, 2000.

20. Shu J, Lv X, Jiang S, Zhang Y, Zhang C, Meng Y, Situ A, Xu H and Song L: Genetic analysis of the UBPI gene in two new Chinese families with beta-ureidopropionase deficiency and the carrier frequency of the mutation c.977G>A in Northern China. Childs Nerv Syst 30: 2109-2114, 2014.

21. Nagai Y, Iwade Y, Hayakawa E, Nakano M, Sakai T, Mitari S, Katayama M, Nosaka T and Yamaguchi T: High resolution melting curve assay for rapid detection of drug-resistant Mycobacterium tuberculosis. J Infect Chemother 19: 1116-1125, 2013.

22. Sirous M, Khosravi AD, Tabandeh MR, Salmanzadeh S, Ahmalkhosravi N and Amin S: Molecular detection of rifampin, isoniazid, and ofloxacin resistance in Iranian isolates of Mycobacterium tuberculosis by high-resolution melting analysis. Infect Drug Resist 11: 1819-1829, 2018.

23. Kramer D, Thunnissen FB, Gallegos-Ruiz MI, Smit EF, Postmus PE, Meijer CJ, Snijders PJ and Heideman DA: A fast, sensitive and accurate high resolution melting (HRM) technology-based assay to screen for common K-ras mutations. Cell Oncol 31: 161-167, 2009.

24. Chou LS, Lyon E and Wittwer CT: A comparison of high-resolution melting analysis with denaturing high-performance liquid chromatography for mutation scanning: Cystic fibrosis transmembrane conductance regulator gene as a model. Am J Clin Pathol 124: 330-338, 2005.

25. Bombow HL, Fox SB, Mitchell G, Scott RJ, Beshay V, Wong SQ; kConFab Investigators and Dobrovic A: A high-throughput protocol for mutation scanning of the BRCA1 and BRCA2 genes. BMC Cancer 16: 205, 2016.

26. Sirdah MM: Superparamagnetic-bead based method: An effective DNA extraction from dried blood spots (DBS) for diagnostic PCR. J Clin Diagn Res 8: FC01-FC04, 2014.

27. Natoli ME, Rohrman BA, De Santiago C, van Zyl GU and Richards-Kortum RR: Paper-based detection of HIV-1 drug resistance using isothermal amplification and an oligonucleotide ligation assay. Anal Biochem 544: 64-71, 2018.

28. Martasek P: MECP2 mutations in Czech patients with Rett syndrome and ethambutol resistance in Mycobacterium tuberculosis. J Infect Chemother 19: 1116-1125, 2013.

29. Sirous M, Khosravi AD, Tabandeh MR, Salmanzadeh S, Ahmalkhosravi N and Amin S: Molecular detection of rifampin, isoniazid, and ofloxacin resistance in Iranian isolates of Mycobacterium tuberculosis by high-resolution melting analysis. Infect Drug Resist 11: 1819-1829, 2018.

30. Kramer D, Thunnissen FB, Gallegos-Ruiz MI, Smit EF, Postmus PE, Meijer CJ, Snijders PJ and Heideman DA: A fast, sensitive and accurate high resolution melting (HRM) technology-based assay to screen for common K-ras mutations. Cell Oncol 31: 161-167, 2009.
29. Ohtomo R, Oka N and Morimoto S: PCR-denaturing gradient gel electrophoresis as a simple identification tool of arbuscular mycorrhizal fungal isolates. Microbes Environ 34: 356-362, 2019.
30. Er TK and Chang JG: High-resolution melting: Applications in genetic disorders. Clin Chim Acta 414: 197-201, 2012.
31. Tong SY and Giffard PM: Microbiological applications of high-resolution melting analysis. J Clin Microbiol 50: 3418-3421, 2012.
32. Reed GH and Wittwer CT: Sensitivity and specificity of single-nucleotide polymorphism scanning by high-resolution melting analysis. Clin Chem 50: 1748-1754, 2004.
33. Chen C, Li S, Lu X, Tan B, Huang C and Qin L: High resolution melting method to detect single nucleotide polymorphism of VKORC1 and CYP2C9. Int J Clin Exp Pathol 7: 2558-2564, 2014.
34. Słomka M, Sobalska-Kwapis M, Wachulec M, Bartosz G and Strapagiel D: High resolution melting (HRM) for high-throughput genotyping-limitations and caveats in practical case studies. Int J Mol Sci 18: 2316, 2017.

35. Fidlerova J, Kleiblova P, Kormunda S, Novotny J and Kleibl Z: Contribution of the β-ureidopropionase (UPB1) gene alterations to the development of fluoropyrimidine-related toxicity. Pharmacol Rep 64: 1234-1242, 2012.
36. Johnson MR and Diasio RB: Importance of dihydropyrimidine dehydrogenase (DPD) deficiency in patients exhibiting toxicity following treatment with 5-fluorouracil. Adv Enzyme Regul 41: 151-157, 2001.
37. Thomas HR, Ezzeldin HH, Guarcello V, Mattison LK, Fridley BL and Diasio RB: Genetic regulation of beta-ureidopropionase and its possible implication in altered uracil catabolism. Pharmacogenet Genom 18: 25-35, 2008.