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SCIENTIFIC OPINION

Rapid Molecular Diagnosis of Genetic Diseases by High Resolution Melting Analysis: Fabry and Glycogen Storage 1A Diseases

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For inborn errors of metabolism, high resolution melting analysis (HRMA) is a rapid, efficient, simple, and inexpensive method for mutation/rare variant screening. HRMA is a recent molecular technique for genotyping single-nucleotide polymorphisms without using probes. Here we apply HRMA to the α-galactosidase a (*GLA*) and glucose-6-phosphatase-alpha (*G6PC*) genes for mutation detection of patients with Fabry disease (MIM 301500) and glycogen storage disease type 1A (GSD1A; MIM 232200), respectively. To evaluate the procedure, genomic DNAs were blindly tested for known *GLA* mutations (c.658C > T, c. 679C > T, c.772G > A, c.796G > A, or c.718-719delAA) in three affected males and two obligate heterozygotes with Fabry disease, a *G6PC* mutation (c.247C > T) in a patient homozygous for that lesion, and 10 healthy control Turkish individuals. HRMA clearly detected the mutant amplicons and discriminated them from all wild-type *GLA* or *G6PC* amplicons. HRMA proved to be a sensitive, specific, and cost-effective mutation screening method for the rapid molecular diagnosis of these inborn errors of metabolism, indicating that the technique can be readily adapted to other genetic diseases.

Introduction

Significant progress has been made in the development and application of new molecular techniques for the accurate diagnoses of genetic disorders (Longo, 2006; Ezgu *et al*., 2008). Gene sequencing is currently accepted as the gold standard to identify the specific molecular lesions in disease-causing genes. However, sequencing is time-consuming and relatively expensive, especially for large genes (Karger and Guttman, 2009). For more rapid and cost-effective sequencing, screening methods such as denaturing gradient gel electrophoresis (DGGE), single-strand conformation polymorphism (SSCP), and denaturing high-performance liquid chromatography (DHPLC) have been employed (Balogh *et al*., 2004; Costabile *et al*., 2006). Recently, high resolution melting analysis (HRMA), a simple real-time PCR-based method for detecting sequence variations was developed (Erali *et al*., 2008). This technique detects changes in the melting curve of DNA duplexes for genotyping and variant scanning. Here, we apply this technique to detect known lesions in the α-galactosidase a (*GLA*) gene causing X-linked Fabry disease (MIM 301500) and in the glucose-6-phosphatase-alpha (*G6PC*) gene, which causes glycogen storage disease type 1A (GSD1A; MIM 232200), an autosomal recessive inborn error.

The *GLA* gene, chromosomally located at Xq22.1, has seven exons and encodes the lysosomal enzyme α-galactosidase a (α-Gal A) (Chou and Mansfield, 2008; Martins *et al*., 2009). More than 600 *GLA* mutations have been reported in unrelated families with Fabry disease (Stenson *et al*., 2009) (www.hgmd.cf.ac.uk). The *G6PC* gene, localized to the chromosomal region 17q21, consists of five exons and encodes the G6PC enzyme, which catalyzes the hydrolysis of glucose-6-phosphate to glucose and phosphate in the terminal step of gluconeogenesis and glycogenolysis (Terzioglu *et al*., 2001; Chou and Mansfield, 2008). In this study, we evaluate the use of HRMA for the rapid molecular diagnosis of known mutations causing Fabry disease and GSD1A.

Materials and Methods

Samples and study design

Genomic DNAs from three affected males and two obligate heterozygotes with Fabry disease, and a patient with GSD1A, who were previously confirmed to have specific *GLA* or *G6PC* mutations, as well as from 10 healthy Turkish controls (five males, five females), were studied. Each sample was assigned a random digit number, and a technician blindly analyzed all samples for *GLA* and *G6PC* by HRMA. Exons that had

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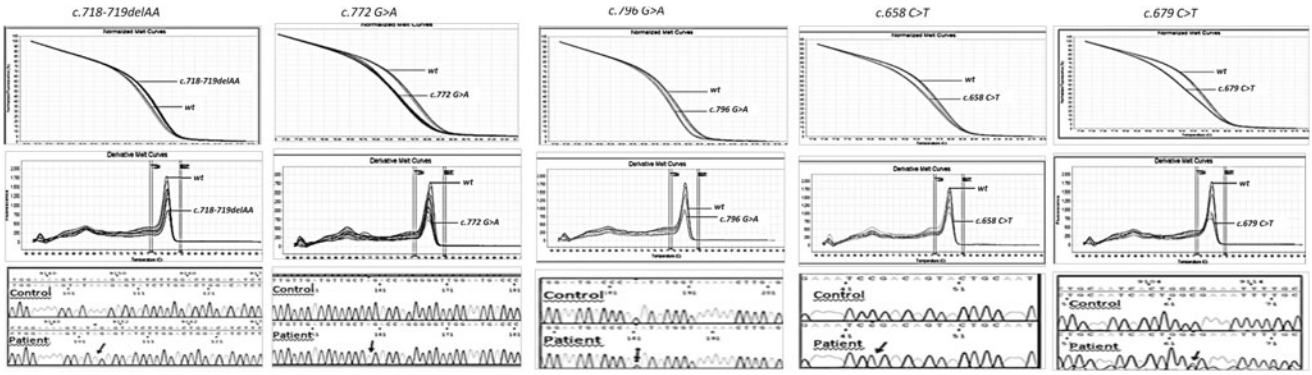

FIG. 1. The normalized melt curves (top), derivative melt curves (middle), and electropherograms (bottom) for each α-galactosidase a (GLA) mutation. Note that mutations c. 679C > T and c.796G > A, were in heterozygous females.

abnormal HRMA curves were sequenced and the detected mutation was identified. Only then was the sample code broken.

DNA extraction

Genomic DNAs were extracted from peripheral blood collected in EDTA by the NucleoSpin® Blood Genomic DNA Extraction Kits (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's instructions.

Gene amplification

Primer sequences for *GLA* and *G6PC* genes were previously reported (Rodríguez-Marí *et al*., 2003; Angaroni *et al*., 2004). The amplicons covered all the exons and intron–exon junctions or flanking sequences of the respective genes. PCR was performed with 15–30 ng of genomic DNA in 50 mM Tris, pH 8.3, with 10 μL MeltDoctorTM HRMA MasterMix (Applied Biosystems, Foster City, CA) containing SYTO9® Green Fluorescent Nucleic Acid Stain, and 1.2 μL of each primer at a 10 pmol/μL concentration. PCR was performed in a 7500 Fast Real-Time System (Applied Biosystems). PCR had an initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

Melting analysis

After PCR, 96-well plates were centrifuged (1500 *g* for 3–5 min), read in a 96-well 7500 Fast Real-Time PCR System (Applied Biosystems), and analyzed using Applied Biosystems® HRMA Software. The plates were heated at 0.1°C/s, and the fluorescence signal was recorded from 60°C to 95°C.

Sequencing

Amplicons showing abnormal HRMA profiles were sequenced using the BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's recommendations with an ABI 3130 Genotype Analyzer.

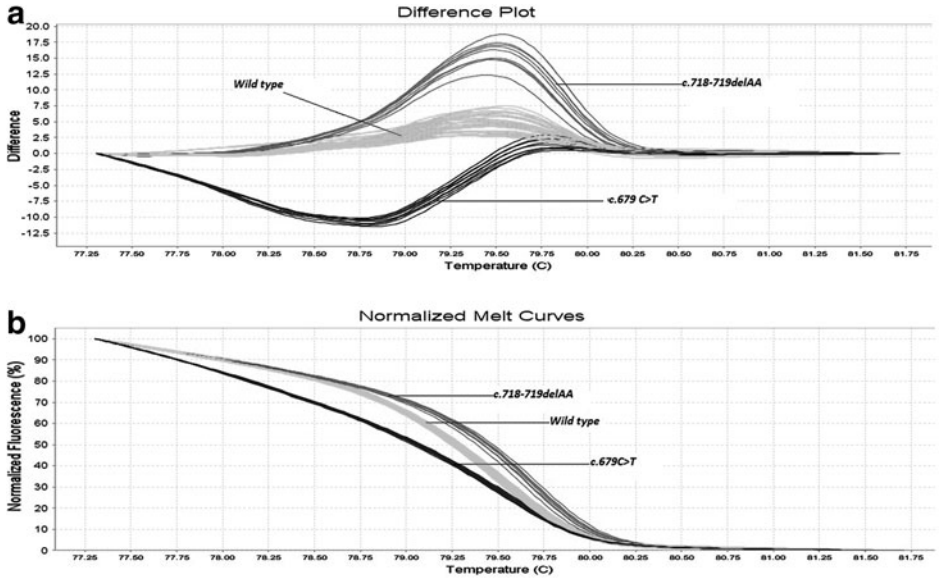

FIG. 2. **(a)** Difference plot; **(b)** normalized melt curve of the GLA amplicons carrying the c.718-719delAA and c.679C > T mutations as well as the wild-type GLA amplicon.

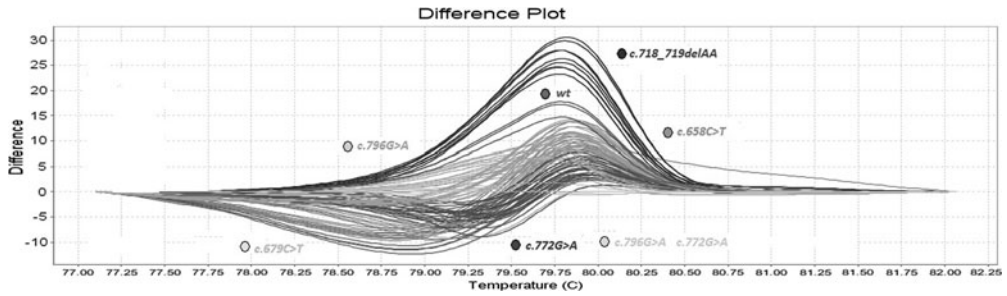

FIG. 3. Difference plot of all the *GLA* amplicons carrying the mutations as well as the wild-type amplicon.

Results

DNAs from the affected males and heterozygous females with Fabry disease, the affected patient with GSD1A, and 10 healthy controls were all blindly evaluated by HRMA. Amplicons showing abnormal HRMA profiles were then sequenced. All PCR amplifications for the *GLA* and *G6PC* genes were successful. HRMA detected abnormal melt curves for the three affected males and two heterozygotes with Fabry disease. The melt curves for the normal *GLA* exons were readily distinguished from those with the four *GLA* missense mutations and the 2 bp deletion. The normalized, derivative melt curves, difference plots, and electropherograms are shown in Figures 1–3.

The four *GLA* missense mutations at different positions in the same amplicon (exon 5) were chosen purposely to investigate the sensitivity and specificity of HRMA to detect single-nucleotide polymorphisms in this X-linked gene. HRMA readily detected the two base deletion and all four exon 5 missense mutations from the wild-type amplicons. In addition, the mutations, c.679C>T and c.796G>A, in the two heterozygotes were readily detected by HRMA as shown in Figure 1.

The *G6PC* gene mutation, c.247C>T, in exon 2 was reported to be the most common mutation causing GSD1A among Turkish patients (Terzioglu *et al.*, 2001). HRMA clearly identified the amplicon carrying the mutation and readily discriminated it from the *GLA* mutations and wild-type control amplicons. The normalized and derivative melt curves are shown in Figure 4.

Discussion

Rapid diagnosis is the most important starting point for the appropriate treatment of inborn errors of metabolism. The difficulty in establishing and performing the assays, the duration and the need for having the right sample from the right tissue, even sometimes requiring biopsies, are the disadvantages of biochemical methods (Longo, 2006; Ezgu *et al.*, 2008). Although the molecular methods, especially DNA sequence analysis, provide accurate diagnosis and do not need tissue

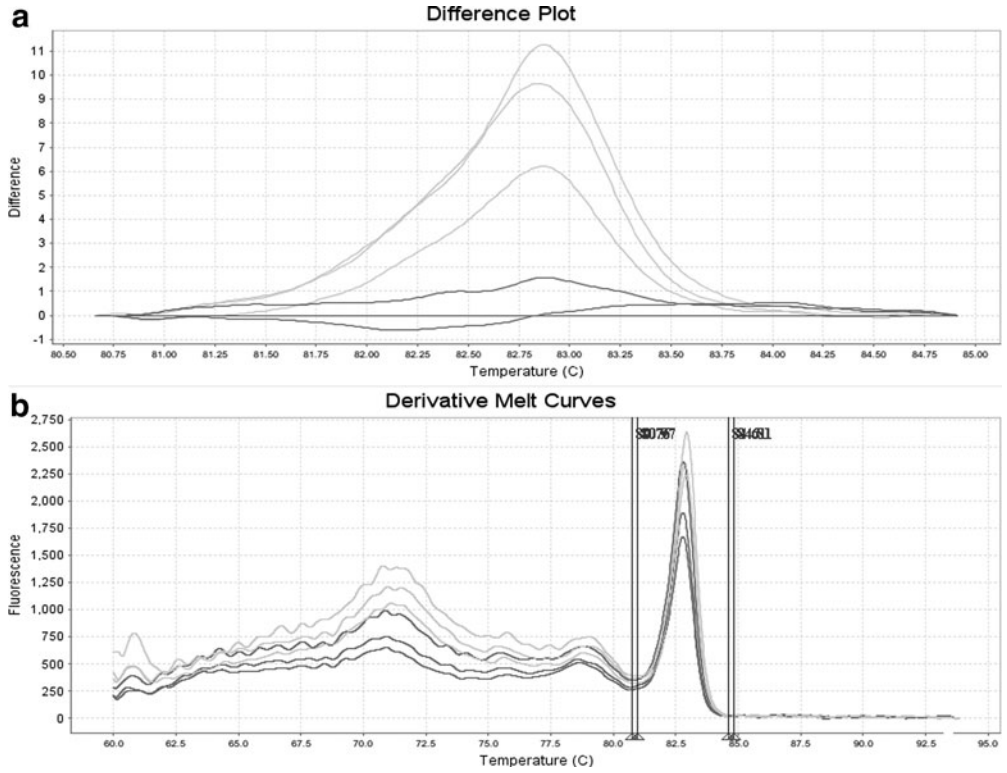

FIG. 4. **(a)** The normalized glucose-6-phosphatase-alpha (G6PC) melt curve; **(b)** derivative melt curve showing the melting profiles of the amplicon having the c.247 C>T mutation and the wild-type G6PC amplicon.

samples other than whole blood from which DNA can be extracted easily, the cost of the analysis has made researchers look for rapid molecular screening techniques. Previously, several methods such as DHPLC, DGGE, and SSCP for presequencing screening have been used effectively to identify amplicons for resequencing (e.g., Sevilla *et al*., 2002; Tabone *et al*., 2006). Recently, HRMA was employed successfully for mutation screening (Vossen *et al*., 2009).

In this study, we evaluate HRMA to rapidly and inexpensively screen for mutations in X-linked Fabry disease and autosomal recessive GSD1A. HRMA of amplicons depends on their DNA melting curves in the presence of saturating DNA binding dyes. As the temperature of the solution is increased, the specific sequences of the amplicon (primarily the GC content and the length) determine the melting behavior. When the fluorescence signal is plotted against the temperature, the fluorescence intensity decreases as the double-stranded DNA becomes single stranded and the dye is released. The melting temperature (Tm) at which 50% of the DNA double stranded is calculated by taking the derivative of the melting curve (Erali *et al*., 2008; Vossen *et al*., 2009).

In this study, the application of HRMA to two inborn errors of metabolism, Fabry disease and GSD1A, was investigated, as their respective biochemical diagnoses have significant challenges. For Fabry disease, the determination of leukocyte α-Gal A activity in males is diagnostic, but heterozygous females can have very low to high normal activities due to random X-inactivation (Rodríguez-Marí *et al*., 2003). Thus, sequencing the *GLA* gene and the identification of disease-causing mutations is required for the accurate diagnosis of suspect female heterozygotes and for genotype/phenotype correlations in affected males. Given the fact that enzyme replacement therapy is available, molecular diagnosis is essential for confirming all affected males and to accurately identify heterozygotes (Zarate and Hopkin, 2008; Germain and Fan, 2009). Previously, presequencing mutation screening of the *GLA* gene with DHPLC was reported (Shabbeer *et al*., 2005). HRMA of the *GLA* gene clearly proved effective in identifying the amplicons that carry the mutations, even in ones that lie very close to each other in both affected males and heterozygous females. HRMA discrimination was not only clear in affected males, but also in the amplicons from female heterozygotes. In addition, the 2 bp deletion in exon 5 was clearly demonstrated.

The biochemical diagnosis of GSD1A relies on the determination of glucose-6-phosphatase levels from liver samples obtained by biopsy. The need for hospitalization to perform this invasive method as well as the need for rapid diagnosis for this treatable disorder also makes molecular diagnosis preferable over biochemical methods. Previously, DHPLC for presequencing screening and microarray technology for screening for common mutations have been used for *G6PC* mutation analyses (Forsyth *et al*., 2005; Xu *et al*., 2010). In this study, HRMA of the *G6PC* gene was performed on genomic DNA from a previously confirmed GSD1A patient. Although others have noted difficulty in detecting homozygous mutants from the wild-type profile even with HRMA (Erali *et al*., 2008), homozygosity for the c.326C > T mutation was clearly discriminated from the normal control samples.

Compared with other presequencing mutation screening methods such as DHPLC, HRMA may be more sensitive and specific, even for homozygous mutant samples. HRMA is a closed-tube method, which can be directly performed after PCR without any additional procedural steps. In addition, it is a much more rapid technique compared with other mutation screening methods. Another advantage of HRMA is that after completing the analysis, the same samples can be directly used for sequencing, thereby eliminating the need for a new amplification reaction (Chou *et al*., 2005; Aguirre-Lamban *et al*., 2010). Finally, the lower cost for mutation detection emphasizes the advantages of HRMA for mutation screening. Thus, HRMA should be considered when screening in other genetic diseases.

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Author Disclosure Statement

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