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The Use of High Resolution Melting Analysis to Detect Fabry Mutations in Heterozygous Females via Dry Bloodspots

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Abstract

Background: As an X-linked genetic disorder, Fabry disease was first thought to affect only males, and females were generally considered to be asymptomatic carriers. However, recent research suggests that female carriers of Fabry disease may still develop vital organ damage causing severe morbidity and mortality. In the previous newborn screening, from 299,007 newborns, we identified a total of 20 different Fabry mutations and 121 newborns with Fabry mutations. However, we found that most female carriers are not detected by enzyme assays._

Methods: A streamlined method for high resolution melting (HRM) analysis was designed to screen for *GLA* gene mutations using a same PCR and melting programme. Primer sets were designed to cover the 7 exons and the Chinese common intronic mutation, IVS4+919G>A of *GLA* gene.

Results: The HRM analysis was successful in identifying heterozygous and hemizygous patients with the 20 surveyed mutations. We were also successful in using this method to test dry blood spots of newborns afflicted with Fabry mutations without having to determine DNA concentration before PCR amplification.

Conclusion: The results of this study show that HRM could a reliable and sensitive method for use in the rapid screening of females for *GLA* mutations.

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Keywords:

Fabry disease; *GLA*; high-resolution melting analysis; heterozygous female

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1. Introduction

Fabry disease (MIM 301500) is an X-linked recessive lysosomal storage disorder resulting from deficient α-galactosidase A (α-Gal A) activity. It has been estimated that this disease affects 1 in ~50,000 males in the general population [1-2]. α-Gal A is an enzyme involved in the metabolic breakdown of globotriaosylceramide (GL-3) and deficient activity of this enzyme results in GL-3 accumulation in the walls of small blood vessels, nerves, dorsal root ganglia, renal glomerular and tubular epithelial cells, and cardiomyocytes. It is a complex multisystemic disorder characterized clinically by peripheral neuropathic pains (chronic burning and acute episodes of severe pain), gastrointestinal disturbances, characteristic skin lesions (angiokeratomata), progressive renal impairment, cardiomyopathy, and early stroke [1].

During the past decade, several variants of Fabry disease have received attention from doctors and researchers. Three primary variants have been identified, respectively targeting the cardiac, renal, and neurological systems. Patients with the cardiac variant lack the classic symptoms of Fabry disease, presenting hypertrophic cardiomyopathy in the 5th-8th decades of life [3-6]. Previous studies reported that 1-4% of patients with left ventricular hypertrophy (LVH) or hypertrophic cardiomyopathy (HCM) had undiagnosed Fabry disease [3-5]. Patients with the renal variant also lack classic symptoms, but instead develop proteinuria and later-onset

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end-stage renal disease after 50 years of age. Screening of plasma α-Gal A activities showed that the prevalence of Fabry disease in patients undergoing hemodialysis was 0.25-1% [7-9]. Patients with neurologic variant also lack the classic symptoms, but develop cerebrovascular disease at around forty years of age. The prevalence of Fabry disease in young patients (18-55 years old) with cryptogenic stroke was reported to be as high as 4.9% in men and 2.4 % in women [10].

Recently, the authors conducted a study that revealed a surprisingly high incidence of the cardiac variant *GLA* mutation IVS4+919G>A (~1 in 1,500-1,600 males) in the Taiwan Han Chinese population [11]. Via family studies of newborns with the IVS4+919G>A mutation, the authors evaluated the clinical manifestations in the adults older than 40 years with this mutation. We found that 47 out of 93 subjects (51%) had left ventricular hypertrophy (LVH), including 28 males (28/39; 72%) and 19 females (19/54; 35%). We also found a positive correlation between disease-onset rate and age of the patient (Figure 1). In additon, none of the 19 female subjects had α-Gal A enzyme activity less than 3.1 μmol/h/L (25% of the normal mean; 25% is our cutoff value of newborn screening). Very similar finding was observed for our female patients who have classic type mutations and significant systemic involvement. Only 2 out of these 12 females had α-Gal A enzyme activity less than 3.1 μmol/h/L. We also analyzed the enzyme activity of 35 young females (age > 40 years old) carrying

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IVS4+919G>A mutation, who did not suffer from left ventricular hypertrophy and found that around 89% of these females had enzyme activity greater than 25% of the normal mean (figure 2). These findings showed that current newborn screening techniques are insufficient in identifying female carriers of Fabry mutations. Considering that most female carriers even with sufficient residual enzymatic activity could still suffer from significant systematic disease, we aimed to develop a new method of newborn screening for Fabry mutations that would be able to detect female carriers.

High-Resolution Melting Analysis

It has long been noted that high-resolution melting (HRM) analysis provides a simple, reliable and cost-effective method to identify sequence variants [12-15]. The procedure is conducted firstly by a PCR amplification in the presence of an appropriate DNA binding dye, followed by the formation of heteroduplex molecules, and a final melting and analysis step. Through this study, we aimed to develop a streamlined method for HRM analysis of the 7 exons (including the flanking intronic sequences) and the Chinese common intronic mutation, IVS4+919G>A of *GLA* gene using the same PCR and melting programme. We also successfully used this method with dry blood spot extracts of the newborns with Fabry mutations without the necessary step of determining DNA concentration before PCR amplification.

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2. Materials and Methods

2.1 Subjects

From Jan 2008 to Dec 2010, a total of 299,007 (156,179 males) newborns were screened for Fabry disease at our cooperative newborn screening centers (Taipei Institute of Pathology and Chinese Foundation of Health). From this screening, we identified 121 (106 males) newborns carrying Fabry mutations. Thereafter, we identified 218 family members (including male and female subjects) carrying Fabry mutations via the family study. A total of 20 different mutations, were identified in these patients (figure 3). Aside from the c.274G>T mutation, which was only identified in one heterozygous (female) patient, all other mutations were identified in both male and female patients enrolled in the HRM analysis study. Thirteen unaffected individuals were analyzed as normal controls in this study.

2.2 Methods

Genomic DNA samples were extracted from whole blood or dry blood spots using MagCore HF16 Automatic DNA/RNA Purification system (RBC Bioscience Corp., Taiwan) with MagCore Tissue Genomic DNA Extraction Kit (RBC Bioscience Corp., Taiwan). DNA concentrations were determined using a Nanodrop spectrophotometer (Infinigen, USA). The sequences of primer sets, annealing temperatures and fragments sizes of each amplicon used in PCR and HRM analysis are listed in Table 1. Primer

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sets were designed using GenBank accession number NM_000169.2 as a reference sequence. The primer sets were used to amplify the sequences of seven *GLA* exons and the region including IVS4+919G>A. The PCR mixture used contained 1x Roche LighCycler High Resolution Melting Master, 2 pmol of each primer and 6 ng of genomic DNA for a total volume of 20-μl. For the dry blood sample tests, 2 μl of out of 30 μl extracts, which were extracted from 3 punched (5 mm in diameter) dried blood spots, were substituted for the 6 ng of genomic DNA. The polymerase chain reaction and HRM analyses were performed using a Roche LightCycler® 480 system. The amplification was performed with an initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 15 s and extension at 72°C for 12 s. To facilitate heteroduplex formation, all the PCR products were heated to 95°C for 1 min and cooled down to 40°C. Melting curves were generated by heating the samples from 65 to 95°C at a ramp rate of 1°C/s. The melting curves were normalized by selecting linear regions of pre- and post-melting transition, and defined as 100% and 0%. The melting curves were displayed as melting peaks. Mutations were identified through a change in melting curve position, shape or deviated melting curve shape.

All the sequence variations are described according to the guidelines for mutation nomenclature recommended by Human Genome Variation Society

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(http://www.hgvs.org/mutnomen/) using the cDNA sequence NM_000169.2 as the reference. PCR products of normal genotype are described as "c.[=]+[=]", while hemizygous PCR products are described as "c.[variation]" and heterozygous PCR products are denoted as "c.[variation]+[=]".

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3. Results

Initially we used the original sequence primers (total 8 primer sets, Table 1) which had been used for sequencing in previous studies [11, 16-21], to cover all seven exons of the *GLA* gene and the intronic IVS4+919G>A mutation. However, two mutations, c.1172A>C and c.1194delA, both located at the 3' region of exon 7, were not identified in heterozygous or hemizygous patients (figure 4b). The IVS4+919G>A mutation was not identified in hemizygous patients.

The amplicons of exon 7 and IVS4+919G>A mutation were the two largest amplicons (352 and 446 bp) in these 8 original sequence primer sets (241 – 446 bp, Table 1). Because the ideal amplicon length is less than 250 bp for HRM analysis, we designed two new primer sets (exon7-1 and 7-2, table 1 and figure 4) to cover exon 7 and a new IVS4 primer set (IVS4-1) to cover the IVS4+919G>A mutation (table 1). All new designed primer sets created smaller amplicons (220, 233 and 121 bp, respectively). Thereafter, both the heterozygous and hemizygous patients of these mutations could be identified by HRM analysis (Figure 4C, 6). The HRM curves of the identified mutations (excluding IVS4+919G>A) are shown in figure 5.

The MagCore HF16 Automatic DNA/RNA Purification system was then used to extract DNA samples from dry blood spots. We found that the DNA concentration extracted from blood spots via this DNA extraction system were usually within the

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acceptable concentrations (around 6 ng/ul) for HRM analysis. Therefore, this method was successful in identifying the mutations from the dry blood spots of the newborns without determining DNA concentration before the PCR amplification. In order to examine the discrimination ability, we performed HRM analysis with 30 samples of different genotypes. The results of 30 dry blood spot samples, including 13 normal, 7 hemizygous and 10 heterozygous individuals with IVS+919G>A mutation, in one HMR analysis are shown in figure 6.

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4. Discussion

The results of our study have demonstrated that HRM is a reliable and sensitive method for use in rapid screening of females or even males carrying known *GLA* mutations in Taiwan. Recently, HRM analysis for detection of known and unknown mutations has grown in popularity, as HRM analysis does not require post-PCR manipulation of samples, unlike DNA sequencing technologies and conventional gel-based or HPLC-based scanning methods [15]. The cost of the reagents used in this study was less than $1 (U.S.) per sample per amplicon, making HRM a cost-effective gene variation analysis technique. In addition to PCR, HRM analysis takes only 15 minutes, amplifying as high as 384 wells at one time for melting analysis. Therefore, HRM has the potential to be an effective alternative method for Fabry newborn screening, especially when considering the fact that current screening methods are not reliable in females.

Although, in our study, all the hemizygous mutations could be easily identified in our study, the detection rates of hemizygous mutations were only around 75% in several studies [22-23]. In situation like this, it has been suggested that mixing the normal male DNA with the hemizygous male DNA could produce artificial heterozygotes, which would in turn increase the detection rate of hemizygous mutations. However, the increased time required to add the same amount and

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concentration of DNA to each male PCR tube and the fact that the current high-throughput enzymatic method for identifying male Fabry patients is highly reliable, make the HRM method to be the preferable choice only in identifying female Fabry patients this time.

The interpretation of mutation analysis via HRM is a challenge owing to the sensitivity of HRM profiles to variable concentrations of nucleic acids or salts [24-25]. It is therefore recommended that DNA samples that have been prepared using a common extraction procedure be used for HRM. In our study, DNA was extracted from dried blood spots via a steady automatic DNA extraction system, which ensured the consistency of the DNA concentration (around 6 ng/ul). In addition, the isolation reagents used to prepare DNA contain little salt, making the determination of DNA concentration unnecessary for dry-blood spot analysis. Hence, the unique advantages of HRM analysis in blood spot analysis may make HRM a possible choice for disease screening in the near future.

Another important factor to consider in the usage of HRM screening is the efficiency of any such screening operation. Each year, around 100,000 female newborns are born in Taiwan. There are 3 newborn screening centers in Taiwan, based respectively in the National Taiwan University Hospital, Taipei Institute of Pathology, and Chinese Foundation of Health. If we divide these 100,000 female babies by 200

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working days and three newborn screening centers, there are around 167 female babies to be screened for Fabry disease per center each day. Therefore, with the use of an appropriate automated nucleic acid extraction system and high throughput melting analyzer, each center could screen all 167 daily female newborns for the IVS4 mutation within 2 hours (including PCR) with one analyzer. Within 8 hours, each center (with two melting analyzers) could easily screen for all exons of the *GLA* gene and the Chinese common intronic IVS4 mutation. Therefore, we propose that, with an appropriately designed system, HRM analysis could be used as a simple, rapid and reliable method in female newborn screening for Fabry mutations. This method may also be viable in the detection of heterozygous Fabry patients within female patient populations suffering from HCM, renal impairment, or stroke.

A possible concern regarding HRM analysis, however, may still be its sensitivity. 2 mutations were missed in the initial screening using the original primer sets, raising concerns that the method established so far is not sensitive enough to identify all Fabry mutations, especially those located at exons 2 and 6 with their amplicons are greater than 300bp. Therefore, it will be necessary to enlarge the sample size of Fabry mutations in future studies through cooperation with other Fabry centers.

In conclusion, considering that a large percentage of Fabry female patients could not be identified with the current screening method, HRM analysis may be well-suited

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for use as a rapid newborn screening technique for Fabry disease, particularly in identifying female Fabry patients.

Abbreviations

GL3 globotriaosylceramide

LVH left ventricular hypertrophy

HCM hypertrophic cardiomyopathy

HRM high resolution melting

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Figure legend

Figure 1. Age-onset of patients with the IVS4+919G>A mutation

The age-onset of male and female patients with the IVS4+919G>A mutation were showed in panel A and B, respectively. 72% percentage of male adults with IVS4+919G>A mutation, who were older than 40 years old, had developed hypertrophic cardiomyopathy. Disease onset rate is positively correlated with the age of the patient. The disease onset rate of male Fabry patients increased from 50% to 64% and then to 87%, as the age progressed from forties to fifties and then to sixties (figure 1a). The disease onset rate of female Fabry patients increased from 18% to 100%, as age progressed from forties to seventies. One woman at the age group of 80 did not show any sign of hypertrophic cardiomyopathy (figure 1b).

Figure 2. Residual α-galactosidase A activity of female adults carrying IVS4+919G>A or classical mutations identified in Taiwan.

A: females with IVS4+919G>A mutation, but without HCM (n = 31); B: females with IVS4+919G>A mutation and HCM (n = 16); C: females with classical mutations and major organ involvement (n= 10). Activity is expressed as percentage of the mean of normal activity (12.4 ± 2.25 nmol/h/ml plasma). The line indicates 25% of the mean

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enzymatic activity of the normal control.

Figure 3. Schematic representation of the exon/intron organization of the *GLA* gene with indication of positions of mutations identified in Taiwanese Chinese populations. A total of 20 *GLA* mutations, identified in Taiwanese patients, including missense (blue); nonsense (red), deletion (green) and splicing site mutations (black) were examined in this study.

Figure 4. HRM analysis of exon 7 of the *GLA* gene.

The primer set 7 was first used in this HRM analysis. With this primer set, however, two mutations, c.1172A>C and c.1194delA, both of which are located at the 3' terminal of exon 7, were not identified in heterozygous or hemizygous patients (figure 3b). The amplicon of this primer set (352 bp) was larger than the ideal amplicon length for HRM analysis (less than 250 bp). Therefore two primer sets (exon7-1 and 7-2) covering the entire exon 7 coding region sequence(table 1 and figure 3a) were designed to replace primer set 7. The primer set "exon 7-1" covered the 5' region of exon 7 and was designed to amplify a fragment of 220 bp. The primer set, exon 7-2, covered the 3' region of exon 7 and was designed to amplify a fragment of 233 bp. Using this new primer set 7-2 for HRM analysis, the samples with c.1172A>C and

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c.1194delA mutations were easily identified (D).

Figure 5. HRM curves of the mutations which were identified at exons in this study. Panels A through F show the normalized difference plots for each amplicon. (A) exon 1, containing the wild type and mutant c.157A>G; (B): exon 2, containing the wild type and mutants c.274G>A, c.331G>T, c.332G>A and c.335G>A; (C) exon 3, containing the wild type and mutants c.394G>A and c.427G>A; (D) exon 4, containing the wild type and mutant c.612G>A; (E) exon 5, containing the wild type and mutants c.656T>C and c.695T>C; (F) exon 6, containing the wild type and mutants c.886A>T and c.902G>A. (G) exon 7-1, containing the wild type and mutants c.1034C>G, c.1066C>T, c.1067G>A, c.1078G>T and c.1087C>T. Notably, the hemizygous c.1034C>G mutation could be distinguished from the wild-type sequence when analyzed alone (Figure H); however, this discrimination disappeared when the samples were analyzed with all other mutations. The HRM curves of exon 7-2 are shown in figure (figure 3c).

Figure 6. An example of simultaneous screening for the IVS4+919G>A mutation in 30 samples

In order to examine the sensitivity of HRM for Chinese common mutation,

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IVS4+919G>A, in larger sample amount at the same run, 30 samples with the wild type (n = 13), heterozygous (n = 10) and hemizygous (n = 7) IVS4+919G>A mutation were simultaneously screened by HRM analysis. The hemizygous IVS4+919G>A samples (genotype: A) could be distinguished from wild type sample (genotype: G/G) by their T_m variations, while heterozygous samples (genotype: G/A) have a different melting curve shape (A). Thus, individuals with the different genotypes of IVS4+919G>A were clearly distinguishable from the wild type. Samples with the IVS4+919G>A hemizygous genotype are marked in green, heterozygous are in red and wild-type samples are in blue. (A): Normalized melting curve. (B): Difference plot.

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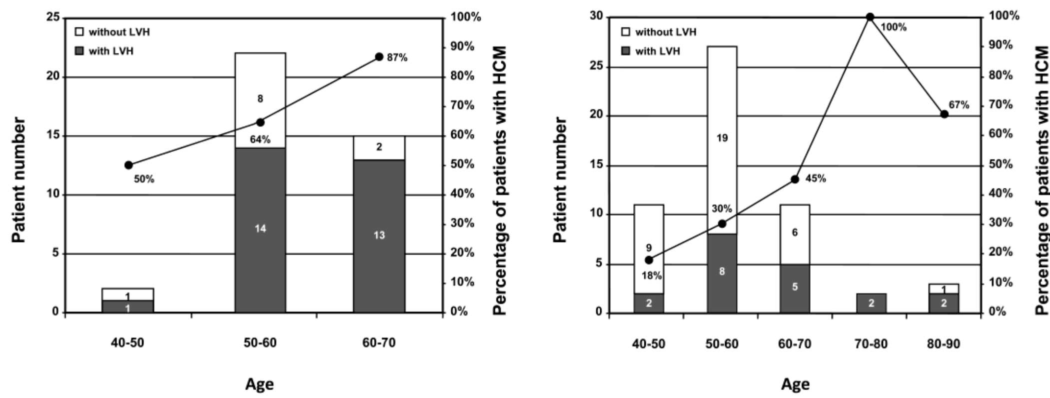

Fig. 1

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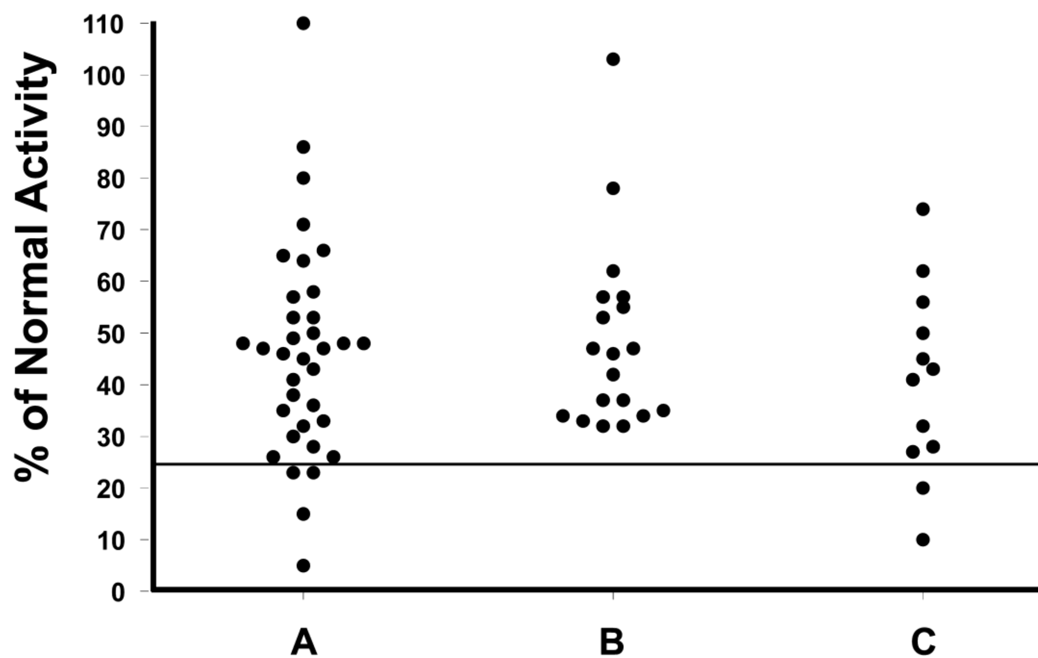

Fig. 2

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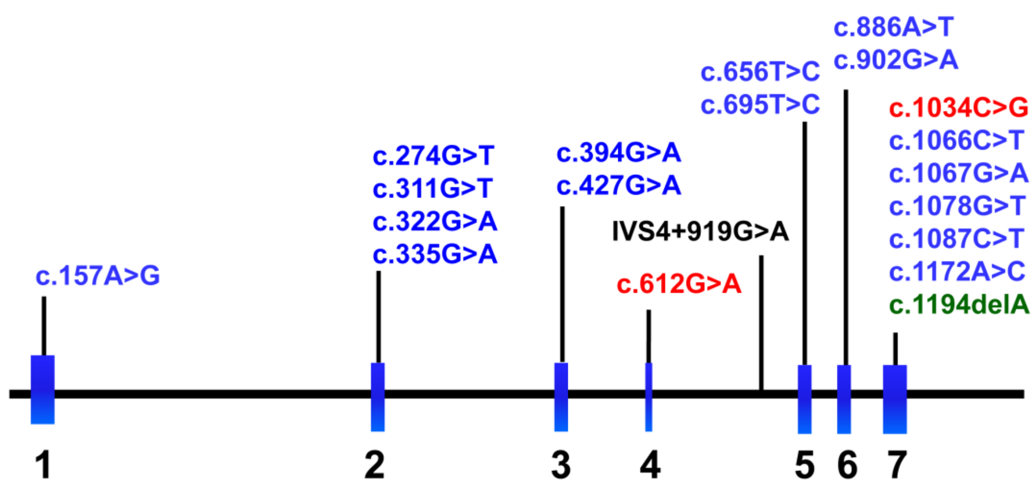

Fig. 3

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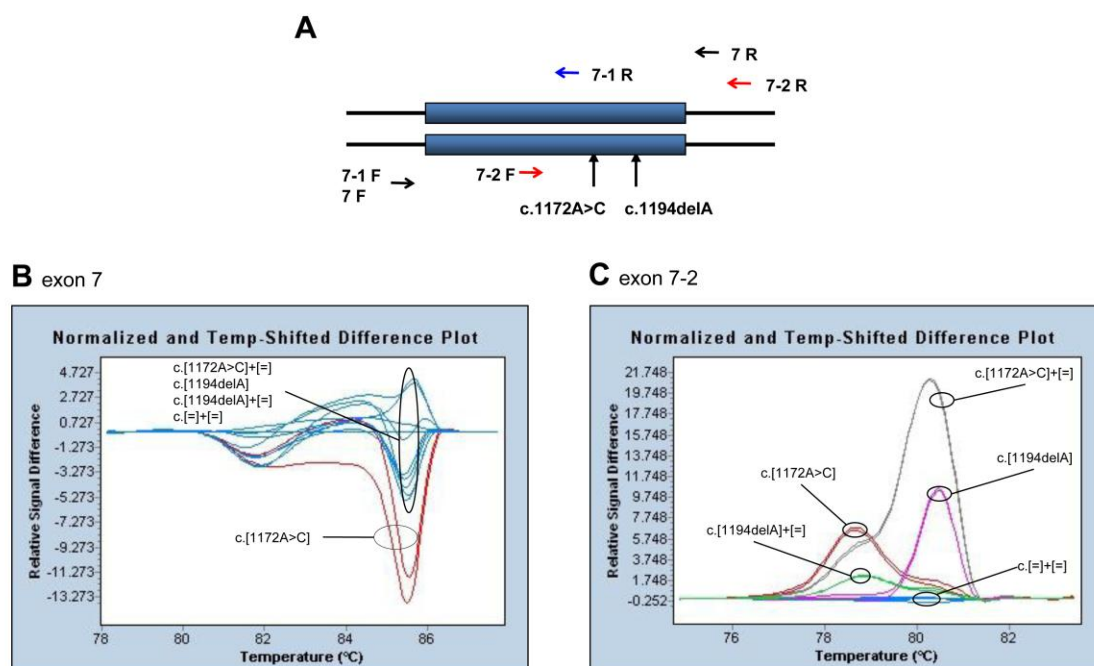

Fig. 4

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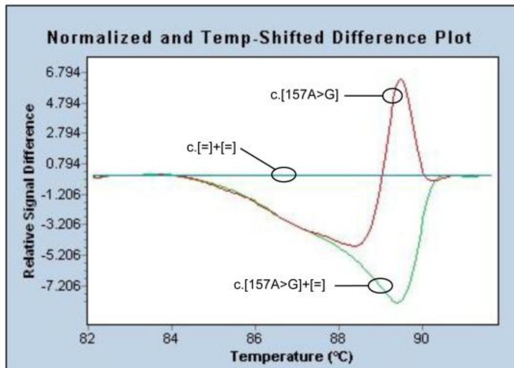
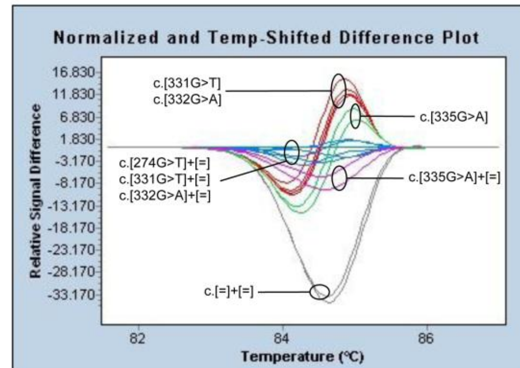
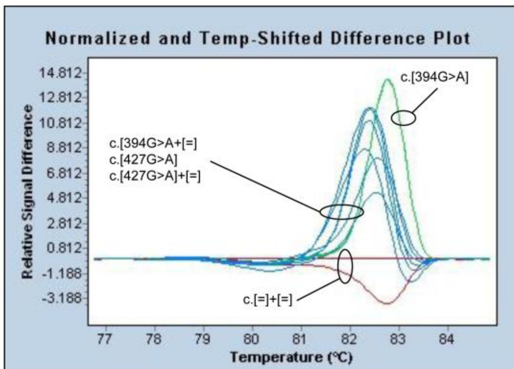
A exon 1



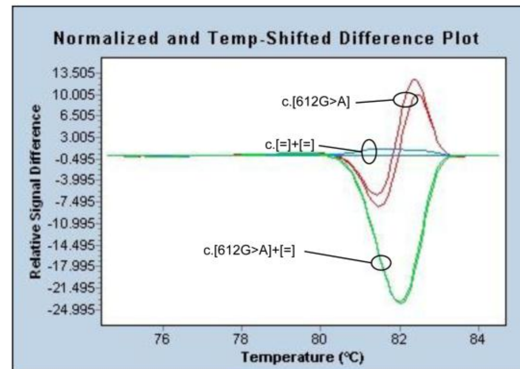
B exon 2



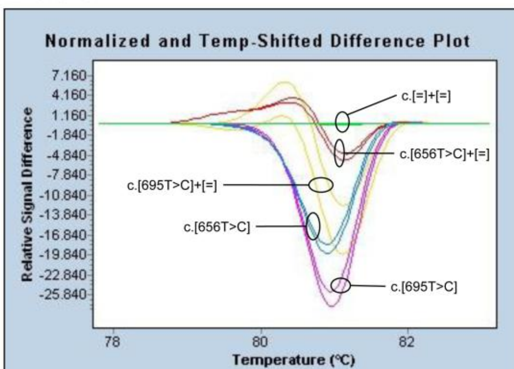
C exon 3



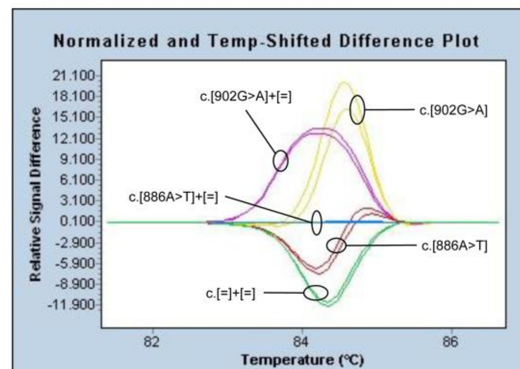
D exon 4



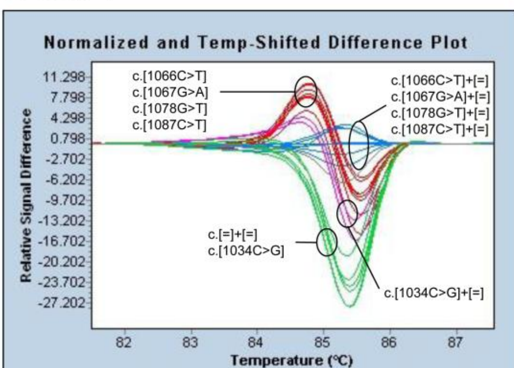
E exon 5



F exon 6



G exon 7-1



H c.1034C>G

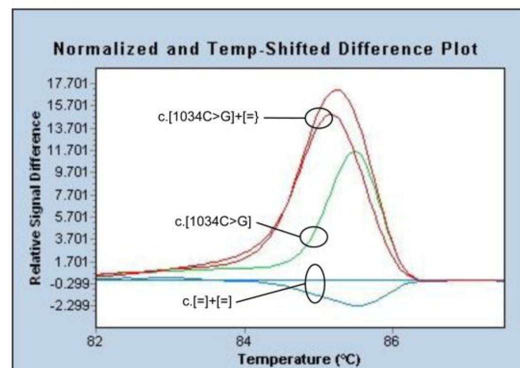


Fig. 5

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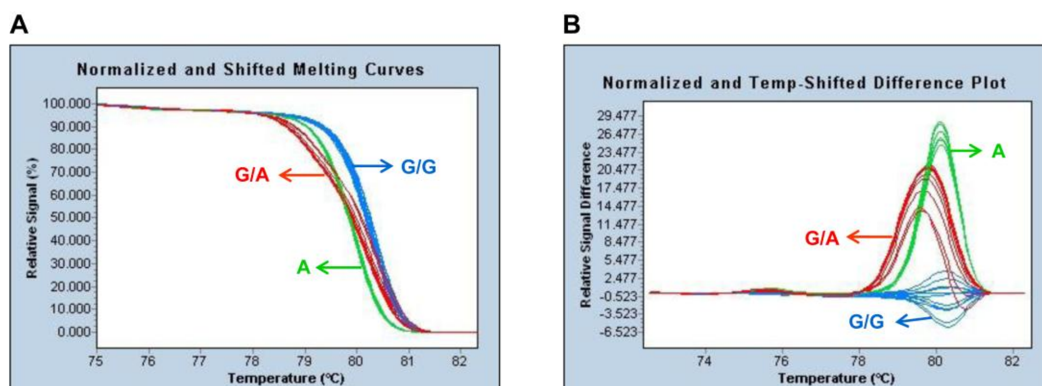

Fig. 6

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Table 1 Primer sets used for HRM

Amplicon	Forward primer	Position	Reverse primer	Position	Anneal temperature	Size (bp)
Exon 1	TTAAAAGCCCAGGTTACCCG	c.1-32_-51	AAAGCAAAGGGAAGGGAG	c.194+16_+33	60	280
Exon 2	AATCCCAAGGTGCCTAATAAA	c.195-61_-81	TACAGAAGTGCTTACAGTCCT	c.369+34_+54	60	310
Exon 3	TCTCTTTCTGCTACCTCACG	c.370-46_-65	TCTTTCCTTTGTGGCTAAATC	c.547+20_+40	60	282
Exon 4	TATAGCCCCAGCTGGAAATTC	c.548-41_-61	GTTGGACTTTGAAGGAGACCT	c.639+68_88	60	241
Exon 5	GAAGGCTACAAGTGCCTCCT	c.640-69_-88	AGCCTACCGCAGGGTCTT	c.801+37_+54	60	293
Exon 6	AAGAATGTTTCCTCCTCTCT	c.802-30_-49	CAAAGTTGGTATTGGGTATAT	c.999+33_53	60	300
Exon 7[a]	GCTAAGCAACCACACTTTCT	c.1000-14_-31	GAAGTAGTAGTTGGCAATA	*12_*30	60	352
Exon 7-1	GCTAAGCAACCACACTTTCT	c.1000-14_-31	GAACCCTAGCTTCCTTTTCACAG	c.1166_1188	60	220
Exon 7-2	GTAATCCTGCCTGCTTCATCA	c.1134_1153	ACCTAGCCTTGAGCTTTTAA	*50-*69	60	233
IVS4 [a]	TCTGTCCCTCAACACTGCAA	c.639+641_+660	TAGGCAGGTGGGATATCAGG	c.639+1067_+1086	60	446
IVS4-1	TTTTCTTCTCAGAGCTCCACA	c.639+854_+874	TGCGAGAGATACAGTCAAAGTCA	c.639+952_+974	60	121

[a] Exon 7 and IVS4 were originally used for sequence analysis and were not suitable for HRM analysis.

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Hightlights

> We developed method for HRM analysis of GLA gene using a same PCR/ melting programme.

>All Fabry mutations in heterozygous or hemizygous patients can be identified with HRM.

>We also develop this HRM method in dry blood spots of newborns with Fabry mutations.