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Short Communication

Simple and efficient screening of patients with Fabry disease with high resolution melting

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ABSTRACT

Background: Fabry disease (FD [MIM: 301500]) is a disorder caused by mutations in the alpha-galactosidase gene (GLA), which presents great allelic heterogeneity. The development of fast screening methods may reduce costs and length of diagnosis, being particularly important for screening programs of high-risk female patients. Therefore, the purpose of this study was to develop a pre-sequencing genetic screening method based on high resolution melting (HRM) analysis.

Methods: We performed HRM analysis in one hundred and three individuals, 79 females and 24 males, with a total of 27 different variants in 30 different genotypes. We standardized a protocol using EvaGreen, a release-on-demand dye specific for HRM, added to the PCR reaction. Amplification was performed in a conventional real-time system with HRM capability.

Results: All genotypes in all amplicons were distinguishable from wild type. In most amplicons it was even possible to differentiate each genotype from the others.

Conclusion: We developed a simple, fast and highly sensitive HRM based protocol that may facilitate genetic screening of FD.

1. Introduction

Fabry disease (FD [MIM: 301500]) is a genetic disorder caused by mutations in the alpha-galactosidase gene (*GLA*), that encodes for the lysosomal hydrolase α-galactosidase A (GLA, EC 3.2.1.22). Its deficiency leads to accumulation of globotriaosylceramide (Gb_3) and other glycosphingolipids [1]. Variants resulting in low residual enzymatic activity are associated with the classical form of the disease, with childhood onset of signs and symptoms and development of cardiac, cerebrovascular and/or renal manifestations. Variants resulting in high residual activity are associated with non-classical mono or oligo-symptomatic phenotypes and later disease onset [2].

Although FD displays X-linked inheritance, women are often symptomatic and can be as severely affected as male individuals. However, diagnosis of female patients must be performed by molecular analysis as enzyme levels can be within normal range. Men, on the other hand, can be reliably diagnosed by low enzymatic activity [1]. Genetic analysis also helps in genotype-phenotype correlation, genetic counselling and screening of family members.

GLA (GeneBank Accession Number: NG_007119.1) is a small gene located at Xq22.1 that spans 12 kb and that is divided in seven exons ranging from 92 to 309 bp [3]. Despite the existence of a few mutational hot-spots, FD is characterized by the presence of private mutations that result in great allelic heterogeneity. Currently, about 1000 *GLA* mutations have been described in the Human Mutation Database [4] and over 1500 entries are listed in the Fabry database [5]. The majority of these variants are missense or affect one or a few bases, which makes Sanger sequencing an appropriated method for molecular diagnosis. Since there are no recurrent pathogenic mutations and variants are mostly homogeneously distributed, the whole gene must be analyzed. Therefore, the development of fast screening methods may reduce costs and length of diagnosis, being particularly important for screening programs of high-risk female patients.

High resolution melting (HRM) analysis is a closed-tube assay with no post-PCR processing that evaluates differences in the dissociation (melting) profiles of amplicons mixed with saturating fluorescent dyes. The position and shape of melt curves are affected by GC content and distribution and by fragment length which are altered by mutations [6].

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Table 1
GLA variants for which HRM protocol was validated.

#	Location	cDNA effect[a]	Protein effect[b]	rsID
1	5′ UTR	c.-30G > A	–	rs3027584
2	5′ UTR	c.-12G > A	–	rs3027585
3	5′ UTR	c.-10C > T	–	rs2071225
4	Exon 1	c.4C > T	p.Gln2Ter	rs869312313
5	Exon 1	c.32delG	p.Gly11Alafs	rs869312278
6	Exon 1	c.44C > A	p.Ala15Glu	rs869312304
7	Exon 1	c.167G > A	p.Cys56Tyr	rs869312258
8	Exon 2	c.195T > C	p.Ser65Ser	rs782803696
9	Exon 2	c.334C > T	p.Arg112Cys	rs104894834
10	Exon 2	c.352C > T	p.Arg118Cys	rs148158093
11	Exon 3	c.398T > A	p.Ile133Asn	N/A
12	Exon 3	c.456C > A	p.Tyr152Ter	N/A
13	Exon 4	c.560T > C	p.Met187Thr	rs869312342
14	Exon 4	c.605G > A	p.Cys202Tyr	rs869312344
15	Exon 4	c.612G > A	p.Trp204Ter	N/A
16	Intron 4	c.640-16A > G	–	rs2071397
17	Exon 5	c.644A > G	p.Asn215Ser	rs28935197
18	Exon 5	c.776C > G	p.Pro259Arg	rs869312399
19	Exon 5	c.790G > T	p.Asp264Tyr	rs190347120
20	Exon 6	c.805G > A	p.Val269Met	rs869312427
21	Exon 6	c.812G > C	p.Gly271Ala	rs869312429
22	Exon 6	c.870G > A	p.Met290Ile	rs869312438
23	Exon 6	c.982G > A	p.Gly328Arg	rs104894832
24	Exon 7	c.1025G > A	p.Arg342Gln	rs28935493
25	Exon 7	c.1033_1034delTC	p.Ser345Argfs	rs398123198
26	Exon 7	c.1066C > T	p.Arg356Trp	rs104894827
27	Exon 7	c.1102G > A	p.Ala368Thr	rs144994244

[a] GenBank NM_000169.2.
[b] GenBank NP_000160.1.

Due to its low cost, high-throughput capability and sensitivity, HRM has been increasingly applied for mutation specific genotyping or mutation screening. However, no protocols were validated for FD HRM screening in the Brazilian population. Therefore, the purpose of this study was to develop a pre-sequencing genetic screening method based on HRM analysis suitable for the mutation profile seen on Brazilian FD patients.

2. Materials and methods

2.1. Samples

One hundred and three individuals (79 females and 24 males) with FD and previous molecular analysis of the *GLA* gene performed by Sanger sequencing were analyzed. In total 27 different variants were studied (Table 1). This study was approved by the Ethics Research Committee of our institution (#03-441 and #15-0196) and the patients gave written informed consent.

Stored DNA from whole blood samples were quantified with Nanodrop spectrophotometer (Thermo Fischer Scientific, USA) and diluted with TE buffer. To facilitate screening of males, artificial heterozygotes were created by mixing samples with male sequenced controls in a 1:1 ratio.

2.2. PCR and HRM

Amplification of the seven *GLA* exons was performed in 8 separate PCR reactions (exon 7 was amplified in two overlapping fragments due to its size). Reactions contained 20 ng of genomic DNA, 1 × PCR buffer, 1 × EvaGreen (Biotium, USA), 0.1 mM of dNTPs, 0.1 μM of each primer and 1 U of Platinum Taq DNA polymerase (Thermo Fischer Scientific, USA) in a final volume of 20 μL. Amplicon length ranged from 214 to 280 bp and Magnesium Chloride ($MgCl_2$) concentration for each fragment was empirically determined to provide the best resolution of melt curves. Detailed information on primer sequences, amplicons sizes, $MgCl_2$ and melt temperature for each amplicon are described in Supplementary Table S1. All reactions were made at least in duplicates in a StepOne Real-Time PCR instrument (Thermo Fischer Scientific, USA). PCR cycling conditions were 1 cycle of 95 °C for 5 min followed by 45 cycles at 95 °C for 15 s, Tm for 30 s and 72 °C for 30 s.

Immediately after PCR cycling, HRM was performed in the same instrument according to manufacturer's recommendations. Amplicons were heated to 95 °C for 10 s, cooled to 60 °C for 1 min, followed by melt curve generation by heating until 95 °C with 0.3% continuous ramp rate. Melt curves were analyzed with High Resolution Melt Software v3.0.1 (Thermo Fischer Scientific, USA). For each amplicon, from one to two samples from each genotype were identified as controls and software automatically classified the remaining.

3. Results and discussion

At least 2 variants were tested for each exon and all presented distinct melt curves from wild type controls (Fig. S1). However, allele discrimination is easier using normalized difference plots, shown in Fig. 1. For all amplicons and all of the tested variants it was possible to discriminate between wild type and heterozygotes, either in female or artificial male subjects. The highest allelic heterogeneity was seen in the amplicon including exon 1 and part of 5′UTR (Fig. 1A). We analyzed samples with eight different variants and nine different genotypes, since variants c.-10C > T and c.-12G > A were detected together in three patients. All genotypes were distinguishable from wild type. However, with increase number of concomitant genotypes per analysis, differences between mutated alleles were less evident. Exon 7, on the other hand, despite a reduced number of variants, all located within the first amplicon, did not yield such a clear discrimination plot (Fig. 1G). Even though all four variant genotypes showed different curves from wild type, it was also not possible to differentiate clearly between different genotypes. A similar result for this region was described in a study that utilized LightCycler reagents and instrument [7]. This indicates the observed result is related to the sequence characteristics and not a specific limitation of the system used.

We used a conventional PCR reaction together with a “release-on-demand” fluorescent dye instead of more expensive master-mixes. This represents an increase of less than USD 0.05 in reagents per standard PCR reaction to perform a high sensitivity assay capable of, in most cases, genotype specific mutations. Moreover, it leads to a significant reduction in sequencing costs, since only amplicons with altered patterns of melting would have to be further analyzed. Additionally, the equipment utilized was an affordable regular peltier block based real-time system with HRM capability but not specifically design for HRM analysis. Other specialized equipment such as LightScanner (BioFire Defense, USA) and LightCycler (Roche Molecular Systems, USA) have increased number of data points collection per degree and higher melting rates, which drastically decreases melting time [8]. Therefore, they can increase throughput but demand higher initial investment.

In addition to intrinsic sequence features as GC length and content, other factors such as DNA quality/purity and ionic strength are known to affect melting profiles [9]. Therefore, it is recommended that all samples undergo the same extraction protocol. In this study, we used DNA from peripheral blood collected in EDTA tubes. Although DNA from these samples was extracted with three different protocols (standard in house salting-out, commercial kits with manual or automatic extraction), after dilution with the same buffer the differences in DNA extraction methods did not influence results. On the other hand, we were unable to use samples from stored filter paper, as they resulted in high degree of variation in melt curves when compared to EDTA samples. Therefore, these samples had to be excluded from analysis and are not accounted for in any section of this report. However, samples included represent 82% of the variants already identified by our group in Brazilian patients with FD [10,11].

Finally, our protocol allowed differentiating each genotype from the others and consequently genotyping multiple variants simultaneously. However, it is not possible to exclude the possibility that a different