Benchmarks

Detection of interruptions in the GAA trinucleotide repeat expansion in the *FXN* gene of Friedreich ataxia

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Friedreich ataxia is a neurodegenerative disorder caused by the expansion of a GAA trinucleotide repeat sequence within the first intron of the *FXN* gene. Interruptions in the GAA repeat may serve to alleviate the inhibitory effects of the GAA expansion on *FXN* gene expression and to decrease pathogenicity. We have developed a simple and rapid PCR- and restriction enzyme-based assay to assess the purity of GAA repeat sequences.

Friedreich ataxia (FRDA) is an autosomal recessive disorder characterized by neurodegeneration and cardiomyopathy, and is the most common inherited ataxia. About 98% of individuals with FRDA are homozygous for an expansion of a GAA trinucleotide repeat sequence within the first intron of the *FXN* gene. The remaining individuals are compound heterozygotes for a GAA expansion and a point mutation. Pathogenic GAA expansion alleles are in the size range of 60 to >1300 repeats. The presence of a GAA repeat expansion results in the inhibition of *FXN* gene expression, reduced levels of full-length *FXN* transcript, and an insufficiency of the mitochondrial protein frataxin (1–4). Overall, an inverse correlation has been found between the size of the smaller GAA expansion and transcript levels, the amount of residual frataxin produced, and the age of onset of disease symptoms (5–9).

The asymmetric R-Y configuration of the GAA repeat expansion has been

shown to form stable R-R-Y or Y-R-Y triple helical DNA structures (10–13). The non-B DNA structure has been implicated in the inhibition of *FXN* gene transcription (4). In addition, the formation of a persistent RNA/DNA hybrid during transcription of the GAA repeat sequence appears to impede transcription elongation (14). It is now apparent that the GAA repeat expansion also generates a heterochromatin-mediated gene silencing effect (15,16). Age-dependent and tissue-specific somatic instability of the GAA repeat expansion may also be a determinant of the progressive pathology of FRDA (17–19).

There are a number of reports that describe variants of the GAA trinucleotide repeat expansion that contain interrupted sequences. There is evidence that such interruptions inhibit the formation of triplex DNA structures, alleviate the inhibitory effects on *FXN* gene expression, and reduce genetic instabilities (10–12,20–25). It remains

unclear to what extent these interruptions influence disease parameters such as age of onset and disease severity (21,25), but in some individuals they may account for discrepancies between the size of the GAA repeat expansion and clinical manifestations.

The confirmation of a clinical diagnosis of FRDA is usually limited to a genetic test for the presence of a GAA repeat expansion in intron 1 of the *FXN* gene. The diagnostic methods used (PCR, triplet repeat–primed PCR, and Southern blotting) do not distinguish between pure GAA sequences and those containing interruptions. Taking into consideration the possible repercussions of interrupted GAA repeats on clinical parameters, it would be beneficial to screen for such variants.

Since it is not technically feasible to entirely sequence long tracts of repetitive DNA, we have developed a simple and rapid PCR and restriction enzyme based assay to assess the purity of GAA repeat sequences. The two expanded alleles are amplified by PCR and subjected to restriction endonuclease digestion with *MboII*, which recognizes and cleaves a GAAGA sequence. Pure GAA repeat tracts are digested, leaving only flanking regions. The presence of extended flanking regions or additional bands indicates the presence of non-GAA sequences adjacent to or within the GAA expansion region. This restriction enzyme was used in the initial identification of a GAA repeat expansion as the causative mutation of FRDA (26).

Genomic DNA from four individuals with Friedreich ataxia was extracted and purified from blood samples using standard techniques. The GAA trinucleotide repeat expansion region of the *FXN* gene was amplified by PCR using the Expand Long Template PCR Kit (Roche Applied Science, Castle Hill, NSW, Australia) and primers GAA-B-F (5'-AATGGATTTCCTGGCAGGACGC-3') and GAA-B-R (5'-GCATTGGCGATCTTGGCTTAA-3'). The thermal profile was: 94°C for 5 min; 10 cycles of 94°C for 20 s, 61°C for 30 s, and 68°C for 5 min; 20 cycles of 94°C for 20 s, 62°C for 30 s, and 68°C for 5 min with a 20 s extension per cycle; and a final cycle at 68°C for 10 min. The synthesized PCR products contained the GAA trinucleotide repeat expansion with flanking sequences of 157 bp at the 5' end and 125 bp at the 3' end. PCR products were examined by electrophoresis on 0.8% agarose gels (Seakem GTG, Lonza, Mt. Waverley, Victoria,

Figure 1. Analysis of the GAA trinucleotide repeat expansion in the *FXN* gene of individuals with Friedreich ataxia. (A) The length of the GAA repeat region in each allele of the *FXN* gene was determined by long-range PCR and agarose gel electrophoresis. Patient 1, 770 and 870 repeats; Patient 2, 760 and 1170 repeats; Patient 3, 790 and 940 repeats; Patient 4, 650 and 1140 repeats; L: DNA size ladder. (B) PCR products following digestion with *Mbo*II. Complete digestion of pure GAA repeat sequences leaves flanking regions of 157 bp and 125 bp. The occurrence of an additional band of ~680 bp in lane P4 indicates the presence of non-GAA sequence within the GAA trinucleotide repeat expansion region.

Australia) and visualized with ethidium bromide (Figure 1A). All four individuals were homozygous for a GAA trinucleotide repeat expansion in each *FXN* allele but of different sizes between alleles. Each PCR product contained 282 bp DNA flanking the GAA trinucleotide repeat region. The number of GAA trinucleotide repeats in each *FXN* allele was calculated using the size of the PCR product (S_{PCR} , measured in bp), via the formula $(S_{PCR} - 282) \div 3$. The approximate sizes of the GAA trinucleotide repeat expansion were: Patient 1, 770 and 870 repeats; Patient 2, 760 and 1170 repeats; Patient 3, 790 and 940 repeats; Patient 4, 650 and 1140 repeats.

DNA was then subjected to restriction endonuclease digestion with *Mbo*II (New England Biolabs, Ipswich, MA, USA). *Mbo*II is a type II enzyme that has a recognition/cleavage sequence of 5'-GAAGA (8/7)-3' (the numbers in parentheses refer to the distance to the cleavage site on the top and bottom strands, respectively) (27,28). Pure GAA repetitive sequences are composed of multiple overlapping *Mbo*II restriction sites and are completely digested. The flanking sequences of the PCR products do not contain any *Mbo*II restriction sites. Digestion products were subjected to electrophoresis on agarose gels composed of a mixture of 1% Seakem GTG and 1% Metaphor (Lonza), and visualized with GelRed (Biotium, Hayward, CA, USA).

The complete digestion of pure GAA repeat tracts left only flanking regions of 157 bp and 125 bp as was the case in Patients 1–3 (Figure 1B, lanes P1–P3). The occurrence of extended flanking regions or additional bands would indicate the presence of non-GAA sequences adjacent to or within the GAA trinucleotide expansion region. In the case of Patient 4, an additional band of ~680 bp was observed (Figure 1B, lane P4).

In order to determine which of the two *FXN* alleles contained the sequence refractory to *Mbo*II digestion, the original undigested PCR products were separately excised from an agarose gel and purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) according to the manufacturer's protocol. The extracted products were used as template DNA for a second round of semi-nested PCR using primers GAA-B-F and GAA-Seq-R (5'-ATCTTGGCTTAATGCAACCTCTGCC-3'). The thermal profile was: 94°C for 5 min; 10 cycles of 94°C for 20 s, 63°C for 30 s, and 68°C for 5 min; 20 cycles of 94°C for 20 s, 65°C for 30 s, and 68°C for 5 min with a 20 s extension per cycle; and a final cycle at 68°C for 10 min. The synthesized PCR products contained the GAA trinucleotide repeat expansion with flanking sequences of 157 bp at the 5' end and 115 bp at the 3' end. PCR products were digested with *Mbo*II and subjected to

electrophoresis. The additional fragment of ~680 bp was found to be the product of the *FXN* allele containing the longer GAA expansion (data not shown).

The PCR product containing the non-pure GAA repeat was subjected to DNA sequencing in both directions. The sequence of the repetitive region was found to be (GAA)₂₁(GGAGAA)₅(GGAGGAGAA)₇₀(GAA)_n. The length of the sequencing read in either direction was not sufficiently long to determine the exact length of the pure GAA tract at the 3' end. However, *n* is estimated to be

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~870 based on the size of the PCR product. The (GGAGAA)₅(GGAGGAGAA)₇₀ sequence that was not cleaved by *Mbo*II was 660 bp in length and corresponds in size to the additional fragment observed on agarose gels.

The method is sufficiently sensitive to detect as little as 20–30 additional bp attached to the 157-bp and 125-bp flanking regions. Very small internal interruptions consisting of <50 bp may not be detected. However, small changes are less likely to affect pathogenicity. It has been demonstrated that GAA repeat tracts incorporating <11% of interrupted sequence are able to form triplexes similar to the uninterrupted sequences (22). The sensitivity of the assay can be increased by radiolabeling PCR products and detection of restriction fragments by autoradiography. Interrupted sequences that contain a high degree of a repeated GAAGA motif will be cleaved by *Mbo*II and not be detected by the assay. Such interrupted sequences have been described (24), although most naturally occurring interruptions to the GAA repeat expansion that have been reported do not fall into this category.

Given the possible impact of interruptions to the GAA repeat expansion in the *FXN* gene on pathogenicity, we believe that diagnostic tests for FRDA or studies investigating the correlation between GAA repeat size and the molecular and/or clinical parameters of disease should consider the incorporation of an assay for the integrity of GAA repeat sequences, such as the simple and rapid method that we have developed.

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Competing interests

The authors declare no competing interests.

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