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Short report

FXN gene methylation determines carrier status in Friedreich ataxia

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ABSTRACT

Background Friedreich ataxia (FRDA) is typically caused by homozygosity for an expanded GAA triplet-repeat (GAA-TRE) in intron 1 of the *FXN* gene. Some patients are compound heterozygous for the GAA-TRE and another *FXN* pathogenic variant. Detection of the GAA-TRE in the heterozygous state, occasionally technically challenging, is essential for diagnosing compound heterozygotes and asymptomatic carriers.

Objective We explored if the FRDA differentially methylated region (FRDA-DMR) in intron 1, which is hypermethylated in *cis* with the GAA-TRE, effectively detects heterozygous GAA-TRE.

Methods *FXN* DNA methylation was assayed by targeted bisulfite deep sequencing using the Illumina platform.

Results FRDA-DMR methylation effectively identified a cohort of known heterozygous carriers of the GAA-TRE. In an individual with clinical features of FRDA, commercial testing showed a paternally inherited pathogenic *FXN* initiation codon variant but no GAA-TRE. Methylation in the FRDA-DMR effectively identified the proband, his mother and various maternal relatives as heterozygous carriers of the GAA-TRE, thus confirming the diagnosis of FRDA.

Conclusion *FXN* DNA methylation reliably detects the GAA-TRE in the heterozygous state and offers a robust alternative strategy to diagnose FRDA due to compound heterozygosity and to identify asymptomatic heterozygous carriers of the GAA-TRE.

INTRODUCTION

Friedreich ataxia (FRDA; OMIM 229300) is an autosomal recessive condition characterised by progressive ataxia, cardiomyopathy and premature mortality.[1] Most patients are homozygous for an expanded GAA triplet-repeat (GAA-TRE) in intron 1 of the *FXN* gene.[2] Compared with <30 GAA triplets in non-FRDA alleles, GAA-TREs ranged from 100 to 1500 triplets, although patients with FRDA typically have both alleles with >500 triplets. The GAA-TRE induces epigenetic silencing of the *FXN* gene,[3 4] which causes deficiency of *FXN* transcript[5] and frataxin protein,[6] and deficient mitochondrial function.[7] Heterozygous carriers, who have only one GAA-TRE allele, have ~50% transcript and protein levels and remain asymptomatic. An epigenetic signature of the GAA-TRE involves DNA hypermethylation of the FRDA differentially methylated region (FRDA-DMR) in intron 1, which occurs in *cis* with the GAA-TRE, and is highly predictive of *FXN* transcriptional deficiency and age of onset in FRDA.[8–10] FRDA individuals homozygous for typical GAA-TREs with >500 triplets have >90% methylation in the FRDA-DMR, and non-FRDA individuals with both GAA alleles of <30 repeats have <10% methylation.[8] A minority of patients (~5%) are compound heterozygotes, having one *FXN* allele with a GAA-TRE and the homologous allele with another intragenic *FXN* pathogenic variant.[11 12] Deficiency of frataxin protein (or its function) in such patients is typically caused by a combination of epigenetic silencing due to the GAA-TRE and loss-of-function due to the pathogenic *FXN* variant.

The GAA-TRE is usually identified by long-range PCR[2 13] or triplet-primed PCR (TP-PCR),[14–16] and occasionally by Southern blot.[2 15] Intragenic *FXN* pathogenic variants are typically detected by sequencing the five coding exons plus flanking intronic regions,[2 11 12] and MLPA has been used for detecting deletion of one or more exons.[17] Molecular diagnosis of asymptomatic carriers of the GAA-TRE, and of individuals with FRDA due to compound heterozygosity, requires detection of the GAA-TRE in the heterozygous state. However, the latter is fraught with technical difficulties.[14–16 18] Some laboratories use protocols that incorporate more than one technique in order to avoid misdiagnosing heterozygous carriers.[14–16] Assuming that heterozygous carriers of GAA-TREs should have ~50% methylation in the FRDA-DMR,[8] we explored if this epigenetic readout could effectively identify GAA-TREs in the heterozygous state. In a series of known heterozygous carriers of the GAA-TRE, we found that methylation of the FRDA-DMR is a reliable way to identify heterozygosity. In a family where the proband had clinical features consistent with FRDA and tested positive for a paternally inherited *FXN* initiation codon deletion (c.2delT), commercial testing failed to detect a GAA-TRE. Methylation of the FRDA-DMR was used to establish the molecular diagnosis of FRDA in the proband and to identify his mother and various maternal relatives as heterozygous carriers of the GAA-TRE. Thus, methylation status of the FRDA-DMR is a robust alternative strategy for identifying the GAA-TRE in the heterozygous state.

PATIENTS AND METHODS

Study participants

Blood samples were collected in EDTA tubes at the Children's Hospital of Philadelphia (CHOP) and shipped overnight for analysis at the University of Oklahoma Health Sciences Center (OUHSC).

Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen).

Long-range PCR

GAA repeat lengths were measured using a long-range PCR kit (AccuStart Long Range SuperMix kit, QuantaBio) using primers 104F and 629R and previously described cycling conditions.[13]

DNA methylation analysis

The DNA methylation assay, analysis and validation were as previously described.[8] Briefly, genomic DNA was bisulfite treated and prepared for targeted deep sequencing. An amplicon encompassing CpG dinucleotides in the FRDA-DMR (n=11, numbered 72–82; upstream of the GAA-TRE in intron 1) was dual-indexed and pooled to create a library which was sequenced using the Illumina MiniSeq platform. Methylation panels depicting FRDA-DMR methylation were generated by stacking individual sequence reads (n=300 rows), with columns representing the n=11 CpG dinucleotides in the FRDA-DMR. Coordinates were marked black if methylated and white if unmethylated (individual reads (rows) were sorted for highest methylation at the bottom).

Statistical analysis

Statistical tests were performed using GraphPad, Prism V.9.2.

RESULTS

Detection of heterozygous GAA-TRE by *FXN* DNA methylation

To explore if DNA methylation of the FRDA-DMR can effectively detect heterozygous carrier status, we tested a cohort of known asymptomatic heterozygous carriers (n=32) and patients with FRDA known to be compound heterozygous for the GAA-TRE and another *FXN* pathogenic variant (n=5). All 37 individuals were tested with both long-range PCR and bisulfite deep sequencing (figure 1A–C). Long-range PCR detected the GAA-TRE in all 37 individuals, which ranged in size from 385 to 1350 triplets (figure 1B; only 15 of 37 carriers are shown). While all individuals were clearly identified as heterozygous carriers of the GAA-TRE, several expanded alleles were associated with some smearing (figure 1B), consistent with somatic instability of the GAA-TRE in FRDA.[19 20] Bisulfite deep sequencing (n=300 sequences) of the FRDA-DMR, a region located upstream of the GAA-TRE with 11 CpG sites (figure 1A),[8] revealed DNA methylation levels ranging from 25.4% to 70% (median=53.7%, 95% CI 50.6 to 59.3) in the 37 heterozygous individuals (figure 1C; the same 15 of 37 carriers in figure 1B are shown). The prevalence of fully methylated epialleles (ie, individual sequences with methylation at all 11 CpG sites, as indicated in figure 1C, ranged from 2.3% to 41.3% (median=22.7%), and correlated significantly with the length of the GAA-TRE in heterozygous carriers (n=37, R^2=0.44, p<0.0001; figure 1D), consistent with DNA methylation being in *cis* with the expanded allele. The level of DNA methylation seen in heterozygous carriers was compared with non-FRDA controls (with no expanded alleles; n=10) and patients with FRDA (homozygous for GAA-TREs; n=73; the latter data are from Ref. 8). Patients with FRDA were separated into two groups; those with both GAA-TREs >500 triplets (n=57) and with one GAA-TRE <500 triplets (n=16). Heterozygous carriers are clearly distinguishable from non-FRDA controls and both cohorts of patients with FRDA (figure 1E; in each case, p<0.0001; Kolmogorov-Smirnov test). While there is some overlap at the upper end of the range of heterozygous carriers and FRDA patients with a short GAA-TRE, the level of *FXN* methylation allowed confident separation of heterozygous carriers from non-FRDA controls and from typical patients with FRDA homozygous for long GAA-TREs. These data indicate that *FXN* DNA methylation in the FRDA-DMR is a reliable indicator of heterozygous carrier status in FRDA.

FXN DNA methylation detects heterozygous GAA-TRE when long-range PCR fails

A male child presented with ataxia, hypertrophic cardiomyopathy and scoliosis, whose condition progressed, with the need for wheelchair assistance and scoliosis correction in the mid-teens. He was diagnosed with diabetes and had a single episode of ketoacidosis. His heart remained hypertrophic with no evidence of systolic dysfunction. There have been no changes in vision or hearing. Commercial genetic testing (long-range PCR and sequencing of coding exons plus splice sites) revealed a paternally inherited c.2delT pathogenic variant in one *FXN* allele, and the GAA repeat length was reported as seven triplets on both alleles (ie, a GAA-TRE was not detected). A GAA-TRE was also not detected in his mother by the same commercial testing service. Testing for the GAA-TRE was repeated through another commercial laboratory (long-range PCR and Southern blot), which also indicated that the proband did not have a GAA-TRE. A lateral flow assay revealed low levels of frataxin protein in the proband's buccal cells, leucocytes and platelets, consistent with the diagnosis of FRDA. This suggested that the GAA-TRE in the proband (and presumably also his mother) was likely missed.

Long-range PCR analysis of the proband, his parents and several maternal relatives revealed multiple smeary bands in the expanded allele range in several individuals (range of 455–1220 triplets), suggestive of an unusually high level of somatic instability of a large GAA-TRE (figure 2A). However, this by itself could not be considered definitive evidence of heterozygous carrier status (eg, compared with typical heterozygous carriers in figure 1B) and may explain why the GAA-TRE was missed by two independent commercial testing laboratories.

Bisulfite deep sequencing of the FRDA-DMR was performed for all family members, which revealed DNA methylation levels consistent with heterozygous carrier status in the proband, his mother, grandmother and two maternal uncles (range 58%–69%; figure 2B). It is noteworthy that these were the same individuals who also showed the smeary band patterns on long-range PCR (see individuals 9.2 to 9.6 in figure 2A and B), suggesting that the smear represents a somatically unstable GAA-TRE. As expected for heterozygous carriers of the GAA-TRE, these individuals also showed substantial quantities of fully methylated epialleles (median=38.7%; 95% CI 37 to 46.7). The proband's father (who carries the c.2delT variant) and the two maternal aunts had low levels of methylation (<5%) consistent with GAA repeat lengths in the non-FRDA range and absence of a GAA-TRE. These data provide a molecular diagnosis of FRDA in the proband, who is compound heterozygous for the GAA-TRE and the c.2delT pathogenic variant.

DISCUSSION

Detection of heterozygous carriers of the GAA-TRE can be challenging. Long-range PCR, which is highly effective at diagnosing homozygous GAA-TREs, has a high false-negative rate for heterozygous GAA-TRE.[14–16] Possible reasons include preferential PCR amplification of the normal allele (allele dropout)[14] and high levels of somatic instability of the GAA-TRE,[19 20] both of which are more likely with long GAA-TREs (>1000 triplets). Long-range PCR also has a false-positive rate, where a heteroduplex formed between normal alleles of variable length

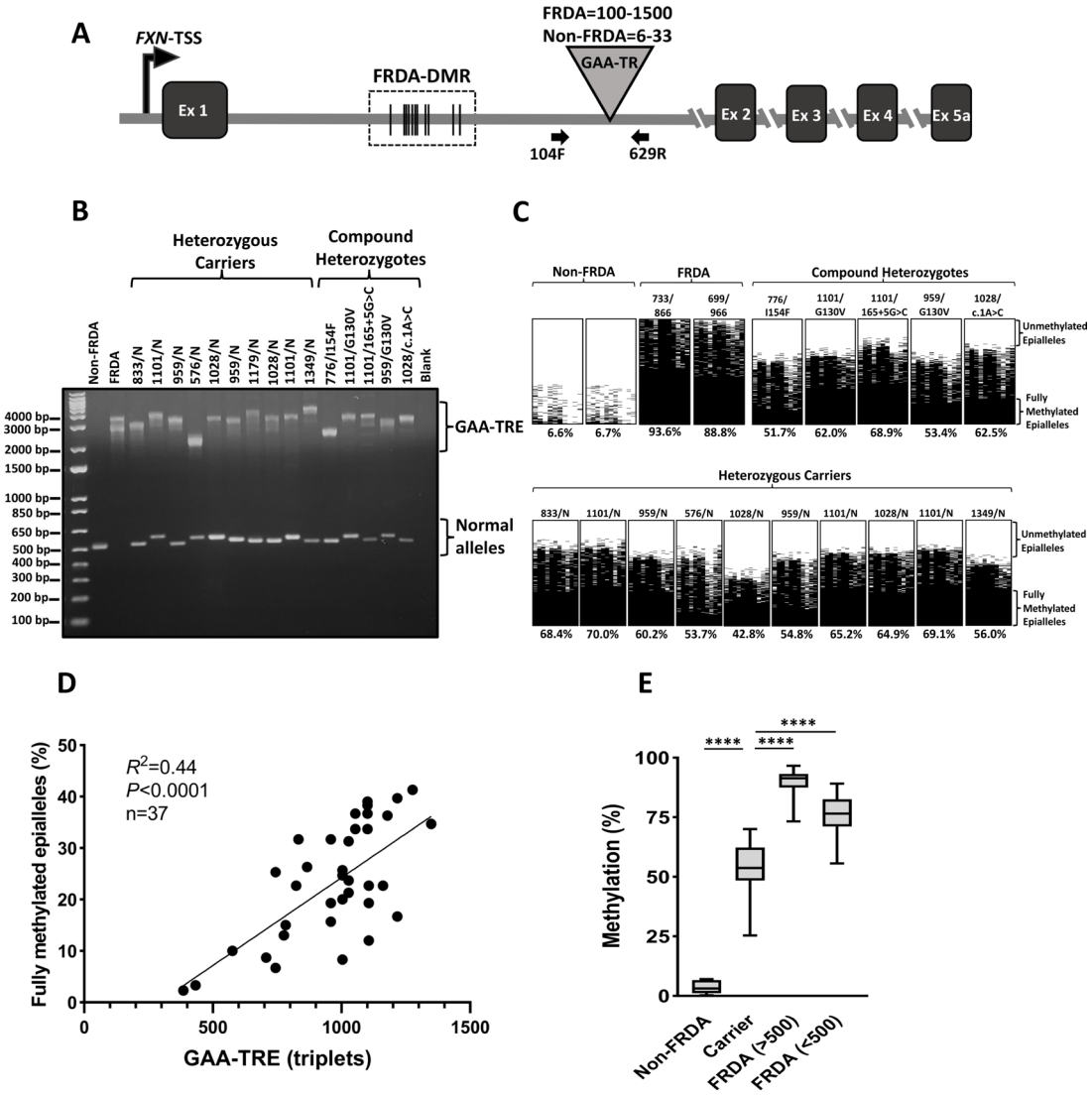

Figure 1 Detection of heterozygous GAA-TRE by *FXN* DNA methylation. (A) The *FXN* gene, showing all five coding exons (Ex1-Ex5a), the expanded GAA triplet-repeat in intron 1 (GAA-TR; triangle), primer sequences used for long-range PCR, and the FRDA-DMR upstream of the GAA-TR (dashed box; vertical lines represent 11 CpGs, per Ref. 8). (B) Long-range PCR detection of GAA-TREs and normal alleles in heterozygous carriers and compound heterozygotes (GAA-TREs are indicated in triplets; identity of pathogenic *FXN* variants is indicated; n=non-FRDA allele). Controls include non-FRDA, FRDA and zero template (blank). (C) Methylation of the FRDA-DMR in heterozygous carriers, compound heterozygotes, and FRDA and non-FRDA controls. Total per cent methylation, as well as methylated and unmethylated epialleles, is indicated. (D) Correlation of repeat length of the expanded allele (in triplets) in heterozygous carriers with DNA methylation in the FRDA-DMR (% of fully methylated epialleles). (E) DNA methylation (%) in the FRDA-DMR in non-FRDA controls (n=10), heterozygous carriers (n=37), and FRDA patients with both alleles >500 triplets (n=57) and those with one allele <500 triplets (n=16). Box plots show the entire range, median, 25th and 75th percentiles. ****p<0.0001. FRDA, Friedreich ataxia; FRDA-DMR, FRDA differentially methylated region.

masquerades as a GAA-TRE.[18] In a cohort of 310 individuals (with 38 homozygotes and 55 heterozygous carriers), long-range PCR mischaracterised 25 individuals; 14 heterozygous carriers were identified as not having a GAA-TRE, and 11 individuals without a GAA-TRE were identified as being heterozygous carriers.[14] TP-PCR is efficient at detecting the presence of an expanded allele,[14–16] although it typically finds that the GAA-TRE is above a certain threshold length without providing an accurate estimate of the length of the GAA-TRE. Southern blot, while accurate at detecting GAA-TREs in the heterozygous state, is tedious and requires large quantities of DNA. Some resort to using more than one test to effectively identify heterozygous carriers.[14–16]

We show that the methylation pattern of the FRDA-DMR allows accurate identification of heterozygous carriers of the GAA-TRE, in both asymptomatic carriers and compound heterozygotes. It is noteworthy that the GAA-TRE that was missed by two independent commercial labs, and showed multiple smeary bands in our long-range PCR assay, was readily diagnosable via methylation levels in the FRDA-DMR. Since most modern molecular diagnostic labs have next-generation sequencing capabilities, the bisulfite deep sequencing test used here offers a robust alternative method to accurately identify or rule out the GAA-TRE in the heterozygous state. While DNA methylation in the FRDA-DMR provides a reliable way to diagnose most heterozygous carriers of the GAA-TRE, it does have certain shortcomings. First, DNA methylation does not detect the GAA-TRE per se, but the epigenetic signature of the GAA-TRE, and so, like TP-PCR, this assay does not specify an actual expanded repeat length. Second, it is possible that the methylation levels in FRDA homozygotes and heterozygous carriers of a GAA-TRE could

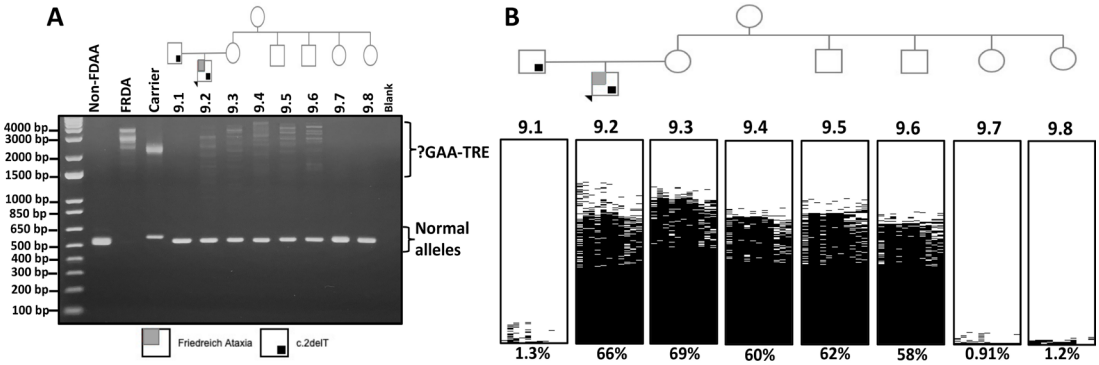

Figure 2 *FXN* DNA methylation detects heterozygous GAA-TRE when long-range PCR fails. (A) Long-range PCR of the family of the proband (arrowhead) showing smeary band patterns suggestive of GAA-TREs in individuals 9.2 through 9.6. Controls include non-FRDA, FRDA, heterozygous carrier, and zero template (blank). (B) Methylation of the FRDA-DMR in members of the proband's family indicating that individuals 9.2 through 9.6 are heterozygous carriers of the GAA-TRE (per cent methylation is indicated). FRDA, Friedreich ataxia; FRDA-DMR, FRDA differentially methylated region.

overlap, especially when a patient has one (or both) relatively short GAA-TRE(s). Third, in rare compound heterozygotes with a GAA-TRE and a large *FXN* gene deletion encompassing the FRDA-DMR, the resulting pattern of methylation would represent the hemizygous GAA-TRE, and depending on the length of the GAA-TRE, it could resemble a homozygous or heterozygous individual.

Contributors CL, KMG and LNR performed experiments. SB was involved in planning and supervision of the project. CL, KMG, LNR and SB processed and analysed data. CL and SB drafted the manuscript and designed the figures. DRL aided in interpreting the results and worked on the manuscript. DRL and KAS provided key patient-derived samples along with clinical information. SB procured funding for the project. All authors discussed the results and commented on the manuscript.

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Competing interests None declared.

Patient consent for publication Consent obtained directly from patient(s)

Ethics approval Research protocols were approved by the institutional review boards at both institutions: Children's Hospital of Philadelphia (IRB# 01-002609) and the University of Oklahoma Health Sciences Center (IRB# 8071). Participants gave informed consent to participate in the study before taking part.

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