

## Research Article

# Molecular Diagnosis of Friedreich Ataxia Using Analysis of GAA Repeats and *FXN* Gene Exons in Population from Western India

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The diagnosis of Friedreich ataxia is based on the clinical symptoms and GAA repeats expansions. In our experience, checking *FXN* gene exons for mutations along with GAA repeat analysis may give better clue for its diagnosis. In the present study, total 49 suspected Friedreich ataxia patients were analyzed for GAA repeat expansion. Eleven patients have normal number of GAA repeats, thereby termed as FRDA negative patients. Thirty-eight patients showed no amplification using GAA repeat analysis. Since no conclusion was possible based on these results, these patients were designated as uninformative. We have analyzed 5 exons of the *FXN* gene in FRDA negative and uninformative patients to check for possible mutations. It was observed that there were no mutations found in any of FRDA negative and most uninformative patients. We further used long range PCR to check for deletion of exon 5a. It was found that 18 patients showed expression for exon 5a PCR but none in long range PCR. Five patients showed no expression for exon 5a PCR as well as long range PCR indicating that these 5 patients may be positive FRDA patients. These findings need to be correlated with clinical history of these patients for confirmation.

## 1. Introduction

Friedreich ataxia (FRDA) is an autosomal recessively inherited neurodegenerative disease, caused due to expansion of the intronic GAA trinucleotide repeats or mutations in the *FXN* gene on chromosome 9q13 [1, 2]. The *FXN* gene consists of seven exons—1, 2, 3, 4, 5a, 5b, and 6. Frataxin, a highly conserved 210 amino acid protein with functions in iron-sulfur cluster biosynthesis, is produced by expression of exons 1–5a [1–6]. The gene *FXN* contains a GAA trinucleotide repeat in the first intron. The size range of the repeat is between 6 and 34 repeats in the wild-type gene [1, 7]. An expansion of the repeat in disease conditions increases the repeat size to 66–1700 or more repeats [1, 7]. It affects the expression of the gene, resulting in the reduced levels of frataxin mRNA and protein [3, 8]. Formation of sticky DNA, DNA-RNA hybrid, and epigenetic changes are the proposed mechanisms for disruption of *FXN* gene expression [9–11].

Most cases of FRDA are homozygous having expansion of the GAA trinucleotide repeat in the first intron of

the *FXN* gene, whereas few cases can be heterozygous, in which there is GAA expansion in one allele and point mutation or deletion in the other allele of *FXN* gene [1, 2]. The onset of FRDA is usually before 25 years of age [1, 12]. However, the disease onset after 25 years of age is known as late onset Friedreich ataxia (LOFA) [1]. The clinical features of FRDA include progressive gait and limb ataxia, scoliosis, proprioception, cardiomyopathy, diabetes mellitus, and foot deformity [1, 12].

Clinical features can be used as primary criteria for the diagnosis of FRDA [12]. However, since many neurological disorders share similar symptoms, it is necessary to use molecular diagnostic tests to confirm the diagnosis. Many diagnostic techniques for FRDA are available based on analysis of DNA [2, 13–19] as well as measurement of frataxin mRNA [20] and protein levels [21–24]. Also, many mutations have been identified in the *FXN* gene [2, 14, 25–29]. These mutations include point mutations [2, 14, 25, 26], deletion of few nucleotides [27], deletion-insertion mutations [28], and deletion of complete exons [14, 24]. Complete deletion of

TABLE 1: This table describes the clinical features of some suspected Friedreich ataxia patients, whose samples were analyzed in this study.

Sr. number	Patient	Age	Sex	Clinical symptoms
1	Uninformative	18	F	Unsteadiness of gait, slurring of speech since 6 years
2	FRDA negative	23	F	Imbalance while walking
3	FRDA negative	12	M	Imbalance while walking
4	FRDA negative	17	M	Imbalance while walking
5	Uninformative	32	M	Wide-based gait, positive cerebellar signs
6	FRDA negative	31	F	Imbalance while walking
7	Uninformative	16	F	Imbalance while walking
8	Uninformative	11	M	Imbalance while walking
9	FRDA negative	52	M	Imbalance while walking
10	Uninformative	20	M	Imbalance while walking
11	FRDA negative	12	F	Mild to severe cerebellar ataxia

the *FXN* gene has also been found to occur in FRDA [29]. It is therefore important to check for mutations in all the exons of the *FXN* gene along with GAA repeat analysis.

## 2. Materials and Methods

**2.1. Samples.** Blood samples were collected from normal individuals, and patients were referred to Jaslok Hospital and Research Centre, Mumbai, India, and used for further analysis as per the guidelines of Ethical Committee of Jaslok Hospital and Research Centre. DNA was extracted from the blood samples by using Qiagen (Qiagen, Hilden, Gmbh) QIAamp DNA extraction kit as per their established protocol. The clinical features of some of the suspected patients are described in Table 1.

**2.2. Molecular Analysis.** Molecular analyses of DNA samples were performed using PCR followed by agarose electrophoresis. DNA sequencing was used for further analysis of *FXN* gene exons.

**2.2.1. Analysis of Expanded GAA Repeats.** GAA repeat analysis was performed for 58 samples including 9 normal individuals and 49 patients. Analyses for expanded GAA repeats were performed using PCR using the following primer pair described by Campuzano et al. [2]: forward: 5'-GGAGGGAACCGTCTGGGCAAAGG-3'; reverse: 5'-CAATCCAGGACAGTCAGGGCTTT-3'. Conditions used for FRDA primers were as follows: 20 cycles of 94°C for 20 seconds, 68°C for 2 min 30 seconds, followed by 17 cycles in which the 68°C step was increased by 15 seconds per cycle, followed by a final extension of 72°C for 7 min. The PCR products obtained were analysed by electrophoresis, on a 0.8% agarose gel stained with ethidium bromide.

Patients were classified as "FRDA negative" and "uninformative" based on results obtained from GAA repeat expansion analysis. 11-patient samples in which GAA repeats were detected to be normal were classified as "FRDA negative" while 38 patients for whom no results were obtained were classified as "uninformative."

**2.2.2. Analysis of Exons of *FXN* Gene.** Exons of the *FXN* gene were amplified in using primers described by Zühlke et al. [14]. The following PCR conditions were used for analysis of *FXN* gene exon 1: 95°C for 5 min, which was followed by 35 cycles of 94°C for 30 seconds, and 72°C for 1 min, 72°C for 30 seconds, followed by 72°C for 10 min. PCR conditions for analysis of *FXN* gene exons 2, 3, 4, and 5a were as follows: 94°C for 4 min, followed by 35 cycles of 94°C for 30 seconds, 65°C for 30 seconds, 72°C for 45 seconds, followed by and 72°C for 10 min. The PCR products obtained from exon PCR were analysed by electrophoresis on a 2% agarose gel.

Long PCR was used for analysing a segment of the *FXN* gene consisting of a part of intron 4 and part of exon 5a. The primers used in this PCR were described by Zühlke et al. [14]. PCR conditions for analysis of *FXN* gene long PCR were as follows: 94°C for 5 min, followed by 10 cycles of 94°C for 10 seconds, 63°C for 30 seconds, and 68°C for 8 min, followed by 20 cycles in which the 68°C step was increased by 20 seconds per cycle followed by 68°C for 10 min. The PCR products obtained from long PCR for exon 5a deletion were analysed by electrophoresis, on a 0.8% agarose gel stained with ethidium bromide.

**2.2.3. Sequencing.** The products obtained by amplification of exons were further analysed by DNA sequencing on 3100 Genetic Analyzer by Applied Biosystems.

The sequences obtained by sequencing were compared with *FXN* gene mRNA cds sequence (Accession ID: U3747) which was numbered from 1 starting with A of ATG start codon from NCBI database. This comparison was performed using BioEdit Sequence Alignment Editor Software.

## 3. Results

**3.1. GAA Repeat Analysis.** The expected amplicon size from GAA repeat PCR is about 1.3 kb in normal individuals. The clinically suspected patients were expected to give amplicon size either similar to normal individuals, indicating normal GAA repeats, or larger than normal size, indicating presence of expanded GAA repeats [2]. Figure 1 shows the results obtained from GAA repeat analysis. Nine normal individuals

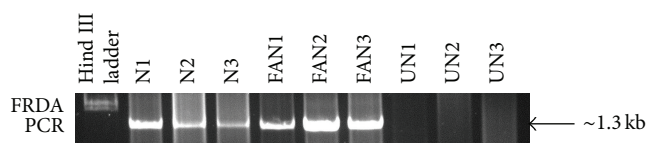


FIGURE 1: Shows results obtained from PCR for detection of expanded GAA repeats. Lanes N1, N2, and N3 represent normal patient's samples. Lanes FAN1, FAN2, and FAN3 represent FRDA negative patients and Lanes UN1, UN2, and UN3 represent uninformative patients showing no band.

and 11 clinically suspected cases of FRDA gave PCR products of expected size of 1.3 kb indicating that these patients could be negative for FRDA. Hence, these patients were termed as “FRDA negative” patients, whereas other 38 patients did not give any amplification in GAA repeat analysis as shown in Figure 1. Therefore it was very difficult to mark these patients as positive or negative for FRDA. Hence, these patients were termed as “uninformative” patients.

**3.2. PCR Analysis of Exons of *FXN* Gene.** Results from GAA repeat analysis are not sufficient to determine whether uninformative patients were positive for FRDA. Therefore, we decided to analyze exons of the *FXN* gene in the patients using primers described by Zühlke et al. [14]. It was observed that all exons were amplified in all normal individuals and all FRDA negative patients giving expected product sizes as shown in Figures 2(a) and 2(b). However, in case of uninformative FRDA patients UN2 and UN3 & UN5 did not give amplification in exon 4 and exon 1, respectively (Figure 2(c)). The amplified exons were further analyzed by DNA sequencing in order to check for presence of mutations.

**3.3. Sequencing of *FXN* Gene Exons.** We further analyzed the 5 *FXN* exons by DNA sequencing. We observed that there were no mutations found in normal individuals, all FRDA negative patients, and most of the uninformative patients. However, we found two novel mutations, that is, A226C and Ins246T in *FXN* exon 2 in one of the uninformative patients. The mutation A226C results in a change of the amino acid methionine to leucine at position 76 (M76L), while the mutation Ins246T results in a frame shift mutation in the frataxin protein leading to termination of protein at position 92. The sites of mutations are highlighted in Figure 3 along with the corresponding electropherogram as shown in Figure 4. Therefore, this uninformative patient may be positive for FRDA.

**3.4. Long PCR Analysis for Exon 5a.** We further analyzed *FXN* exon 5a using primers for long range PCR, described by Zühlke et al. These primers gave a PCR product of 3983 kb including coding region of exon 5a and a part of intron 4 [14]. In our study, it was observed that all normal, FRDA negative individuals, and 13 uninformative patients gave the expected PCR product size of ~4 kb as represented in Figure 5. However, 23 uninformative patients did not give any results in long range PCR. It was further observed that 18

of these 23 uninformative patients gave positive expression for coding region of *FXN* 5a gene. However, 5 uninformative patients were negative for *FXN* 5a long range PCR as well as coding region of *FXN* 5a indicating that these 5 patients may be FRDA positive patients. These patients should be correlated with their clinical history to confirm the status of FRDA in these uninformative patients.

## 4. Discussion

Extensive research has been carried out for understanding and diagnosis of Friedreich ataxia. Homozygous expansion of GAA repeats is the cause of 98% of FRDA cases [2]. Therefore, the presence of expanded GAA repeats in first intron of the *FXN* gene is used as basis for molecular diagnosis of FRDA in most of the cases. Many techniques have been explored for estimating GAA repeat size [2, 13–19]. Occurrence of FRDA has been reported to be less frequent in Indian population [30]. In our research, we found that many patients showed no expansion of GAA repeats, and, hence, they may be called normal. Therefore, these patients were termed as “FRDA negative” patients. The remaining 38 patients studied in our lab did not give any amplification of GAA repeats as shown in Figure 1, and, hence, it was difficult to conclude the disease status of these patients. Therefore, these patients were termed as “uninformative patients.” This situation has not been reported elsewhere.

There is a need of an additional test to determine the status of uninformative patients. Hence, we decided to analyze five exons of the *FXN* gene for presence of possible mutations. Two percent of FRDA cases are heterozygous for a mutation in the *FXN* gene exons [2]. Various types of mutations have been described, that is, point mutations, deletion of exons, and even complete deletion of the *FXN* gene in heterozygous patients [2, 14, 20–29]. In our analysis, we found that the exons of the *FXN* gene were normal in all FRDA negative patients and most of the uninformative patients. However, in one uninformative patient, two novel point mutations were observed in exon 2. Hence, this patient may be positive for FRDA.

We further analyzed exon 5a using long range PCR described by Zühlke et al. [14]. Zühlke et al. identified a heterozygous deletion of 2776 bp including exon 5a using this method [14]. We observed that in long range PCR studies all FRDA negative and 13 out of 38 uninformative patients showed expression of ~4 kb band indicating that all these patients were normal. However, remaining 23 uninformative patients showed no expression of exon 5a in long range PCR. This may be due to deletion or mutations in the intron 4 or exon 5a region. It shows that there is a need of examining exon 5a in long range PCR for confirmation of uninformative patient as FRDA positive patient. Overall, this suggests that all of these 23 patients may be FRDA patients and this needs to be confirmed by correlation with their clinical symptoms.

## 5. Conclusions

Friedreich ataxia is an autosomal recessive neurodegenerative disorder that occurs commonly due to expansion of the GAA

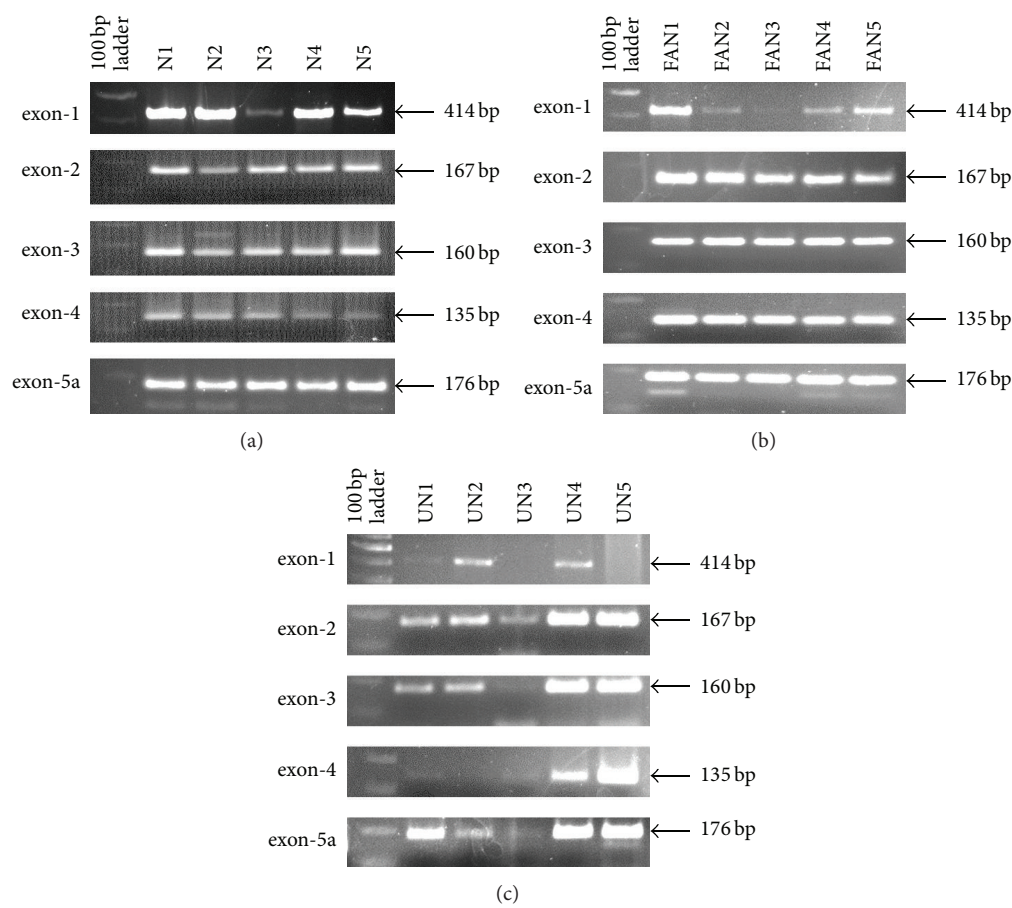


FIGURE 2: Shows exon PCR results for (a) normal samples represented by N1–N5, (b) FRDA negative samples represented by FAN1–FAN5, and (c) uninformative samples represented by UN1–UN5.

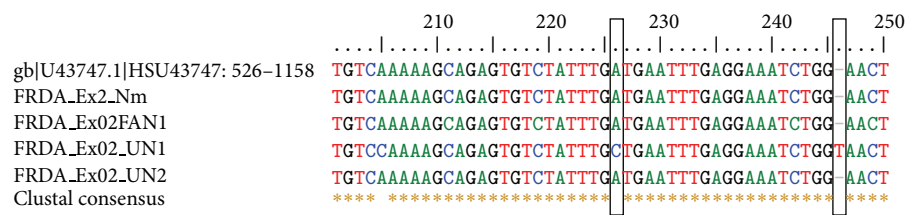


FIGURE 3: Shows BioEdit analysis of exon 2 of the *FXN* gene in normal individual, FRDA negative patient and one uninformative patient, which showed mutation at A226C and Ins246T. The sites of mutation in exon 2 have been highlighted by boxes.

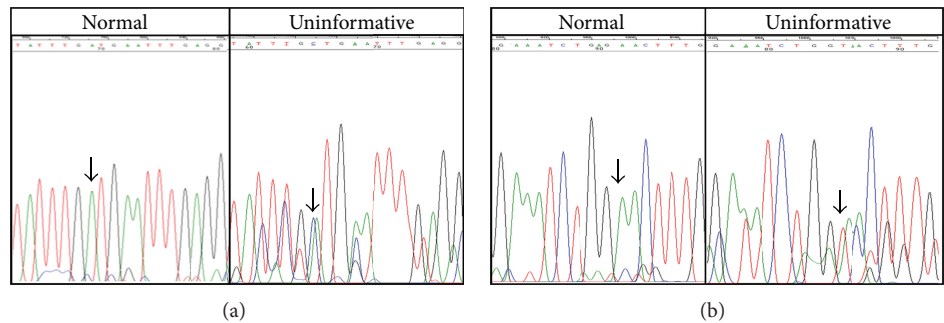


FIGURE 4: Shows electropherograms for 2 novel mutations (A226C and Ins246T) seen in one of the FRDA uninformative patient. The mutation sites are indicated by arrows.

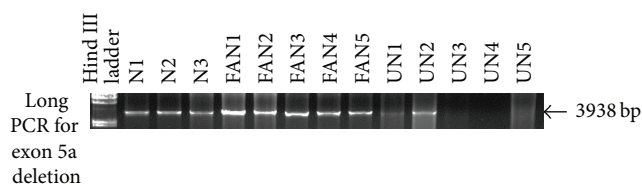


FIGURE 5: Shows results of long PCR for exon 5a analysis in normal individuals represented by Lanes N1–N3. FRDA negative patients are represented by Lanes FAN1–FAN5, and uninformative patients are represented by Lanes UN1–UN5.

trinucleotide repeats in first intron of *FXN* gene. Molecular diagnostic tests for FRDA are mainly based on detection of the GAA repeats. However, we found in our study of the West Indian patients that an additional test may be required to diagnose FRDA in cases which may give negative result for GAA repeat analysis. This may be due to heterogeneity in *FXN* gene. We, therefore, studied additional techniques such as mutation analysis in five exons of the *FXN* gene and exon 5a in long PCR to confirm these patients for FRDA. We found that there was no expression of exon 5a in long range PCR, which clearly confirmed the diagnosis of FRDA. Therefore, exon 5a in long range PCR is an essential test for FRDA in western Indian population. This study, thus, highlights the significance of examining *FXN* gene exons by mutation analysis and exon 5a in long range PCR and correlating the results with the clinical symptoms for a conclusive diagnosis of FRDA in patients from western India.

## Conflict of Interests

The authors declare no conflict of interests.

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