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Agarose-based long-range polymerase chain reaction assay for genotyping Turkish patients with Friedreich's ataxia

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ABSTRACT

Objective: An agarose-based long-range polymerase chain reaction (PCR) assay was performed on patients with a preliminary diagnosis of Friedreich's ataxia (FA). It aimed to determine the repeat number in intron 1 of the *FXN* gene and to investigate the genotype-phenotype correlation.

Patients and Methods: Nineteen cases participated in the study. Long-range PCR was performed under appropriate conditions using two custom-designed primer pairs (F1-R1 and F2-R2). The PCR products were run on an agarose gel, and guanine-adenine-adenine (GAA) repeat numbers were determined.

Results: Biallelic GAA repeat expansions were detected in 6 patients. Four patients had typical FA. One had late-onset FA (LOFA). Another had very late-onset FA (VLOFA). All patients diagnosed with FA had ataxia and areflexia. The detected repeat numbers were approximately 190-1120 for F1-R1 and 170-1100 for F2-R2. The patient with LOFA had 190/1000 GAA repeats. The VLOFA patient had 290/290 GAA repeats.

Conclusion: An earlier clinical onset correlated with higher GAA repeats. An agarose-based long-range PCR assay is a quick, low-cost, and effective way to determine GAA repeats. This study shows it can diagnose the disease and identify carriers.

Keywords: FA, Long-range PCR, *FXN*, GAA repeats, agarose-based

1. INTRODUCTION

Friedreich's ataxia (FA, OMIM: #229300) is a rare genetic disorder. It mainly affects the nervous system. The typical clinical findings include cerebellar signs like ataxia (in almost all patients) and dysarthria (95%) [1], ophthalmologic signs like optic nerve atrophy (25%) [2], cardiac manifestations like cardiomyopathy (in about two-thirds of individuals) [1], and endocrinologic findings such as impaired glucose tolerance (49%) and diabetes mellitus (30%) [3]. Complications related to cardiac disease are the leading cause of death, responsible for about 60% of all fatalities [4]. Although FA typically manifests in childhood or early adulthood (mean age: 5-15), there is considerable variation in both the age of onset and the severity experienced by individuals [1]. Approximately 75% of individuals with FA exhibit "typical FA", while around 25% present with "atypical FA" [2]. The atypical clinic comprises late-onset FA (LOFA) with disease onset after age 25; very late-onset FA (VLOFA), with disease onset after age 40; and FA with retained reflexes (FARR) [2].

Friedreich's ataxia is the most common early-onset inherited ataxia, which follows an autosomal recessive inheritance pattern. The prevalence of FA is 0.5 per 100,000 based on clinical and molecular diagnoses [5]. Its carrier frequency ranges from 1 in 60 to 1 in 100 [5]. The *FXN* gene (OMIM: *606829, NM_000144.4) is on chromosome 9q21.11, consists of 5 exons, and has a 2,608 bp transcript. FA is caused by an expansion of a guanine-adenine-adenine (GAA) trinucleotide repeat in intron 1 of the *FXN* gene [6]. Repeat numbers between 5 and 33 indicate normal alleles, whereas repeat numbers between 34 and 65 GAA classify as premutation alleles. Borderline alleles fall within the range of 44 to 65 GAA. When the GAA repeat numbers exceed 66, they are classified as expanded alleles [2]. Approximately 96% of patients with FA have biallelic GAA repeat expansions in the

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FXN gene. In 4% of patients, a compound heterozygous variant in the *FXN* gene occurs, along with increased GAA repeats [7]. Biallelic pathogenic *FXN* single nucleotide variants have not been reported in the literature except for a single family. In the reported family, a Charcot–Marie–Tooth disease-like phenotype was observed in three affected children [8].

In this study, we applied an agarose-based long-range polymerase chain reaction (PCR) assay to patients with a preliminary diagnosis of FA to accurately determine the repeat number and investigate the genotype-phenotype correlation.

2. PATIENTS and METHODS

Patients

Informed consent was obtained from the patients or their parents. A total of 19 patients with a preliminary diagnosis of FA from 17 families participated in the study. Additionally, the mothers, fathers, and siblings of patients from three of these families were included. An agarose-based long-range PCR assay was conducted on a total of 26 individuals. The demographic characteristics, neurological examinations, cardiac evaluations, ophthalmological examinations, X-ray images, fasting blood glucose, and HbA1c levels of the participants were recorded. This study received approval from the Ethics Committee of Marmara University School of Medicine, Turkey (approval number:09.2021.02/date:08.01.2021).

Molecular studies

Peripheral blood samples were collected from a total of 26 individuals. Genomic deoxyribonucleic acid (DNA) was isolated from 20 of these using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. For the remaining 6 cases, DNA was extracted from leukocyte fractions using the phenol-chloroform extraction method, a standard manual technique. DNA purity and concentration were assessed using the Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA). DNA concentrations for the samples processed with the commercial kit ranged from 10.1 ng/μL to 44.2 ng/μL, with a mean of 21.59 ng/μL. Samples processed using the phenol-chloroform method yielded higher concentrations, ranging from 77.1 ng/μL to 454.4 ng/μL, with a mean of 227.1 ng/μL. The overall mean DNA concentration was 80.2 ng/μL. Absorbance ratios (A260/A280 and A260/A230) were measured for all samples and found to be within the acceptable range of 1.7 to 2.0, indicating sufficient purity for downstream analyses.

To detect GAA trinucleotide repeat expansions in the *FXN* gene, long-range PCR was performed using two custom-designed primer pairs (F1-R1 and F2-R2), both located in intron 1 of the gene. The sequences of the primers were as follows:

F1: 5′-GGGATTGGTTGCCAGTGCTTAAAGTTAG-3′

R1: 5′-ATCTAAGGACCATCATGGCCACACTTG-3′

F2: 5′-AGGGATCCGTCTGGGCAAAGG-3′

R2: 5′-CAATCCAGGACAGTCAGGGCTTT-3′

Each PCR reaction was carried out in a total volume of 25 μL, containing: 0.5 μg of genomic DNA, 5 μL of 5×GC buffer, 0.5 μM of each forward and reverse primer, 0.2 mM deoxynucleotide triphosphates (dNTPs), 1 unit of Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Waltham, MA). The PCR thermocycling conditions were as follows:

- Initial denaturation at 98 °C for 30 seconds,
- 35 cycles of: – Denaturation at 98 °C for 10 seconds
  – Extension at 72 °C for 5 minutes
- Final extension at 72 °C for 10 minutes

The same amplification protocol was applied for both primer pairs. Agarose gel electrophoresis and visualization of PCR products were separated on a 1% agarose gel using GeneRuler DNA Ladder (Thermo Fisher Scientific, Waltham, MA), which includes DNA size markers up to 10,000 bp. Electrophoresis was carried out at 100 volts for 45 minutes using the EC330 power supply (Thermo Fisher Scientific, Waltham, MA). The DNA bands were visualized using the GAS700XX/71XX Gel Imaging System (Biolab, United Kingdom). The expected product sizes for normal (non-expanded) alleles were: 640 bp for the F1-R1 primer pair and 1560 bp for the F2-R2 primer pair. In individuals carrying expanded GAA repeats, the total amplicon size increases proportionally to the length of the repeat region. The number of GAA repeats in each allele was estimated based on the size of the PCR product, as follows: The total amplicon size was measured from the agarose gel (using the DNA ladder as a reference). For the F1-R1 product, 640 bp was subtracted from the total size. For F2-R2, 1560 bp was subtracted from the total size. The remaining base pair length corresponds to the length of the GAA repeat region. Since each GAA repeat consists of 3 base pairs, the remaining length was divided by 3 to calculate the approximate number of repeats.

To detect sequence variations in the *FXN* gene, all five exons of the gene were amplified using appropriate primers and analyzed by the Sanger sequencing method. Sanger sequencing was performed using ABI Prism 3500 Genetic Analyzer (Thermo Fisher Scientific, Mass, USA).

Statistical Analysis

For statistical analysis, Statistical Package for the Social Sciences (SPSS) software (version 26.0) (IBM Corp., America) was used. Descriptive statistics were used to summarize the data. Continuous variables were expressed as mean ± standard deviation or median (minimum–maximum), as appropriate. Categorical variables were presented as numbers and percentages.

3. RESULTS

Among the FA pre-diagnosed cases, nine were female and ten were male. The ages of the cases ranged from 10 to 65 years (mean: 31.65 years). All cases had ataxia. Biallelic GAA repeat expansions were detected in six (37.5%) cases, and monoallelic expansion was detected in two (12.5%) cases. Of six patients with FA, three (H2, H3, H4) had the same repeat number expansion in both alleles (homozygous). The other three (H1, H5, H6) had different

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expansions in each allele (compound heterozygous). The minimum and maximum repeat numbers detected were approximately 190 and 1120 for F1-R1, and 170 and 1100 for F2-R2 (Figure 1a, b).

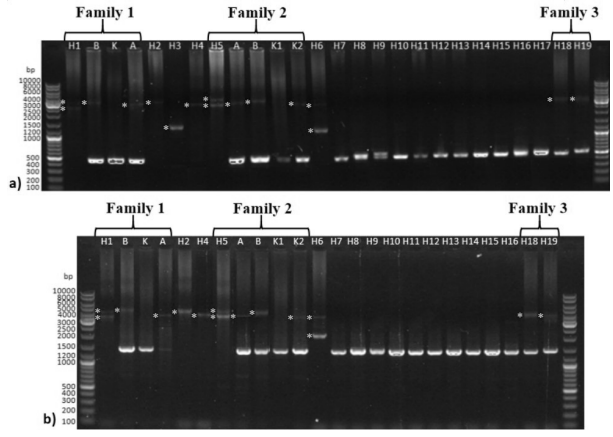

Figure 1. *Agarose-based long-range PCR assay results using: a) the F1-R1 primer set, and b) the F2-R2 primer set. Biallelic GAA repeat expansions were detected in 6 cases (H1, H2, H3, H4, H5, H6), and monoallelic expansion was detected in 2 cases (H18, H19). Of six patients with FA, three (H2, H3, H4) had the same repeat number expansion in both alleles (homozygous). The other three (H1, H5, H6) had different expansions in each allele (compound heterozygous).*
The maximum and minimum repeat numbers detected were approximately 190 and 1120 for F1-R1 and 170 and 1100 for F2-R2. Alleles showing expansion are marked with a yellow asterisk. (The expected band sizes for the F1-R1 and F2-R2 primer pairs were 640 bp and 1560 bp, respectively. To find the GAA repeat size, subtract 640 (for F1-R1) and 1560 (for F2-R2) bp from the amplicon size. Then divide the result by 3. The size of the repeat number can be estimated within a range of ±30 GAA repeats. H: Patient, B: Father, K: Sibling, A: Mother)

Of the patients with biallelic expansions, three were female and three were male. The ages of the patient group with a molecular diagnosis ranged from 7 to 40 years (mean: 18.5). In four of the six patients, typical FA (66.6%) was noted. One had LOFA (16.6%), and another had VLOFA (16.6%). There were no patients diagnosed with FARR in this study. All patients diagnosed with FA had ataxia and areflexia. Two patients had no additional clinical findings other than ataxia and areflexia. One of these two patients had typical FA, while the other had LOFA. Scoliosis (50%) was detected in three patients. Cardiac involvement (33.3%) and nystagmus (33.3%) were observed in two patients, while diabetes mellitus (16.6%) and dysarthria (16.6%) were noted in one patient (Table I). All patients with scoliosis, cardiac findings, and diabetes mellitus had typical FA phenotypes. Dysarthria was observed only in the patient diagnosed with VLOFA. In the patient with a LOFA (H6) phenotype, 190/1000 GAA repeats were detected, while the patient with VLOFA (H3) had 290/290 GAA repeats (Table I).

Patient H18 was a 48-year-old female presenting with ataxia and areflexia symptoms that began at age 40. Patient H19, a 49-year-old male and the brother of H18, exhibited ataxia and areflexia starting at age 42. There were no additional clinical findings suggestive of FA in either case. There was no consanguinity between their parents. Both cases were referred to our laboratory for molecular testing due to VLOFA. In both cases, an increased number of GAA repeats in the heterozygous state (approximately 1000 GAA repeats) was detected using agarose-based long-range PCR, and no pathogenic variants were identified in the *FXN* gene through sequencing analysis (Table II). Based on these molecular and clinical findings, a presumptive diagnosis of FA was ruled out for these individuals.

Table I. *Demographic and clinical findings of the patients diagnosed with FA*

Patient No.	Age (year)	Gender	Clinical characteristics	Ataxia	Dysarthria	Areflexia	Nystagmus	Scoliosis	Cardiac involvement	Diabetes mellitus
H1	7	Male	Typical FA	+	-	+	+	+	+	-
H2	7	Female	Typical FA	+	-	+	-	+	-	+
H3	40	Male	VLOFA	+	+	+	+	-	-	-
H4	15	Female	Typical FA	+	-	+	-	+	+	-
H5	12	Female	Typical FA	+	-	+	-	-	-	-
H6	30	Male	LOFA	+	-	+	-	-	-	-

FA: Friedreich's ataxia, LOFA: Late-onset FA, VLOFA: very late-onset FA

Table II. *Genotypes of the patients detected using the Agarose-Based Long-Range PCR Assay.*

Patient No.	Clinical characteristics	F1-R1*		F2-R2*	
		GAA1	GAA2	GAA1	GAA2
H1	Typical FA	690	1020	670	1000
H2	Typical FA	1120	1120	1100	1100
H3	VLOFA	290	290	270	270
H4	Typical FA	1120	1120	1100	1100
H5	Typical FA	790	1120	730	1100
H6	LOFA	190	1000	170	1000

**The guanine-adenine-adenine (GAA) repeat size was determined by subtracting 640 (for primer set F1-R1) and 1560 (for primer set F2-R2) bp from the amplicon size and dividing the obtained value by 3. The size of the repeat number can be estimated within a range of ±30 GAA repeats. FA: Friedreich's ataxia, LOFA:Late-onset FA, VLOFA:very late-onset FA*

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4. DISCUSSION

In this study, we investigated the correlation between GAA repeat expansions and clinical manifestations in Turkish FA patients using an agarose-based long-range PCR assay. Our findings demonstrated that higher GAA repeat numbers correlate with earlier disease onset, consistent with previous studies [9].

Biallelic expansion of the GAA trinucleotide repeat in intron 1 of the *FXN* gene is the primary cause of FA. The *FXN* gene encodes the frataxin protein, which consists of 210 amino acids. Frataxin is a mitochondrial iron chaperone encoded by the nuclear genome. Frataxin is crucial for: (1) the synthesis of iron-sulfur clusters, which are necessary for the activity of enzymes in respiratory chain complexes I-III; (2) metabolism, including aconitase; and (3) various proteins involved in DNA, ribonucleic acid (RNA), and protein synthesis [10]. The pathogenic expansion of GAA repeats leads to a reduction in *FXN* gene transcription by preventing the binding of transcription factors and RNA polymerase II to the *FXN* gene and by causing epigenetic changes such as DNA methylation [11]. As a result, the reduction in *FXN* mRNA impairs mitochondrial respiratory function and increases oxidative stress [12].

Our study confirmed the universal presence of ataxia in all FA patients, aligning with previous reports that identify it as the cardinal presenting symptom [1]. However, the prevalence of dysarthria (16.6%) in our cohort was notably lower than the 95% reported in the literature [13]. This discrepancy might be due to several factors, including our patients' age distribution and possible variations in clinical assessments across age groups.

The GAA1 allele is the shorter of the two expanded alleles, while GAA2 is the longer one. GAA1 repeat size negatively correlates with the age of onset and disease severity. A higher repeat size indicates a more severe clinical presentation and an earlier onset of the disease [14]. If the GAA1 repeat number is under 300, it is typically associated with VLOFA. If it is under 500, it is more likely linked to LOFA. Interrupted GAA repeats may also be associated with a milder clinical presentation and later age of onset. In our study, the VLOFA patient had 290 GAA repeats in the GAA1 allele. The LOFA patient had 190 GAA repeats in the GAA1 allele. Although these repeat numbers are consistent with the literature, it was expected that the patient with the VLOFA phenotype would have fewer repeats in the GAA1 allele [14]. This discrepancy might be explained by the presence of interrupted repeats or a potential influence of the GAA2 allele on the clinical phenotype. Nevertheless, in our study, a correlation between higher GAA repeat numbers and earlier clinical onset was demonstrated.

Methods such as Southern blot [2], long-range PCR [5], and repeat-primed PCR [15] can determine GAA repeat numbers. Additionally, Sanger sequencing and next-generation sequencing can detect single nucleotide variants in the *FXN* gene. It is important to emphasize that standard molecular genetic tests do not determine nucleotide interruptions in the GAA tract. However, long-read sequencing is expected to be the future method for detecting repeat numbers and interruptions [16]. The Southern blot technique has long been regarded as the gold standard for detecting repeat sizes. However, its practical use has declined in recent years due to being labor-intensive and time-consuming. The benefits of using an agarose-based long-range PCR assay compared to Southern blot techniques include lower DNA concentration requirements, faster turnaround time, and the absence of the need for radiological materials. Also, this study shows that the agarose-based long-range PCR assay can detect carriers (H18, H19) with different-sized repeats, overcoming allele dropout. The largest allelic expansions documented in the literature range from 1700 to 2000 GAA repeats [5]. In this study, the maximum fragment sizes in the agarose-based long-range PCR method were about 1120 and 1100 GAA repeats for the F1-R1 and F2-R2 primers, respectively.

This study has potential limitations. The single-center design and relatively limited sample size represent important methodological considerations that may affect the generalizability and statistical power of the findings. Data collected from a single institution may reflect region-specific characteristics, referral patterns, or technical practices that do not necessarily represent broader or more diverse populations. Similarly, the small sample size limits the ability to perform subgroup analyses and may reduce the statistical robustness of the conclusions. While the findings are consistent and clinically relevant within the studied cohort, larger multi-center studies would be valuable to confirm these results and enhance their applicability to a wider population. The study's cross-sectional nature prevents establishing causal relationships or observing longitudinal changes over time. Also, agarose-based long-range PCR assays have low resolution. So, size estimates are accurate only within about 30 GAA repeats. Another significant limitation of this method is its inability to detect interrupted GAA repeat sequences, which may have clinical relevance due to their potential phenotypic impact. Despite these limitations, this assay remains a practical and accessible option for diagnostic purposes, especially in laboratories with limited technical or financial resources.

Conclusion

In conclusion, agarose-based long-range PCR assay is fast, effective, and cheap method for determining the GAA repeats in the *FXN* gene. This study demonstrates that it can accurately diagnose the disease and identify carriers. Determining the exact repeat number is crucial for establishing an accurate diagnosis, planning follow-up care, and providing proper genetic counseling.

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Compliance with Ethical Standards

Ethical approval: This study received approval from the Ethics Committee of Marmara University School of Medicine, Turkey (approval number:09.2021.02/date:08.01.2021). Informed consent was obtained from the patients or from their families.

Conflict of interest: The authors declare that there is no conflict of interest.

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Author contributions: CA and AA: Conceptualization, investigation, methodology, formal analysis, writing, PKK: Validation, visualization, and manuscript editing, KU and DT, supervision, reviewing, and editing of the manuscript, All authors approved the final manuscript.

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