Neurogenetics (1998) 1:253–257

Original article

The correlation of clinical phenotype in Friedreich ataxia with the site of point mutations in the *FRDA* gene

Susan M. Forrest · Melanie Knight · Martin B. Delatycki · Damien Paris · Robert Williamson John King · Leone Yeung · Najah Nassif · Garth A. Nicholson

Received: March 24, 1998 / Accepted: May 28, 1998

ABSTRACT

Most cases of Friedreich ataxia (FRDA) are due to expansions of a GAA trinucleotide repeat sequence in the *FRDA* gene coding for frataxin, a protein of poorly understood function which may regulate mitochondrial iron transport. However, between 1% and 5% of mutations are single base changes in the sequence of the *FRDA* gene, causing missense, nonsense, or splicing mutations. We describe three new mutations, *IVS4nt2* (T to G), *R165C*, and *L182F*, which occur in patients in association with GAA expansions. These cases, and a further five reported cases of point mutations causing FRDA, demonstrate that splicing, nonsense, or initiation codon mutations (which cause a complete absence of functional frataxin) are associated with a severe phenotype. Missense mutations, even in highly evolutionally conserved amino acids, may cause a mild or severe phenotype.

Key words Point mutation · Friedreich ataxia · GAA expansion · Phenotype

S.M. Forrest (✉) · M. Knight · M.B. Delatycki · D. Paris R. Williamson
Murdoch Institute for Research into Birth Defects, Royal Children's Hospital, Flemington Road, Parkville, Victoria, 3052, Australia

J. King
Department of Neurology, Royal Melbourne Hospital, Parkville, 3052, Australia

L. Yeung · N. Nassif · G.A. Nicholson
University of Sydney Department of Medicine, Molecular Medicine Laboratory, Concord Hospital, 2137, NSW, Australia

INTRODUCTION

Friedreich ataxia (FRDA) is the most-common autosomal recessive hereditary ataxia, affecting about 1 in 50,000 individuals [1]. The essential diagnostic criteria, as defined by Harding [2], include onset before 25 years and within 5 years of onset there is progressive ataxia of limbs and gait, absent knee and ankle jerks and extensor plantar responses, as well as dysarthria after 5 years of symptom onset. Small or absent sensory action potentials are also required. Patients who do not meet all the essential criteria are defined as atypical.

The 210-amino acid protein, frataxin, which is mutated in FRDA, has homologs in species such as yeast and *Caenorhabditis elegans* [3]. Studies of yeast have shown that the homologuous gene, *YFH1*, encodes a mitochondrial protein which is involved in iron homeostasis and respiratory function [4, 5]. Campuzano et al. [6] demonstrated that human frataxin is located in the mitochondria, consistent with the yeast localization.

Over 95% of mutations which cause FRDA are due to an expansion of a triplet repeat in intron *1* of the *FRDA* gene (originally known as *X25*) [3]. Most affected individuals have repeats in the range from 600 to well over 1,000 GAA repeats, but occasional cases with as few as 66 repeats have been recorded [7, 8]. The range in normal alleles is 6–27 uninterrupted repeats [9]. The expansion causes reduction in the level of frataxin mRNA [3, 10]. In patients homozygous for the expansion, allele size correlates with phenotypic severity, with the smaller of the two alleles being the more significant in conferring a less-severe phenotype [7, 11–14].

Point mutations have been reported in the remaining mutant alleles that are not caused by GAA expansion. Three alterations were initially identified [3]: *L106X*, *I154F*, and an A to G transition that disrupted the acceptor splice site at the end of the third intron, altering the AG of the acceptor site to GG. Bidichan-

dani et al. [15] found a fourth point mutation, *G130V*. Cossee et al. [10] found a single base substitution, G to T, that altered the ATG initiation codon of frataxin. No patient has yet been found with no expansion but two point mutations.

The previously described *I154F* conservative change of a hydrophobic amino acid affects an invariant position within the highly conserved domain shared between human, worm, and yeast from amino acids 141 to 167 [3]. The clinical phenotype observed in the patients compound heterozygous for the *I154F* substitution and a GAA expansion is indistinguishable from that observed in individuals homozygous for the GAA repeat expansion [11]. In addition, all three patients from two unrelated families who are compound heterozygotes for the initiation codon mutation and a GAA expansion have typical FRDA [10]. In contrast, the three patients heterozygous for the *G130V* mutation have a mild atypical phenotype [15].

We report here three novel point mutations that have been identified in our Australian patients, one at the splice donor site in intron *4* (*IVS4 nt 2*, T to G), the second at codon 165 in exon *5a* (*R165C*), and the third at codon 182 also in exon *5a* (*L182F*). The patient with the splice donor site mutation has typical FRDA [2], whilst the other two patients with amino acid substitutions have had a milder disease course.

MATERIALS AND METHODS

Genomic DNA extractions

Whole blood was collected from patients in both lithium heparin and EDTA tubes. Blood leukocytes were extracted using a Progenome II DNA extraction kit (Progen).

Polymerase chain reaction amplification

Patients had their DNA analyzed using the oligonucleotides GAA-F and GAA-R from Campuzano et al. [3] with the GeneAmp XL Long PCR reagent kit (Perkin-Elmer). These primers gave a product of $457+3n$ base pairs (bp) (where n = number of GAA repeats). Reactions contained 25–150 ng genomic DNA, 0.2 mM each dNTP, 1 μM each primer, 1.5 mM magnesium (Mg^{2+}) (EDTA samples) or 3.0 mM Mg^{2+} (lithium heparinized samples), and 2.0 units rTth (*Thermus thermophilus*) DNA polymerase, XL. Other conditions were as recommended by the manufacturer. The cycling reaction involved 95 °C for 5 min, followed by ten cycles of 95 °C for 1 min and 68 °C for 3 min, then ten cycles at 95 °C for 1 min and 68 °C for 7 min, finishing with ten cycles of 95 °C for 1 min and 68 °C for 10 min. The whole polymerase chain reaction (PCR) product was then analyzed on a 0.8% agarose low electroendosmosis gel (LE, Boehringer-Mannheim) and the size of the expansion relative to the molecular weight standard marker C (Boehringer-Mannheim) was determined using EAGLE EYE 3.0 software (Stratagene). Errors in estimation of triplet repeat expansion numbers were ±20 for expanded alleles and ±3 for alleles in the normal range.

Sequencing

Each exon was then amplified for sequencing using PCR primers designed by Campuzano et al. [3]. The annealing temperatures were: exons *2*, *3*, and *4* 55 °C, exon *1* at 59 °C, and exon *5a* at 52 °C. PCRs contained 25–150 ng genomic DNA, 0.2 mM each dNTP, 1 μM each primer, 3.0 mM Mg^{2+}, and 1.25 units of Taq polymerase (Boehringer-Mannheim). PCR products were purified either by spinning through a plugged tip or using the QIAquick PCR purification kit (QIAGEN). Cycle sequencing was performed using standard conditions (Amersham).

Clinical examination

The patients were each examined by one of the authors (G.A.N., M.B.D., or J.K.).

RESULTS

Characterization of clinically affected FRDA patients for the GAA expansion identified five patients who are heterozygous for the expansion (Table 1). Sequencing was performed for each exon of *FRDA* to identify point mutations for the second mutant allele.

Patient 1 is a Fijian Indian male who presented with an onset of symptoms at 9 years of age with asymmetric ataxia. The patient is now 20 years of age. Recent examination revealed spinocerebellar ataxia, pes cavus, and reduced vibration sense. He was areflexic, mildly dysarthric, and plantar responses were both upgoing. Sural nerve biopsy performed at age 13 years showed severe chronic axonal neuropathy. Sensory nerve action potentials measured at the same time showed evidence of a peripheral neuropathy which was predomi-

Table 1 Summary of clinical and genetic analysis (*echo* echocardiogram, *ECG* electrocardiogram, + present, − absent)

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Age onset (years)	9	15	23	8	2
Pes cavus	+	+	+	−	+
Reflexes	All −	Ankle − Knee +	Ankle − Others brisk	Ankle − Others brisk	Ankle − Others brisk
Plantar responses	Upgoing	Upgoing	Upgoing	Upgoing	Upgoing
Heart	Normal echo	Mild ECG changes	Normal echo	Normal echo	Normal echo
Peripheral neuropathy	Reduced	Reduced	Reduced	Reduced	Reduced
GAA repeat size	600	730	380	1330	1080
Point mutation	*IVS4nt2* (T to G)	*L182F*	*R165C*	*G130V*	*G130V*

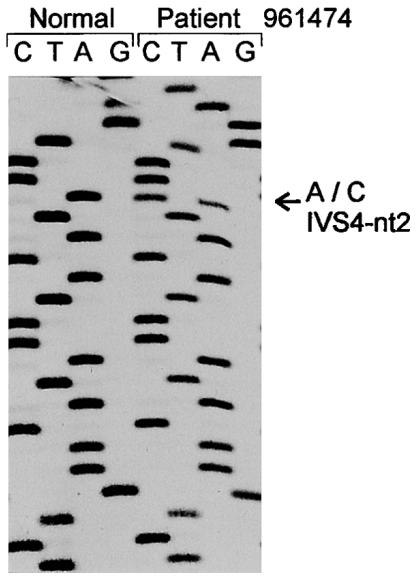

Fig. 1 Sequencing of exon *4* in patient 1 using the reverse primer. Detection of an A to C single base pair substitution at position 2 in intron *4* which translates to a T to G substitution on the sense strand

Fig. 2 Sequencing of exon *5a* in patient 2 using the reverse primer. Detection of a G to A single base pair substitution in codon 182 on the antisense strand. Codon changes from CTC to TTC on the sense strand

nantly sensory. Recent echocardiography was within normal limits.

Patient 1 was found to be heterozygous for an expanded *FRDA* allele of approximately 600 GAA repeats. From sequencing, a point mutation of a T to a G at the second base of the splice donor site in intron *4* was detected (Fig. 1). This mutation creates a *Fok* I site, and was not detected in 40 normal alleles. The most-likely outcome from this mutation is that exon *4* is lost from the mature mRNA. No tissue samples were available from this patient to determine the level of mutant mRNA products or if a cryptic splice site is utilized instead. Assuming a simple deletion of exon *4* from the mRNA, a shortened protein product of 130 amino acids would result because of the introduction of a premature stop codon, thus causing loss of functional activity.

Patient 2 is a Caucasian female, who is currently 27 years of age, with an onset of symptoms at age 15 years with frequent tripping. She has progressively worsening lower limb ataxia and currently uses a walking stick. She has minimal dysarthria and has little upper limb dysfunction and describes her handwriting as "perfect, better than any doctor." Recent examination revealed bilateral pes cavus, mild pyramidal muscle weakness in the lower limbs (4/5) without wasting, and absent ankle jerks with reduced but present knee reflexes and extensor plantar responses. There was reduced vibration sense and two-point discrimination. Electrocardiography revealed inferior T wave changes only.

Patient 2 was also found to be heterozygous for the expansion in *FRDA* with an expanded allele of 730 repeats. Sequencing the genomic DNA from this individual revealed a single base substitution of a C to a T altering the codon at position 182 from leucine to phenylalanine (Fig. 2). This mutation therefore results in the production of full-length protein, but we are unable to assess its functional level in vitro. The mutation removes a naturally occurring *Mnl* I site, and was not detected in 40 normal alleles. The amino acid which is mutated is conserved between human, worm, and yeast.

Patient 3 is also a 27-year-old Caucasian female. Onset of symptoms was at 23 years with gait disturbance, although her mother notes that clumsiness was present from about 12 years of age. On examination there was ataxic gait, bilateral pes cavus, weakness of wrist extension, hip flexion, and ankle dorsiflexion but no dysarthria. Plantar responses were extensor and reflexes were brisk except for the ankle jerks which were absent. Proprioception and vibration sense were reduced in the feet.

Patient 3 had one allele in the expanded range of 381 repeats. Sequencing revealed a single base substitution of a C to a T altering codon 165 from arginine to cysteine (Fig. 3), again resulting in full-length protein. The mutation was not detected in 40 normal alleles. Again the mutated amino acid is well conserved through evolution.

Two brothers from a fourth family who are heterozygous for a GAA expansion were found to have inherited a *G130V* missense mutation from their father (results not shown). This mutation has been reported pre-

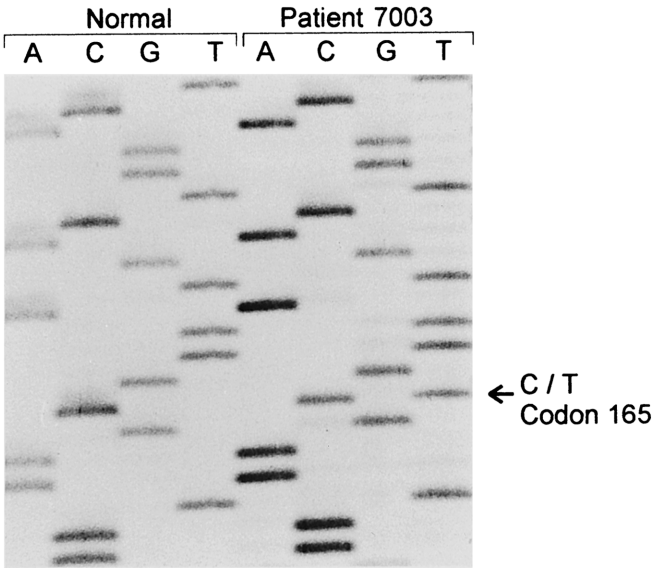

Fig. 3 Sequencing of exon *5a* in patient 3 using the forward primer. The single base substitution of C to T alters the codon at 165 from CGT to TGT

viously in unrelated individuals [15], and destroys a restriction site for the enzyme *Bsi Y*1. These patients are of Caucasian origin. Currently 15 and 12 years old, their ages of onset were 2 and 8 years. They have atypical FRDA as defined by the diagnostic criteria of Harding [2], in that they have brisk knee, biceps, and triceps jerks, but absent ankle jerks in addition to moderately severe limb and gait ataxia, reduced proprioception and vibration sense, and very mild dysarthria. Neither has scoliosis, cardiomyopathy on echocardiography, or diabetes mellitus. Both are ambulant.

DISCUSSION

FRDA is the only autosomal recessive disorder shown to be due to expansion of a trinucleotide repeat. An inverse correlation was observed by Campuzano et al. [6] between the amount of frataxin protein and the size of the GAA repeat on the smaller allele. Previous observations have identified a correlation between the length of the smaller allele and phenotypic severity [7, 11–14]. Thus, the milder clinical presentation in individuals with smaller GAA repeat sizes is likely to be due to higher levels of frataxin present in such patients. As the GAA expansion alters protein levels rather than function, examination of the few patients that have point mutations in their *FRDA* gene will help to elucidate the key functional domains of frataxin. It is assumed that the expanded allele will contribute to the phenotype in patients that are compound heterozygotes for an expansion on one allele and a point mutation on the other, but we are unable to quantify this contribution.

Our patient with the splicing mutation (*IVS4 nt 2*, T to G) has a clinical phenotype consistent with that observed in individuals homozygous for the GAA expansion. This mutation should result in the loss of exon *4*, codons 129 to 160, deleting a major proportion of the highly conserved domain and causing premature termination and loss of functional activity.

The exon *5a* mutation *L182F* is a conservative change of a hydrophobic amino acid at an invariant position in the protein. This region is downstream of the extended conserved domain where the *I154F* substitution is located. Our patient has had a milder course and has atypical FRDA as defined by Harding [2], indicating that mutations in position 182 disrupt function to a lesser extent than mutations at position 154.

The *R165C* mutation is nonconservative, altering a basic amino acid which is hydrophilic to a hydrophobic noncharged sulfur-containing amino acid. The mutation occurs at an invariant position in the protein and within the conserved structural domain defined by amino acids 141–167. This patient had a relatively mild presentation in contrast to the published patients with the *I154F* mutation [3, 11] who have typical FRDA phenotype. Including the two brothers we have described, all five patients reported with the *G130V* mutation [15] have atypical FRDA [2]. The *G130V* mutation is proving to be relatively common among point mutations in *FRDA*. The *FRDA* mRNA level in carriers of this mutation is not reduced [15], unlike carriers of the GAA expansion [3]. Frataxin protein studies on a patient who is a compound heterozygote for GAA_{670} and the *G130V* mutation demonstrated no detectable protein in muscle [6], but this may be an artefact as there is loss of the epitope recognized by the monoclonal antibody used, 1G2.

In conclusion, point mutations in *FRDA* which result in an absence of functional frataxin are associated with a severe phenotype. The phenotype associated with missense mutations, even in regions of the gene which show high evolutionary conservation in amino acid sequence, cannot be predicted with certainty, as such patients can have a mild or severe clinical course.

Acknowledgements We thank generous donors from APEX (Elmore, Victoria), the FRDA Support Groups from Victoria and Queensland (S.F.) and New South Wales (G.N.), and the families for their support. The Murdoch Institute is an NH and MRC block-funded institute. We thank Dr. Kathy Williamson for helpful discussions.

REFERENCES

1. Filla A, De Michele G, Marconi R, Bucci L, Carillo C, Castellano AE, Iorio L, Kniahynicki C, Rossi F, Campanella G (1992) Prevalence of hereditary ataxias and spastic paraplegias in Molise, a region of Italy. J Neurol 239:351–353
2. Harding AE (1981) Friedreich's ataxia: a clinical and genetic study of 90 families with an analysis of early diagnostic criteria and intrafamilial clustering of clinical features. Brain 104:589–620

3. Campuzano V, Montermini L, Molto MD, Pianese L, Cossee M, Cavalcanti F, Monros E, Rodius F, Duclos F, Monticelli A, et al (1996) Friedreich's ataxia: autosomal recessive disease caused by an intronic GAA triplet repeat expansion. Science 271:1423–1427
4. Babcock M, De Silva, Oaks R, Davis-Kaplan S, Jiralerspong S, Montermini L, Pandolfo M, Kaplan J (1997) Regulation of mitochondrial iron accumulation by Yfh 1p, a putative homolog of frataxin. Science 276:1709–1712
5. Foury F, Cazzalini O (1997) Deletion of the yeast homologue of the human gene associated with Friedreich's ataxia elicits iron accumulation in mitochondria. FEBS Lett 411:373–377
6. Campuzano V, Montermini L, Lutz Y, Cova L, Hindelang C, Jiralerspong S, Trottier Y, Kish S, Faucheux B, Trouillas P, Authier F, Durr A, Mandel J-L, Vescovi A, Pandolfo M, Koenig M (1997) Frataxin is reduced in Friedreich ataxia patients and is associated with mitochondrial membranes. Hum Mol Genet 6:1771–1780
7. Durr A, Cossee M, Agid Y, Campuzano V, Mignard C, Penet C, Mandel JL, Brice A, Koenig M (1996) Clinical and genetic abnormalities in patients with Friedreich's ataxia. N Engl J Med 335:1169–1175
8. Epplen C, Epplen J, Frank G, Miterski B, Santos E, Schols L (1997) Differential stability of the (GAA)n tract in the Friedreich ataxia (*STM7*) gene. Hum Genet 99:834–836
9. Montermini L, Andermann E, Labuda M, Richter A, Pandolfo M, Cavalcanti F, Pianese L, Iodice L, Farina G, Montticelli A, Turano M, Filla A, Michele GD, Cocozza S (1997) The Friedreich ataxia GAA triplet repeat: premutation and normal alleles. Hum Mol Genet 6:1261–1266
10. Cossee M, Campuzano V, Koutnikova H, Fischbeck K, Mandel JL, Koenig M, Bidichandani SI, Patel PI, Molte MD, Canizares J, De Frutos R, Pianese L, Cavalcanti F, Monticelli A, Cocozza S, Montermini L, Pandolfo M (1997) Frataxin fracas. Nat Genet 15:337–338
11. Filla A, De Michele G, Cavalcanti F, Pianese L, Monticelli A, Campanella G, Cocozza S (1996) The relationship between trinucleotide (GAA) repeat length and clinical features in Friedreich ataxia. Am J Hum Genet 59:554–560
12. Lamont PJ, Davis MB, Wood NW (1997) Identification and sizing of the GAA trinucleotide repeat expansion of Friedreich's ataxia in 56 patients. Clinical and genetic correlates. Brain 120:673–680
13. Montermini L, Richter A, Morgan K, Justice CM, Julien D, Castellotti B, Mercier J, Poirier J, Capozzoli F, Bouchard JP, Lemieux B, Mathieu J, Vanasse M, Seni MH, Graham G, Andermann F, Andermann E, Melancon SB, Keats BJ, Di Donato S, Pandolfo M (1997) Phenotypic variability in Friedreich ataxia: role of the associated GAA triplet repeat expansion. Ann Neurol 41:675–682
14. Monros E, Molto MD, Martinez F, Canizares J, Blanca J, Vilchez J, Prieto F, de Frutos R, Palau F (1997) Phenotype correlation and intergenerational dynamics of the Friedreich ataxia GAA trinucleotide repeat. Am J Hum Genet 61:101–110
15. Bidichandani SI, Ashizawa T, Patel PI (1997) Atypical Friedreich ataxia caused by compound heterozygosity for a novel missense mutation and the GAA triplet-repeat expansion. Am J Hum Genet 60:1251–1256