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ORIGINAL INVESTIGATION

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Genotype and phenotype analysis of Friedreich's ataxia compound heterozygous patients

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Abstract Friedreich's ataxia is caused by mutations in the *FRDA* gene that encodes frataxin, a nuclear-encoded mitochondrial protein. Most patients are homozygous for the expansion of a GAA triplet repeat within the *FRDA* gene, but a few patients show compound heterozygosity for a point mutation and the GAA-repeat expansion. We analyzed DNA samples from a cohort of 241 patients with autosomal recessive or isolated spinocerebellar ataxia for the GAA triplet expansion. Patients heterozygous for the GAA expansion were screened for point mutations within the *FRDA* coding region. Molecular analyses included the single-strand conformation polymorphism analysis, direct sequencing, and linkage analysis with FRDA locus flanking markers. Seven compound heterozygous patients were identified. In four patients, a point mutation that predicts a truncated frataxin was detected. Three of them associated classic early-onset Friedreich's ataxia with an expanded GAA allele greater than 800 repeats. The other patient associated late-onset disease at the age of 29 years with a 350-GAA repeat expansion. In two patients manifesting the classical phenotype, no changes were observed by single-strand conformation polymorphism (SSCP) analysis. Linkage analysis in a family with two children affected by an ataxic syndrome, one of them showing heterozygosity for the GAA expansion, confirmed no linkage to the *FRDA* locus. Most point mutations in compound heterozygous Friedreich's ataxia patients are null mutations. In the present patients, clinical phenotype seems to be related to the GAA repeat number in the expanded allele. Complete molecular definition in these patients is required for clinical diagnosis and genetic counseling.

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Introduction

Friedreich's ataxia (MIM 229300) is the most common hereditary ataxia, affecting 2–4 in 100,000 inhabitants in Caucasians (Romeo et al. 1983; López-Arlandis et al. 1995). Friedreich's ataxia is a mitochondrial disease (Koutnikova et al. 1997) inherited as an autosomal recessive trait. It is characterized by loss of the sensory neurons of the large dorsal root ganglia, with degeneration of the posterior columns of the spinal cord and the presence of sensory axonal neuropathy, with relative preservation of the motor component of peripheral nerves. According to Harding (1981), essential diagnostic criteria are age at onset before 25 years, progressive gait and limb ataxia, absent lower limb tendon reflexes, extensor plantar responses, and motor nerve conduction velocity of more than 40 m/s, with small or absent sensory nerve action potentials. Dysarthria has to be present within 5 years of onset. Most patients also show other features, which may include hypertrophic cardiomyopathy, skeletal deformities, and diabetes mellitus. After mapping the gene locus to chromosome 9q13 (Chamberlain et al. 1988), locus homogeneity for several clinical variants was demonstrated

by linkage analysis. These variants included late-onset Friedreich's ataxia (LOFA; De Michele et al. 1994), Friedreich's ataxia with retained reflexes (FARR; Palau et al. 1995), and Acadian Friedreich's ataxia (Keats et al. 1989), a milder form than the typical disease. In 1996 the *FRDA* gene, also called *X25*, was isolated from the candidate region (Campuzano et al. 1996). The gene has six coding exons, 1–5a/5b. The major mRNA isoform, transcribed from exons 1 to 5a, encodes a 210-amino acid protein called frataxin. Frataxin is a nuclear-encoded protein located within mitochondrial membranes and crests (Campuzano et al. 1997). A defect on mitochondrial iron metabolism has been postulated as a pathogenetic mechanism (Babcock et al. 1997; Rötig et al. 1997). The most frequent mutation found in Friedreich's ataxia is the abnormal expansion of a GAA repeat located within the first intron of the *FRDA* gene (Campuzano et al. 1996). This mutation is detected in most mutated alleles (Filla et al. 1996; Dürr et al. 1996; Monrós et al. 1997; Montermini et al. 1997). Thus, most patients are homozygous for the GAA expansion and only around 5% of patients are compound heterozygotes (Monrós et al. 1997; Schöls et al. 1997). In a number of compound heterozygotes, point mutations have been reported in Friedreich's ataxia (Campuzano et al. 1996; Cossée et al. 1997; Bidichandani et al. 1997; Bartolo et al. 1998; Forrest et al. 1998; Cossée et al. 1999). Most of these mutations represent sequence changes resulting in the premature truncation of frataxin or in an amino acid substitution in the carboxy-terminal half of frataxin that is preserved through evolution (Gibson et al. 1996). Finding a patient manifesting an inherited ataxia with only one GAA expanded mutant allele raises the question of diagnosis. It is likely that Friedreich's ataxia is the correct diagnosis, especially if the clinical picture is the classic one. However, definitive diagnosis requires the finding of the second mutation for the full definition of molecular pathology and genetic counseling. We report here the molecular and clinical characterization of four Friedreich's ataxia patients carrying a compound heterozygous genotype. In three cases, the point mutations have not been described previously. We also address the point about the risk of misdiagnosis in patients with an ataxic syndrome and one expanded allele in the *FRDA* gene.

Patients and methods

Study design and patients

We investigated 241 patients from 210 unrelated families with progressive, unremitting autosomal recessive or idiopathic spinocerebellar ataxia for the GAA trinucleotide repeat genotype in the *FRDA* gene. To define the phenotype of patients showing two or one expanded GAA repeat alleles at the *FRDA* gene, a standardized survey for clinical, electrophysiological, and neuroimaging data was sent to each neurologist or medical geneticist who referred the patients to the Genetics Department at La Fe University Hospital for genetic studies or diagnosis between September 1988 and December 1998. In each case, informed consent before testing was obtained. Patients showing a compound heterozygous genotype with one GAA expanded allele and one GAA allele within the normal range were analyzed for point or small mutations.

Molecular analysis

DNA was isolated from peripheral leukocytes by standard phenol/chloroform and ethanol precipitation methods. The *FRDA* GAA repeat was amplified by a long polymerase chain reaction (PCR) protocol using primers GAA-F and GAA-R as previously reported (Monrós et al. 1997; Cruz-Martínez et al. 1997).

Screening for point mutations was performed by single-strand conformation polymorphism analysis (SSCP). Exons 1 to 5b and flanking sequences were amplified by PCR using primers reported by Campuzano et al. (1996) in a final volume of 50 µl. Five microliters of the PCR product were mixed with 8 µl of loading buffer and 10 µl of dH_2O, and then 5 µl were run on 15% polyacrylamide plus 5% glycerol gels, at 650 W for 18–20 h. PCR fragments were detected by silver stain method.

The sequencing analysis of forward and reverse strands was performed in each case using the same primers as for SSCP analysis. PCR products were purified with Qiaquick PCR Purification Kit (Qiagen, Germany) and automated sequence was performed using the ABI PRISM Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, Calif.). Gel electrophoresis analysis was carried out in a ABI377 DNA Sequencer (Applied Biosystems, Foster City, Calif.; Sistemas Genómicos, Valencia, Spain). Nucleotides were numbered starting at the translation methionin of the *FRDA* cDNA (GenBank no. U43747).

For restriction analysis, 10 µl of the amplified products were digested with 2 U of the corresponding restriction enzyme, in a final volume of 20 µl overnight. Two microliters of the digestion product were run over 3 h on a 10% polyacrylamide gel and silver-stained.

Microsatellite genotyping and linkage analysis

Polymorphic microsatellites linked to the FRDA locus, FR1 (D9S202), FR2 (D9S886), FR8 (D9S888), FR7 (D8S887), and FR5 (D9S889) were analyzed as previously reported (Monrós et al. 1996). FAD1, a single nucleotide polymorphism, was studied by SSCP according to Monrós et al. (1996). Two-pairwise LOD scores were calculated using the FASTLINK 2.1 program. A disease gene frequency of 0.0001 and equal female-male recombination distances were considered for calculations.

Results

FRDA gene point mutations

To identify compound heterozygote subjects for the *FRDA* gene, a cohort of patients with early-onset spinocerebellar ataxia was investigated for the GAA triplet expansion in the *FRDA* gene. The screening of 241patients revealed that 175 of them had the GAA expansion. Seven out of 175 (4%) showed one expanded repeat and one nonexpanded allele. Samples of DNA from these seven patients were amplified by PCR and analyzed for mutations within each of the six coding exons and flanking sequences of the *FRDA* gene. The corresponding PCR products of each patient were screened for mutations by SSCP analysis. We could identify four band changes in exon 1 (patient AF214), exon 3 (patient AF559), and exon 4 (patients AF3 and 96–230). No abnormal bands were observed in the other three patients. Further sequencing analysis of the two DNA strands demonstrated nucleotide mutations – one nonsense, one deletion, one insertion, and one mutation in a splice site – that are likely to result in a translational frameshift and, thus, premature termination of frataxin.

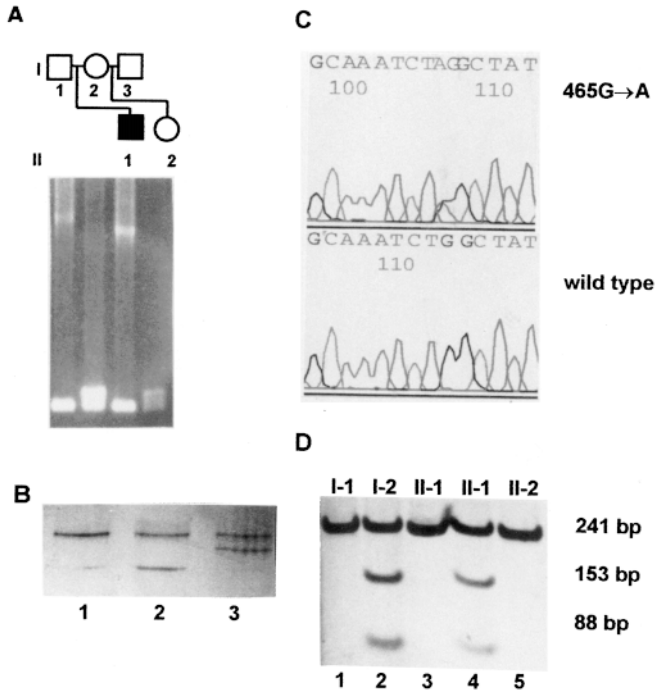

Fig. 1A–D Molecular studies in patient 96-230. **A** Analysis of the GAA triplet repeat: the patient shows one expanded allele of 850 triplet repeats, inherited from the father, and one nonexpanded allele. **B** Single-strand conformation polymorphism (SSCP) analysis of exon 4 amplified product: *Lanes 1* and *2* represent normal control DNAs, and *lane 3* represents the patient's DNA, showing an abnormal band. **C** Direct sequencing analysis of the sense strand using the reverse primer. *At the top*, thymine-to-cytosine transition in the patient's DNA; *at the bottom*, wild-type sequence. **D** Restriction analysis amplified DNA from patient, parents, and sister: *lanes 1* and *3* show the normal 241-bp fragment after paternal DNA digestion with *Alu*I enzyme and patient's undigested DNA; *lanes 2* and *4* represent maternal and patient digestion, showing two extra bands of 153 bp and 88 bp along with the 241 bp, caused by the guanine-to-adenine transition that creates a new *Alu*I site. Normal restriction pattern in the sister (*lane 5*) indicates that she is not a carrier

The first mutation (Fig. 1) was a nucleotide change, 465G→A in exon 4 of the *FRDA* gene, which resulted in a stop codon at frataxin position 155, found in a Cuban patient (96-230) from Florida. This nucleotide substitution generates a new *Alu*I restriction site that allowed us to test the mutation in the family members. This *Alu*I site produces two fragments of 153 bp and 88 bp after digestion of the 241-bp PCR product. Restriction analysis showed that the W155X mutation was inherited from his mother and the expanded GAA allele of 850 repeats from his father. It could be confirmed that both mutations, the GAA expansion and W155X, were in trans. The patient has a half-sister that is not a carrier of the point mutation.

The second mutation is a single-base (T) insertion between nucleotides 296 and 297 of exon 3 of the *FRDA* gene and was found in heterozygosity in patient AF559, who is of Basque origin. This insertion introduces a frameshift at codon E100, 11 novel amino acids, and the premature termination of a 110-residue protein compared with the normal 210-amino acid frataxin. This insertion generates a second new *Alu*I restriction site within the 227 bp of the PCR product. We performed restriction analysis of the mother's DNA and the DNA of two unaffected sisters who did not have an expanded allele. We confirmed the inheritance of the thymine insertion from the mother. The 350-GAA repeat expansion was inferred to be inherited from the deceased father. One of the sisters was a carrier of the point mutation.

The third mutation is a deletion of one nucleotide (118 C) in exon 1 of *FRDA*, found in heterozygosity in a patient (AF214) of Spanish origin. This results in a frameshift after codon R39, the introduction of 35 novel amino acids, and the premature termination of a 74-amino acid frataxin. The cytosine deletion generates the loss of a *Bgl*I restriction site. Both parents and one unaffected brother were investigated for the GAA expansion and the point mutation. The patient inherited an 810-GAA repeat expanded allele from his mother and the nucleotide deletion from his father. His brother was a heterozygous carrier of a 780-GAA triplet expansion.

The fourth mutation is an A-to-G splicing mutation in the 3′ acceptor site of intron 3 at position –2 of the splice site (385–2A→G) and it was found in the patient previously reported by Campuzano et al. (1996). This nucleotide substitution generates a new *Bst*NI restriction site. The 800-GAA repeat expansion was inherited from his mother and the point mutation from his father. One of two brothers was also a heterozygous carrier of the splicing mutation.

Restriction analysis of each point mutation in 50 unaffected control subjects revealed normal restriction patterns, indicating that the *FRDA* mutations detected in this study are specific for the disease and are not common polymorphisms.

Origin of the point mutations in the Spanish population

Three mutations had Spanish origin, two belonging to the general population and one from a Basque family. As all of them were detected only once, we postulated that they were associated with a rare *FRDA* chromosome. By microsatellite analysis we have previously demonstrated that most of the *FRDA* mutations in the Spanish population (85%) are associated with three main *FRDA* haplotypes (Monrós et al. 1996). We constructed FR1-FR2-FAD1-FR8-FR7-FR5 haplotypes in families of patients AF214 and AF3 carrying the 118delC and the 385–2A→G mutations, respectively. In each case, the mutation was associated with a rare haplotype that has not been previously observed in the Spanish FRDA patients (not shown). Since only genetic information of the FAD1 single nucleotide polymorphism within *FRDA* genomic region is available in the Basque population, we investigated only this marker in patient AF559. Point mutation 297insT was associated with the rare allele that is present in 21.5% of the normal Basque population.

Table 1 Clinical features of compound heterozygous FRDA patients (HCM hypertrophic cardiomyopathy, + presence, – absence, *n.a.* not available)

	Patients			
	96-230	AF559	AF214	AF3
Geographical origin	Cuba	Spain (Basque origin)	Spain (Mediterranean)	Spain (Mediterranean)
GAA expanded allele (triplets)	850	350	810	800
Age at onset (years)	4	29	3	3
Age of first examination	4	39	7	10
Age when first in wheelchair (years)	10	Ambulant	14	21
Gait ataxia	+	+	+	+
Limb ataxia	+	+	+	+
Dysarthria	+	+	+	+
Lower limb areflexia	+	Reduced	+	+
Upper limb areflexia	Reduced	–	+	+
Decreased vibration/positional sense	+	+	+	+
Babinski sign	+	+	+	+
Tremor	+	–	+	+
Amyotrophy	–	–	–	–
Optic atrophy	–	–	–	–
Nystagmus	–	+	n.a.	n.a.
Hearing loss	–	–	–	–
Scoliosis	+	–	+	+
Foot deformity	+	–	+	+
Diabetes	–	–	–	–
T-wave inversion in ECG	+	+	+	+
HCM in echo CG	+	–	–	–
Axonal sensory neuropathy	+	+	+	+
Cervical cord atrophy in MRI	–	–	–	+
Cerebellar atrophy in MRI	–	(Mild) +	(Mild) +	–

Clinical features of compound heterozygous patients for the *FRDA* gene

All patients were isolated cases, and no consanguinity was observed in any family. Three out of the four heterozygous patients were men and the other one was a woman. Age at onset was very early in childhood in the three men (AF3, AF214, 96-230) and very late in the woman (AF559). The presenting symptom was gait ataxia in all. In the three early-onset patients, the interval before being confined to a wheelchair ranged from 6 to 18 years, whereas AF559 was ambulant after 12 years of evolution. The overall clinical picture is summarised in Table 1. Patients 96–230, AF214, and AF3 were diagnosed in the first decade of life. All of them showed a classical phenotype as defined by Harding (1981). By contrast, patient AF559 showed a variant phenotype, with late-onset at the age of 29 years. This patient was first examined and diagnosed after 10 years of evolution. No clinical information is available from that period, but she is still ambulant, knee reflexes are reduced but still present, and she does not show any skeletal abnormality.

Disease association with one GAA expanded allele but no linkage to the *FRDA* locus

We did not find a second mutation in three heterozygous patients showing only one expanded allele. One of them, patient AF462 (Fig. 2, II-5), belongs to a nonconsanguineous family with two siblings affected by an early-onset cerebellar ataxia and three unaffected siblings. However, his affected sister (II-3) had two normal nonexpanded GAA alleles. The age at the last examination was 13 years and 29 years, respectively. The disease started very early, before the age of 2 years as gait ataxia. The clinical picture was similar in both siblings. At the age of 9 years, patient AF462 showed gait and limb ataxia, dysarthria, general deep tendon areflexia, indifferent plantar response, limb weakness, normal positional and vibratory senses, dystonic movements of the head, and dystonic position of hands; electrocardiographic activity (ECG) was normal. Electrophysiological studies did not show peripheral neuropathy, but somatosensory evoked potentials showed a demyelinating pattern, with normal visual evoked potentials. Magnetic resonance imaging (MRI) revealed a mild atrophy of left cerebellar hemisphere. His sister was more affected; she had a similar clinical history but she had developed an axonal motor and sensory neuropathy, as was shown on the electrophysiological investigations and sural nerve biospy. Auditory and somatosensory evoked potentials were abnormal. A MRI study showed both cerebellar and cervical spinal atrophy. As both siblings showed a different phenotype to the typical FRDA, we postulated that the presence of an expanded allele in patient AF462 was a coincidence, with no involvement in the pathogenesis of the ataxic syn-

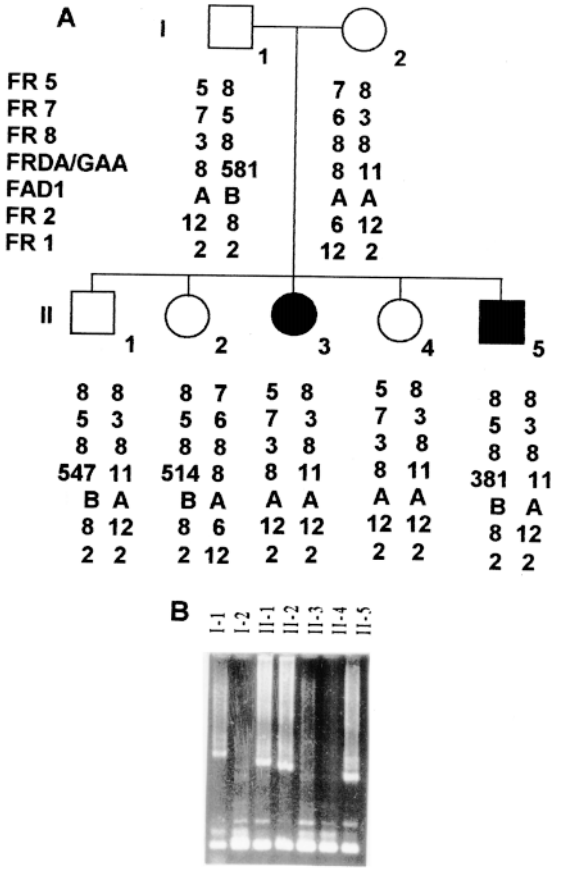

Fig. 2A, B Genetic studies in patient AF462 and family. **A** Haplotype analysis with *FRDA* linked markers: the GAA expanded allele is inherited from the carrier father by patient AF462 (II-5) and his normal sibs II-1 and II-2. Affected sister II-3 and normal sibling II-4 have not inherited the mutant expansion. GAA expansion segregates along with the extended haplotype 2-8-B-8-5-8. **B** Long PCR analysis of the GAA triplet repeat

drome. To confirm this hypothesis, we screened by SSCP analysis the full gene in the patient's DNA and we did not observe any abnormal band. Further linkage studies with *FRDA* locus flanking markers FR1, FR2, FAD1, FR8, FR7, and FR5, excluded the disease gene 1 cM around the *FRDA* locus (max. lod=–2.44 at 0.01 of recombination fraction). The GAA expansion was inherited from the father. This repeat mutation segregated with haplotype 2-8-B-8-5-8, which is probably derived by microsatellite mutation at the FR7 polymorphism from the main FRDA haplotype in the Spanish population, 2-8-B-8-3-8, which represents 50% of *FRDA* chromosomes (Monrós et al. 1996).

Discussion

Due to the high frequency of the GAA trinucleotide expansion in FRDA patients, mutations in the coding sequence seem to be very rare. In a series of 175 patients with spinocerebellar ataxia associated with mutant expansion in the *FRDA* gene, we have found that 168 patients (96%) were homozygous and 7 patients (4%) were heterozygous. These 7 patients were screened for the presence of point or small mutations in the six coding exons of the *FRDA* gene by SSCP analysis. Band shifts were found in four of them. Sequencing of both amplified strands in each case revealed four different point mutations. All of these mutations predict a truncated frataxin by three different mechanisms: (1) G-to-A transition at nucleotide position 465 generates a nonsense change at the tryptophan 155; (2) both 118delC and 297insT mutations are frameshift changes that introduce premature stop codons at 75 and 111 positions, resulting in a short and probably unstable frataxin; and (3) 385–2A→G mutation involves the consensus AG dinucleotide in 3´-splicing acceptor site of intron 3, suggesting exon 4 skipping. Exon 4 encodes the most conserved amino acids throughout evolution (Gibson et al. 1996), so the absence of this exon sequence in the frataxin mRNA may cause a nonfunctional protein.

These point mutations have been observed only once in our series. Genotype analysis using flanking markers of the *FRDA* locus confirmed that the two Spanish mutations, 118delC and 385–2A→G, are associated with very rare, specific haplotypes. Genetic data from FRDA patients strongly suggest that patients carrying two point mutations have to be very uncommon. Since 98% of *FRDA* chromosomes have the GAA expansion, it is expected that only 1 in 2500 FRDA patients is homozygote or compound heterozygote for two nonexpanded alleles. We have searched for point mutations in 66 early-onset cerebellar ataxia patients without GAA expansion and we did not find any abnormal change in the SSCP pattern (data not shown).

It has been postulated that GAA expansions inhibit transcription or maturation of frataxin mRNA (Campuzano et al. 1996; Bidichandani et al. 1998). In patients homozygous for the GAA repeat expansion, the expansion size inversely correlates with age at onset and the time of wheelchair-confinement. In fact, the length of the smaller expanded allele accounts for 30%–50% of the variance in age of onset (Dürr et al. 1996; Filla et al. 1996; Monrós et al. 1997; Montermini 1997), suggesting that late-onset in patients with small expansions might be the consequence of higher levels of frataxin transcript allowing residual frataxin expression in the disease-specific tissues. Since the compound heterozygous patients from the present series have only one expanded allele along with a loss of function point mutation, their study is useful to evaluate the relevance of the GAA expansion size on the clinical phenotype. Clinical pictures of all patients but one, AF559, were typical Friedreich's ataxia. Patients AF3, AF214, and 96–230 had a very early-onset of the disease, at the ages of 3 years or 4 years. The duration of the disease before the patient became confined to a wheelchair varied between 6 and 18 years. Conversely, patient AF559 started the ataxic syndrome at the age of 29 years and after 12 years of evolution she still remains ambulant. As mentioned, all patients were bearing a null point mutation, but molecular differences with regard to the GAA expanded allele sizes were observed. The late-onset pa-

tient AF559 had a small expanded allele of 350 repeats, whereas the three early-onset patients were bearing GAA alleles between 800 and 850 repeats. Most patients who began the disease after the age of 25 years had a smaller allele with 500 or less repeats (Dürr et al. 1996; Monrós et al. 1997). Above a threshold around 500–700 GAA repeats, the residual expression of frataxin may have little influence on the clinical expression of the disease. Although the series is too small, our results strongly suggest that the smaller allele has an important role in the pathogenesis of the clinical expression and the evolution of the phenotype in Friedreich's ataxia patients. Three point mutations, G130V (Bidichandani et al. 1997; Cossée et al. 1999), L106S (Bartolo et al. 1998), and D122Y (Cossée et al. 1999), have been associated with milder and atypical phenotypes in heterozygous patients. In the three cases, an amino acid substitution outside the highly conserved domain of the frataxin protein was associated with a GAA repeat greater than 800 repeats. In these cases, it is likely that the less severe phenotype may be caused by a partially functional frataxin encoded by the nonexpanded mutant allele.

We did not find any abnormal SSCP pattern in three compound heterozygotes. Two patients had severe, typical Friedreich's ataxia and another one showed a very early ataxic syndrome. The absence of point mutation in the nonexpanded allele may be explained by: (1) the analysis of only the coding region and not the regulatory sequences of the *FRDA* gene; (2) possible limits in the ability of SSCP analysis to detect mutations; and (3) the absence of linkage data leaves an open possibility that some patients may have mutations at other genes. With regard to the last point, a second locus, *FRDA2*, has been described in three families associated with Friedreich's phenotype, either classic (Smeyers et al. 1996; Kostrzewa et al. 1997) or with retained reflexes (Kostrzewa et al. 1997). Thus, although extremely rare, locus genetic heterogeneity exists in Friedreich's ataxia. Moreover, ataxia with vitamin E deficiency caused by mutations in the α-TTP gene is also expressed as a Friedreich-like phenotype (Cavalier et al. 1998). In two patients, lack of evidence of a second mutation does not allow a Friedreich's ataxia diagnosis, although the classic phenotype suggests that it is the most likely one. Serum vitamin E levels were normal in one patient and not tested in the other one. In patient AF462, by contrast, we could demonstrate by linkage analysis of the family that association between an ataxic syndrome and one mutant expansion of the GAA repeat is a coincidence, suggesting that the expansion had no pathogenic relation on the generation of the ataxic disorder.

In conclusion, we report molecular and clinical findings in four compound heterozygous Friedreich's ataxia patients. Our findings contribute to confirm the relevance of the GAA expansion size on the pathophysiology of the disease's clinical expression, especially the age at onset. Due to the great variation of the clinical spectrum in Friedreich's ataxia, molecular diagnosis of the GAA repeat has become a basic diagnostic tool for spinocerebellar ataxias. However, in heterozygous patients, complete genetic definition by point mutation detection or by linkage studies is essential for clinical and differential diagnosis, and genetic counseling.

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