Screening for the Presence of FMR1 Premutation Alleles in Women With Parkinsonism

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Background: Fragile X–associated tremor/ataxia syndrome (FXTAS) is a progressive, late-onset neurodegenerative disease that affects older carriers of premutation (CGG) repeat expansions of the fragile X mental retardation 1 (FMR1) gene. Clinical features include intention tremor, gait ataxia, memory loss, peripheral neuropathy, autonomic dysfunction, and parkinsonism. The presence of parkinsonism in FXTAS raises the possibility that some individuals who have Parkinson disease are actually carriers of a premutation FMR1 allele.

Objective: To screen DNA samples from a large cohort of females with Parkinson disease for an excess of expanded alleles of the FMR1 gene.

Design and Patients: We screened a cohort of 595 women with Parkinsonism, the largest screening of a parkinsonism-associated group to date, for the presence of an FMR1 premutation allele (55-200 CGG repeats). The screening protocol uses an enhanced polymerase chain reaction method capable of flagging any FMR1 expanded CGG repeat in women as well as in men.

Setting: Diagnostic assessments were performed at an outpatient tertiary clinic (Parkinson Institute, Milan).

Genotyping was conducted at the University of California, Davis.

Main Outcome Measures: CGG repeat number and clinical/neuroimaging assessments of patients with Parkinson disease were conducted. Two premutation carriers were identified.

Results: Two individuals possessed an FMR1 allele in the premutation range (CGG repeats: 30 and 75; 30 and 115). This carrier frequency (2 of 595 [0.34%]) is not significantly different from estimates of the allele frequency among women in the general population (0.4%-0.8%). Clinical and radiologic features of these 2 patients were similar to those of the general Parkinson disease population; however, 1 patient (115 CGG repeats) had a family history of 2 sons with the fragile X syndrome.

Conclusion: Screening of women within the parkinsonism clinical spectrum is unlikely to be productive in the absence of additional medical or family history suggestive of involvement of the FMR1 gene.

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Fragile X–associated tremor/ataxia syndrome (FXTAS) is a late-onset neurodegenerative disorder associated with a CGG trinucleotide repeat expansion in the fragile X mental retardation 1 gene (FMR1) (OMIM *309550) within the premutation range (55-200 CGG repeats; normal, <45 repeats). Population estimates for the premutation allele frequency range from approximately 1 in 260 to 800 men and 1 in 130 to 260 women.1,3 Symptoms of FXTAS generally begin in adults older than 50 years, with penetrance greater in men than in women,5,3 and include intention tremor and/or gait ataxia, cognitive decline, and parkinsonism.6,10 Additional features include peripheral neuropathy,11 autonomic dysfunction, and, particularly in women, thyroid dysfunction.5,12 Magnetic resonance (MR) imaging shows characteristic hyperintensities in the middle cerebellar peduncles, periventricular and subcortical white matter changes, and whole brain volume loss.7,13 There is also global brain atrophy, particularly in the frontal, parietal, cerebellar, and pontine regions.7,13 Hyperintensities in the middle cerebellar peduncles occur in approximately 60% of patients with FXTAS and are relatively specific (although not unique) to this syndrome.7,13

Although FXTAS occurs more commonly in men, women also have clinical involvement,9,14 albeit with approximately 5-fold lower penetrance than for men.3 Interestingly, female carriers with definite...
or probable FXTAS had a greater medical comorbidity than male carriers, with increased prevalence of thyroid disease, hypertension, seizures, peripheral neuropathy, and fibromyalgia.5

A retrospective study analyzing the initial diagnoses of patients with FXTAS showed that nearly 25% were given a prior diagnosis of parkinsonism.17 This suggests that carriers of the premutation allele could be found in significant numbers within cohorts with parkinsonism. Subsequent studies of male populations with parkinsonism failed to support this hypothesis. However, the assessment of women in these studies was inadequate due to either non-inclusion or a small number of patients in the study. Thus, to address the possibility that a significant number of women diagnosed as having parkinsonism actually have FXTAS, we screened the DNA of 595 women who received a diagnosis involving parkinsonism for the presence of an expanded FMR1 allele. To date, this is the largest study of women who were evaluated at a Parkinson disease (PD) clinic with a diagnosis of either idiopathic PD or atypical parkinsonism.

METHODS

SUBJECTS

A novel, polymerase chain reaction (PCR)–based screening approach was used for the detection of expanded alleles of the FMR1 gene within a cohort of 595 unrelated female patients who had received a diagnosis of either PD or another parkinsonism spectrum disorder and who had contributed DNA samples to the Human Genetic Bank of Patients Affected by Parkinson Disease and Parkinsonisms of the Parkinson Institute–Istituti Clinici di Perfezionamento, Milan, Italy (http://www.parkinson.it/dnbank.html). The cohort was selected on the basis of consecutive clinic visits, regardless of family history of parkinsonism or age at onset of the disease. Written informed consent was obtained from all subjects through the Istituti Clinici di Perfezionamento. Samples were de-identified and coded before they were transferred to the University of California, Davis, site.

Among the 595 female patients screened, 480 fulfilled clinical criteria for idiopathic PD, 7 for dementia with Lewy bodies, 2 for frontotemporal dementia, 28 for multiple system atrophy, 20 for progressive supranuclear palsy, 12 for corticobasal degeneration, and 19 for essential tremor. For the remaining 27 patients, the clinical diagnosis was still uncertain; those patients are reported as having undefined primary parkinsonism. The clinical diagnosis of PD was established according to the UK Parkinson Disease Society Brain Bank criteria,10,19 which require the presence of bradykinesia, the absence of atypical features or other causes of parkinsonism, and at least 1 of the following: resting tremor, rigidity and postural instability, and a positive response to dopaminergic therapy. In patients without PD diagnoses were assigned according to published diagnostic criteria.20,21 None of the patients was related, and they did not represent more than 1 participant from a family.

Among the 480 patients with idiopathic PD, the mean (SD) age at onset was 54.96 (10.64) years (range, 22–85 years), and the mean (SD) disease duration was 13.62 (6.34) years (range, 3–40 years). All of the patients were white, and all except 2 individuals, who were born in South America, were born in Italy.

All patients underwent neurological examination and were assessed with use of the Unified Parkinson Disease Rating Scale.22 In addition, those patients identified with the FMR1 premuta-

REPORT OF CASES

PATIENT 1

A 51-year-old woman presented with a 2-year history of progressive left-sided parkinsonism, which started with left shoulder ache and rest tremor of the left hand. Her medical history included depression and positional paroxystic vertigo, without premature ovarian failure, peripheral neuropathy, or other comorbidities associated with the FMR1 premutation.2 She had 2 sons with fragile X syndrome (Figure 1).

Neurological examination revealed hypomimia, hypophonia, and horizontal first-degree nystagmus on right-directed gaze with saccadic pursuit. Bilateral mild postural tremor of the upper extremities was seen, as well as rest tremor of the left hand. Rigidity and bradykinesia were bilateral, mainly on the left. She had a shuffling gait and reduction of pendular sway of the left upper arm. She did not show ataxia or either pyramidal or autonomic system involvement. There was no impaired toe position, numbness, or absence of pinprick/vibration sensation. She had a good response to levodopa (600 mg/d), showing a significant improvement of all parkinsonian symptoms (Unified Parkinson Disease Rating Scale–Revised score from 29 to 18; −38% at 6-month follow-up). Full-scale intelligence quotient, measured using the Wechsler Adult Intelligence Scale–Revised,24 was within the normal range.

Brain MR imaging revealed mild subcortical and cortical cerebral and cerebellar atrophy, without evidence of the typical white matter abnormalities and middle cerebellar peduncles7,13 (Figure 2). Single-photon emission computed tomography imaging using iodine 123-2-carbomethoxy-8-(3-fluoropropyl)-3-(4-iódophenyl)tropane (FP-CIT) showed moderate presynaptic dopaminergic nigrostriatal terminal loss (Figure 3A). Postsynaptic dopaminergic D2 receptor density and cerebral blood flow were investigated by single-photon emission computed tomography imaging using [123I]iodobenzamide (Figure 3B) and Tc 99m ethyl cysteinate dimer bi-
cisate, respectively, and both receptor density and cerebral blood flow were normal.

DNA molecular testing revealed the presence of normal (30 CGG) and premutation (115 CGG) \textit{FMR1} alleles. The activation ratio was 0.51.

\section*{PATIENT 2}

A 76-year-old woman had an 11-year history of left-sided parkinsonism before her diagnosis of PD, at which time she started taking levodopa and bromocriptine with clinical improvement. After several years of levodopa therapy, she manifested motor fluctuations and dyskinesias. Nevertheless, she still showed a good response to levodopa but with “on-off” motor fluctuations. Her mother had PD, but no other family members had movement disorders, dementia, developmental delay, mental retardation, or any other comorbidity associated with the \textit{FMR1} premutation.\textsuperscript{5}

On neurological examination (“off” motor fluctuations), she showed hypomimia, rigidity, and bradykinesia mainly on the left, and dystonia of the left arm. She presented with a shuffling gait, with freezing and severe balance instability. There was no sign of pyramidal or autonomic system involvement. No cognitive impairment was detected by neuropsychological assessment; however, she did display features of anxiety and depression as described by Goldwurm et al.\textsuperscript{25} Brain MR imaging showed mild subcortical and cortical cerebral atrophy (Figure 2C and D). DNA molecular testing revealed the presence of normal (30 CGG) and premutation (75 CGG) \textit{FMR1} alleles. The activation ratio was 0.29.

\section*{MOLECULAR ANALYSIS}

Genomic DNA was isolated from peripheral blood leukocytes using standard phenol-chloroform extraction methods. DNA from each patient was amplified using an enhanced PCR technique containing the osmolyte betaine and primers \textit{c} and \textit{f}.\textsuperscript{26} Amplified DNA was then visualized by ethidium staining on 2\% agarose gels. Grayzone (45-54 CGG repeats) and premutation alleles were accurately sized on polyacrylamide gels. Hybridization was performed with a digoxigenin end-labeled oligonucleotide probe (CGG)\textsubscript{10}. Repeat sizes were determined using an imaging system (FluorChem 8800 Im-

\begin{figure}[h]
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\caption{Brain magnetic resonance images of patient 1 (A and B) and patient 2 (C and D). T1-weighted sagittal (A) and T2-weighted axial (C) sections showed mild cortical cerebellar atrophy in patient 1 and mild cortical cerebral atrophy in patient 2. Note the absence of the characteristic high signal in the middle cerebellar peduncles in both patients (B and D).}
\end{figure}
age Detection System; Alpha Innotech Corp, San Leandro, California) as described previously. For apparent homozygous women, the possible presence of a second normal allele vs a potential full mutation allele was resolved using the newly developed PCR-based screening tool for expanded \textit{FMR1} alleles, which uses a “chimeric” CGG-targeted primer in conjunction with betaine-based PCR. This method allows rapid determination of the allele status of all men and women, regardless of the number of CGG repeats. Southern blot analysis, as described by Tassone et al, was performed only on the 2 premutation carriers. The activation ratio, which measures the percentage of cells carrying the normal allele on the active X chromosome, was measured using the imaging system. Based on the number of CGG repeats, patients were classified as having a normal allele (6-44 CGG repeats), a gray-zone allele (45-54 CGG repeats), or a premutation allele (55-200 CGG repeats).

**RESULTS**

We screened 595 female patients who had received a diagnosis of PD or another parkinsonism spectrum disorder for the presence of an \textit{FMR1} premutation allele. Through the use of the betaine-based PCR approach, 2 premutation carriers were identified, with allele sizes of 30, 115 (patient 1) and 30, 75 (patient 2) (\textbf{Figure 4}). Approximately 30% of the screened samples showed only a single band on agarose gels after the first PCR screening, because the betaine-based PCR approach is unable to distinguish between women who are homozygous for normal \textit{FMR1} alleles and women with 1 normal allele and a second, full mutation allele that cannot be amplified by PCR. To address this ambiguity in the context of a rapid screening protocol, we have used a newly developed PCR approach that uses a “chimeric” CGG-targeted primer in conjunction with betaine-based PCR; this latter PCR method is capable of flagging all expanded alleles, allowing the distinction to be made between homozygosity and the presence of a large expansion.
This second approach uses the standard $\epsilon$ primer with a second, chimeric PCR primer that anneals randomly within the CGG repeat expansion via a (CCG)$_n$ 3’ portion of the primer. The resulting PCR amplification will produce a smear on the gel whenever an expanded allele is present, whereas in the absence of an expanded allele, no large smear will be detected. An example of the method is presented in Figure 5. Using this second approach, we ruled out the presence of either full mutation or high-end premutation alleles in the cohort.

The observed rate of premutation alleles (2 of 595 [0.34%]) in this group of women is not significantly different from the estimated rate in the general female population (0.4%-0.8%). Notably, the woman with a 115 CGG repeat expansion had 4 children, including 2 sons with fragile X syndrome. Thus, it is likely that at least some of her clinical features may be due to the mechanisms underlying FXTAS. In addition, 14 gray-zone alleles were identified, which corresponds to a rate (2.3%) that does not differ from the frequency within the general female population (3%-4%). The remaining 579 individuals had normal FMR1 alleles (<45 CGG repeats).

The findings of this screening of 595 women who were evaluated at a PD clinic showed that 2 women were carriers of an FMR1 premutation allele and that the frequency of such alleles did not differ from that in the general population. Thus, the present study supports the position that screening of PD or parkinsonism cohorts is not warranted in the absence of additional findings (eg, gait ataxia) or family history (eg, members with learning delays or autism) that might suggest involvement of FMR1 Clinical and radiological analyses of these 2 individuals did not show features suggestive of definite or probable FXTAS. One of the 2 carriers (patient 1) had a positive family history of 2 sons with fragile X syndrome. This patient also showed first-degree nystagmus, although this finding is unlikely to be related to the FMR1 expansion as it is not associated with other typical clinical or radiological features of FXTAS and does not represent a common clinical feature of FXTAS. However, the patient’s nystagmus might be associated with a history of vestibular system impairment.

A review of the clinical histories showed that both women had a fairly representative presentation and progression of PD, including a good response to levodopa and the development of motor complications such as “on-off” motor fluctuations and dyskinesia. In addition, MR and functional single-photon emission computed tomography imaging investigations were consistent with the diagnosis of idiopathic PD. Notably, neither patient presented with any cognitive impairment.

These findings suggest that the presence of the FMR1 premutation does not necessarily increase the severity or alter the presentation of parkinsonism in patients with idiopathic PD. As a corollary, the current results support the notion of different pathogenetic mechanisms for FXTAS and PD that do not appear to act in a synergic manner. This would be consistent with the converse finding, namely, Lewy bodies in patients with FXTAS and PD.

In conclusion, screening of women with parkinsonism is unlikely to be productive without the existence of additional clinical or family history data that are suggestive of a fragile X gene disorder. In this regard, the present study confirms the main findings of a recent screening assessment in a male PD population.

Figure 5. Lanes 1 through 4, Normal alleles from 6 Parkinson disease samples show only small polymerase chain reaction products with the chimeric primer. Lanes 5 and 6, Smear indicating the presence of an expanded allele in both premutation carriers (patients 2 and 1, respectively). Lane 7, Positive control (DNA from a woman with the FMR1 full mutation) for which an extensive smear is produced with the chimeric primer. Lane 8, Normal control. Lanes 9 and 10, Negative control and a size marker, respectively.

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Author Contributions: Drs Cilia and Goldwurm had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Hagerman and Tassone. Acquisition of data: Cilia, Kraff, Canesi, Goldwurm, Tang, and Pan. Analysis and interpretation of data: Cilia, Pezzoli, Goldwurm, Hagerman, and Tassone. Drafting of the manuscript: Kraff, Amiri, Hagerman, and Tassone. Critical revision of the manuscript for important intellectual content: Cilia, Canesi, Pezzoli, Goldwurm, Tang, Pan, and Hagerman. Obtained funding: Pezzoli, Hagerman, and Tassone. Administrative, technical, and material support: Cilia, Canesi, and Goldwurm. Study supervision: Cilia and Tassone.

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REFERENCES