Journal of Molecular Diagnostics, Vol. 11, No. 4, July 2009

DOI: 10.2353/jmoldx.2009.080173

Screening for Expanded Alleles of the *FMR1* Gene in Blood Spots from Newborn Males in a Spanish Population

Isabel Fernandez-Carvajal,* Paulina Walichiewicz,† Xie Xiaosen,† Ruiqin Pan,† Paul J. Hagerman,†‡ and Flora Tassone*†‡

From the Laboratorio de Genética Humana, Unidad de Diagnóstico Genético y Perinatal, Instituto de Biología y Genética Molecular, Universidad de Valladolid-CSIC, Valladolid, Spain; the Department of Biochemistry and Molecular Medicine,† University of California, School of Medicine, Davis, California; and the M.I.N.D. Institute,‡ University of California Davis Health System, Sacramento, California*

Fragile X syndrome, which is caused by expanded CGG repeats of the *FMR1* gene, is associated with a broad spectrum of clinical involvement and is the most common inherited form of intellectual disability. Early diagnosis and intervention are likely to lead to improved outcome for children with fragile X syndrome, but such strategies require better estimates of the frequencies of expanded alleles of the *FMR1* gene. In this study, we report the results of a newborn screening study of 5267 male blood spots collected from the Northwest region of Spain as part of the national newborn screening program. The blood spots were screened using a rapid polymerase chain reaction-based method that is capable of identifying the presence of all expanded alleles for both males and females. The screened samples included 199 gray zone alleles, 21 premutation alleles, and two full mutation alleles (1 in 2633). The frequency of premutation alleles was three times higher (1 in 251) than the quoted value of 1 in 813 from a Canadian population and is fully consistent with the results of large-scale Israeli screening studies. Our results demonstrate that newborn screening for the presence of expanded *FMR1* alleles is an effective means for defining the distribution of expanded *FMR1* alleles in newborn populations; as such, this method is suitable for large-scale newborn screening. *(J Mol Diagn 2009, 11:324–329; DOI: 10.2353/jmoldx.2009.080173)*

Although fragile X syndrome (FXS) is the most common inherited form of intellectual disability, prior estimates of the frequencies of expanded CGG-repeat alleles have varied widely, ranging from ~1/2000 to 1/8000, depending on the nature of ascertainment.[1] Estimates of fragile X syndrome disease prevalence, or *FMR1* full mutation (>200 CGG repeats) allele frequency, derived from screening of special education needs populations will likely miss individuals with mild learning disabilities, particularly in females with favorable X-activation ratios.[2] Indeed, a higher full mutation allele frequency (1 in ~2500 females) was reported by Pesso et al,[3] who screened a large number of Israeli women in the general population.

For premutation alleles (range, 55–200 CGG repeats), allele frequencies are more often estimated through general population screening, where the most solid estimates are for females.[1] However, there remains some uncertainty regarding the premutation allele frequencies[1,3–7] due in part to lingering issues of ascertainment bias,[8] but also to real frequency differences across ethnic and regional populations. For example, in the screens of males and females in Eastern Canada, allele frequencies were estimated to be ~1/800 males and ~1/260 females.[4,6] However, in the Israeli studies,[3,7] the frequency of premutation alleles in women is closer to ~1/130.[7,9] By contrast, in an Asian (Taiwanese) population, the frequency of premutation alleles in males was reported to be much lower (~1/1670).[10]

Within the past decade, there has been increasing recognition of the breadth of phenotypes associated with expanded *FMR1* alleles, especially in the premutation range.[11] In particular, two disorders specific to the premutation range have been described: primary ovarian insufficiency (formerly premature ovarian failure), which occurs in approximately 20% of females with the premutation, as compared with 1% of the general population[12];

Supported by National Institute of Child Health and Development grants 265 HD02274 (to F.T.) and HD040661 (to P.J.H.); by the Federacion Española del Sindrome X fragil; by Dr. Alfredo Blanco Quiros, director of the Department of Pediatric in the Faculty of Medicine, University of Valladolid; and by Junta de Castilla y Leon (newborn screening program) and the Federacion Española de Sindrome X fragil (to I.F.-C.).

Accepted for publication February 6, 2009.

F.T. and P.J.H. collaborated with Asuragen, Inc. for the development of screening methodologies, which involves no financial interest or intellectual property claims on the part of the authors. F.T. and P.J.H. also have a filed patent application 20080113355 for screening methodology.

Address reprint requests to Flora Tassone, Ph.D., Department of Biochemistry and Molecular Medicine, University of California, Davis, School of Medicine, One Shields Avenue, Davis, CA 95616. E-mail: ftassone@ucdavis.edu.

and the late-adult-onset neurodegenerative disorder, fragile X-associated tremor/ataxia syndrome.[13–17] Therefore, it is critical for understanding the broader societal impact of the fragile X family of disorders to obtain an accurate estimate of allele frequencies for both premutation and full mutation alleles, in diverse ethnic and geographical populations.

Previously, large-scale screening of the newborn populations has been hampered by the lack of a rapid, inexpensive screening test that is capable of using blood spots to register all expanded (premutation and full mutation) *FMR1* alleles, for both males and females. However, with the recent development of such a test,[18] screening of blood spots to detect all expanded alleles is now feasible, which in turn permits large-scale screening of newborn populations.

Here we report the results of an anonymous blood spot screening of 5267 newborns (males) collected across the region of Castilla y León, Spain. The screened samples included 199 gray zone alleles (1 in 26; 95% confidence interval, 1/23–1/30), 21 premutation alleles (1 in 251; 95% confidence interval, 1/164–1/385), and two full mutation alleles (1 in 2633; 95% confidence interval, 1/714–1/10,000), which is in line with estimates based on the Israeli population screens.[8]

Materials and Methods

Subjects

Blood spots used in the current study were obtained from an existing archive of bloodspots representing routine newborn screening at designated hospitals of the region of Castilla y León (nine different provinces), with bloodspots routinely collected between the third and fifth day of life. Samples were collected on 903 specimen collection paper (Whatman, Inc., NJ). The dried blood spots were received at the Metabolic Diseases Laboratory of the Institute of Biology and Molecular Genetics of the University of Valladolid, reference laboratory for the newborn screening of metabolic and genetic diseases (hypothyroidism, phenylketonuria, and cystic fibrosis) in the Castilla y León region of Spain. For the current study, a total of 5267 samples (labeled as male), representing consecutive samples received during the first 6 months of 2007, were used. The samples were stripped of all identifiers, patient codes, and/or accession numbers at Institute of Biology and Molecular Genetics, preserving only stated sex and ethnicity of the donor, to ensure that the samples were not traceable to the donors; thus, only completely de-identified samples were sent to the M.I.N.D. Institute molecular laboratory at the University of California, Davis, for genotyping. On reaching the University of California, Davis, each sample was assigned a local accession number. The majority of the subjects were Caucasian and Spanish from the catchments region. Based on regional census figures, approximately 4% of the population was found to be foreign to the catchment region. All protocols involving human subjects were performed under an existing Institutional Review Board for anonymous screening.

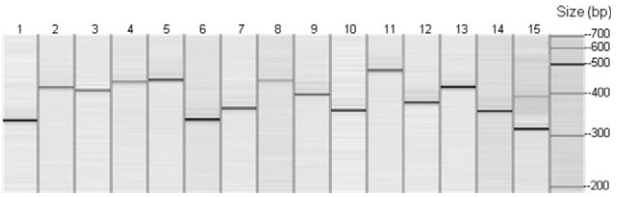

Figure 1. Example of sizing of *FMR1* alleles obtained from blood spots included in this study. Premutation alleles: lanes 2, 3, 4, 5, 8, 9, 11 and 13. Gray zone alleles: lanes 7, 10, and 12; normal alleles: lanes 1, 6, and 14. Lane 15: female control (30, 54 CGG size determined by sequencing). A DNA size marker 100 bp, 3 kb (Qiaxcel) was used to size the different alleles.

Molecular Studies

A disk 1.2 mm in diameter was removed by punch from each dried blood spot and was placed in a clean 0.5-μl polymerase chain reaction (PCR) tube. Two hundred microliters of Qiagen RBC lysis solution (Qiagen, Valencia, CA) was added to the tube followed by incubation for 5 minutes at room temperature. The supernatant was removed, including any excess liquid adhering to the disk, and the disk was left to dry for several minutes before the PCR master mix (FastStart PCR kit; Roche Diagnostics, Indianapolis, IN) was added to each sample. Master mix containing primers c and f[19] was prepared and used according to the manufacturer's instructions; primers c and f yield amplicons of 221 + 3 × CGG repeat number bp. Using the applied Biosystems 9700 thermocycler, the PCR conditions were: 10 minutes initial denaturation at 95°C, 10 cycles of 95°C for 35 seconds, 64°C for 35 seconds, 68°C for 4 minutes; followed by 25 cycles of 95°C for 35 seconds, 64°C for 35 seconds, 68°C for 4 minutes (with 20 seconds increase each cycle); followed by a final extension of 10 minutes at 68°C. The PCR products were stored at 4°C until analysis or were immediately analyzed using the Qiaxcel genetic analyzer (Qiagen), which utilizes a preassembled cartridge (cartridge type Qiaxcel DNA high-resolution cartridge, injection time 10 seconds, Qiaxcel DNA size marker 100 bp, 3 kb) to simultaneously run samples and collect data. Using conditions as recommended by the manufacturer, Figure 1 shows PCR products derived from 14 bloodspots using the Qiaxcel capillary system. Data were analyzed on a PC running BioCalculator software, which saves the data collected by the unit and allows CGG repeat size analysis after collection. Using DNA size marker as indicated in Figure 1, alleles were classified as normal (<45 CGG repeats), gray zone (45-54 CGG repeats), premutation (55-200 CGG repeats), and full mutation (>200 CGG repeats). Samples that did not yield a band after the first round PCR with primers c and f were subjected to a secondary CGG-primer-based PCR screening as previously described.[18] Figure 2 shows PCR products obtained from secondary screening of blood spots using the chimeric CGG-targeted primer for the detection of large CGG repeat expansions run on a 2% agarose gel. An extensive smear is produced with the chimeric primer

Figure 2. Detection of large CGG repeat expansions using a CGG-targeting PCR primer. Lanes 1 and 4, the two full-mutation alleles identified in this study; lanes 2 and 3, normal alleles identified in this study; lanes 5 and 6, normal and full mutation controls; lane 7, negative control.

when an expanded allele is present, as shown in lanes 1 and 4 for the two full mutation samples, while no smear is visible in the presence of a normal allele as shown in lanes 2 and 3. Blood spots that underwent the CGG primer-based PCR screening were washed two times for 15 minutes in 1 ml of ddH2O and used immediately in a PCR reaction.[18] PCR products were run on a 2% agarose gel. Isolation of DNA from blood spots was performed on the two full mutation samples to rule out the possibility that the two samples were indeed large premutation alleles (see Results). DNA was obtained by using a 1 × 3 mm punch bloodspot directly into a 500-μl Eppendorf tube containing 60 μl of cell lysis solution (Qiagen) and 3 μl of 20 mg/ml proteinase K (Roche Diagnostics, Indianapolis, IN). Spots were incubated at 55°C overnight and then treated with 2.5 μl of RNase A (5 mg/ml) at 37°C for 15 minutes. Proteins were precipitated by adding 200 μl of protein precipitation solution (Qiagen). The solution was spun down at 12,000 rpm for 5 minutes and the DNA was precipitated from the supernatant with 1 volume of isopropanol and 1 μl of glycogen solution (20 mg/ml). The DNA pellet was washed with 70% ethanol, dissolved in DNA hydration solution (Qiagen), and stored at −20°C until use.

Results

Of the 5267 total male blood spots screened, 32 (0.6%) showed two main bands using two different sets of primers and were not analyzed further. Although some of those samples may have been mislabeled with respect to the sex of the infant, one would expect that 5 to 10 samples would have come from Klinefelter subjects. Given the anonymous nature of the sample, no follow-up was possible. It should be noted that all bands in those 32 samples were in the normal range (data not shown). Some of the blood spots were run twice if they failed to amplify the first time. Of the remaining 5235 alleles, 5013 had a CGG repeat number within the normal range; 199 were gray zone alleles (1 in 26; 95% confidence interval, 1/23–1/30), 21 were premutation alleles (1 in 251; 95% confidence interval, 1/164–1/385), and two were presumptive full mutation alleles (1 in 2633; 95% confidence interval, 1/714–1/10,000) (Table 1, Figure 3). With the CGG primer approach, a smear is detectable also in the presence of a large premutation allele. Therefore, to rule out the possibility that the two samples harbored alleles in the upper premutation range, which we failed to amplify using the first PCR step (with primer c and f), we mixed the DNA isolated from the two blood spots separately with the same amount of DNA from a known premutation carrier harboring premutation alleles of 144 and 185 CGG repeats. After PCR with primer c and f, only the two premutation bands corresponding to the known premutation carrier were detected and visualized on the agarose gel (data not shown). These findings reinforce our position that two full mutation alleles were detected.

Whereas the frequency of gray zone alleles (1/26) is not significantly different from literature values for the

Table 1. Allele Frequencies Within the Screened Sample

Allele class (range)	Number of samples	Frequency*	95% confidence interval
Normal (6-44 CGG)	5013		
Intermediate (45-54 CGG)	199	1/26	1/23–1/30
Premutation (55-200 CGG)	21	1/251	1/164–1/385
Full mutation (>200 CGG)	2	1/2633	1/714–1/10,000
Samples with two bands	32		
Total	5267		

*Frequencies are based on the total sample size, which includes the 32 samples ejected for sizing.

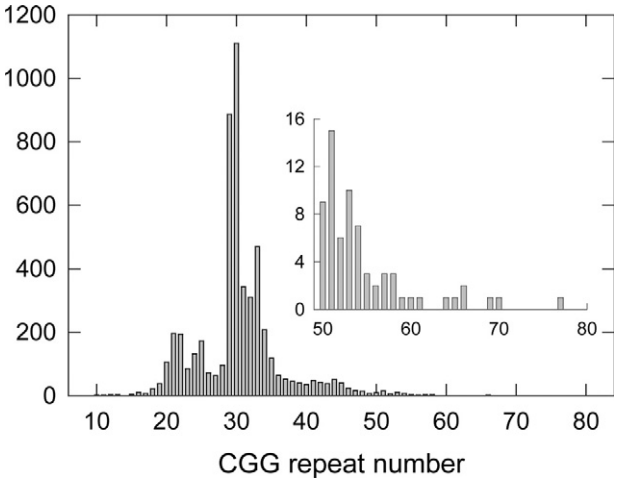

Figure 3. Distribution of the sizes of the 5233 *FMR1* alleles analyzed in the current study.

Canadian cohorts,[6,20] the value of 1/251 for premutation alleles is three times larger than the value of 1/813 previously reported in males by Dombrowski et al,[6] and is in close agreement with the expectation derived from the female Israeli cohorts.[8] The premutation alleles ranged in size from 55 to 77 CGG repeats; thus, all were toward the small end of the premutation range, again consistent with previous observations.[21]

Using the current PCR screening method, we could not establish whether any of the 21 identified premutation subjects were actually size and/or methylation mosaic individuals; that is, that they were also carrying a full mutation allele that we did not detect. Were any one of these 21 cases actually an undetected mosaic, the current study would have underestimated the true frequency of full mutation alleles, which would then yield 1/1756 for the frequency of full mutation alleles (ie, for three full mutations, not two), which is much higher than other estimates for full mutation frequency. The presence of mosaics and full mutation alleles can be established by Southern blot analysis; however, given the limited amount of DNA that can be extracted from the filters, such an analysis is not possible. Methylation analysis (ie, using long PCR in combination with bisulfite modification) of the promoter region of the *FMR1* region could also be used to identify methylated (and hence full mutation) alleles; again, the method requires much larger quantities of DNA than can be obtained from the filters. We are currently attempting to improve the sensitivity of the current PCR approach.

Discussion

In this work we have used a newly developed methodology to answer a number of questions regarding *FMR1* allele frequency, size distribution, and feasibility of newborn screening on a large scale. Previous studies, aimed at establishing allele frequencies in different populations, have yielded dissimilar results mainly due to screening selection bias. Moreover, no studies have demonstrated the practicability of large-scale population screening for all expanded *FMR1* alleles (for both males and females) from newborn blood spots. Our current findings underscore the feasibility of large population screening, as for example newborn screening. The availability of an easy, rapid, and inexpensive test may facilitate the introduction of newborn screening for *FMR1* mutations into the established public health infrastructure for existing newborn screening programs, and thus early childhood developmental intervention strategies could be enhanced for children who are diagnosed with *FMR1* mutations.

One important outcome from this study is that the frequency of premutation alleles (1/251) in an unbiased sample of male newborns in Spain appears to be three times higher than the frequency most often quoted in the literature (1/813).[6] This higher frequency of premutation alleles has important implications for the prevalence in Spain of developmental and behavioral problems (eg, attention deficit hyperactivity disorder and autism spectrum disorders[22,23]) that are frequently observed in children who carry premutation alleles, and for fragile X-associated neuroendocrine (fragile X primary ovarian insufficiency) and neurodegenerative (fragile X-associated tremor/ataxia syndrome) disorders among adult carriers. However, the current results speak more broadly of the potential for significant differences in allele frequencies across different populations. It should be kept in mind that founder effects could contribute to the discrepancies in allele frequencies observed in different population groups.

The estimated frequency of full mutation alleles in this Spanish cohort of 5267 males, 1/2633, is in line with results from recent population screening studies (see[1,8]); however, since this estimate was based on only two samples, the confidence interval is quite large. Assuming that the observed frequency is correct, a sample size of approaching 50,000 would be needed to reduce the upper limit of the confidence interval to within about 25% of the mean value. Previously reported frequencies for full mutation alleles (~1 in 2500 to 1 in 8000)[3,5,24–26] have generally been based on screening of target populations with significant developmental problems extrapolated to the general population. Such projections tend to underestimate the disease prevalence, and hence allele frequencies, since individuals with only mild or no apparent learning difficulties would be excluded. Interestingly, a recent report[8] that used an average of the known frequency for premutation females (1/126)[3,7] to estimate the expected frequencies of full mutation and (male) premutation alleles yielded frequencies of 1/2355 (males and females) for full mutation alleles and 1/282 for premutation alleles (male); remarkably close to the observations of the current study.

The current distribution of *FMR1* alleles indicates that the most common alleles in this population are 29 and 30 CGG repeats (Figure 3). Previous studies have indicated that the most common alleles vary of few repeats in different populations including 28 CGG repeats,[20] 29 CGG repeats in Taiwan,[27] 30 CGG repeats,[28] 29 and 30 CGG repeats in Spain,[29] and 29 CGG repeats in a Chinese population.[30] However, it should be noted that small differences among studies (1–2 CGG repeats) may be a consequence of experimental errors in various labs in the absence of single repeat precision and sequenced CGG standards.

In a previous screen for expanded *FMR1* alleles using blood spots, Rife et al[31] presented a general population screening of 4937 newborn blood spots from males collected throughout the Catalonia region in Spain. The screening yielded a frequency of 1/2466 full mutation males and 1/1233 premutation carriers. Whereas the frequency of full mutation alleles observed in the Rife et al study is in agreement with previous studies,[32,33] their frequency of premutation alleles was lower than the value of 1/813 reported in the Canadian study[6]; a paradoxical result both in terms of the expectation from the genetic model for transmission,[1,8] and given the fact that both the Rife et al study and the current investigation used newborn samples from Spain, albeit from different regions. This discrepancy could be explained by the genotype methodology used by this group. Two additional stud-

ies[20,34] reported screens of newborns for fragile X using blood spots. Dawson et al[20] analyzed cohorts of 1000 males and 1000 females; however, the number of samples successfully analyzed in that study was quite small, owing to a high (~25%) failure rate on PCR. In the second study, Holden et al[34] reported blood spot screening results for 2050 newborn males; however, their study also suffered from difficulty with PCR amplification for alleles larger than ~75 CGG repeats. Finally, a recent study by Saul et al[35] reported an equal frequency of both premutation and full mutation (1:730), which most likely reflect the low number of subject screened (1459 newborn blood spots).

The current pilot screening study for expanded *FMR1* alleles, using newborn blood spots, demonstrates the applicability of our methodology to large-scale newborn screening. The methodological approach satisfies the principal requirements for a screening tool: that it reliably detect expanded alleles at least through the upper portion of the premutation range; that it be both rapid and cost effective (ie, less than $3 for reagents per test); and that it works effectively with the small amount of DNA typically yielded from a portion of a single blood spot, the principal resource for newborn screening. It should be noted that although the CGG-primer-based screening method is capable of registering large expansions in the full mutation range, it is not designed to determine the actual sizes of full mutation alleles. Rather, full mutation alleles flagged by the screening method would be sized by Southern blot analysis as part of a newborn follow-up assessment and early intervention program. In this context, newborn screening would provide parents the opportunity to learn about their child's fragile X status and their own reproductive risk, in addition to other likely benefits provided by accessing early intervention programs, which have been shown to positively influence child development and provide support to families of children with fragile X syndrome.[36–38]

Acknowledgments

This work is dedicated to the memory of Matteo.

References

1. Song FJ, Barton P, Sleightholme V, Yao GL, Fry-Smith A: Screening for fragile X syndrome: a literature review and modelling study. Health Technol Assess 2003, 7:1–106
2. Hagerman RJ, Hagerman PJ, eds: Fragile X Syndrome: Diagnosis, Treatment, and Research, 3rd ed. Baltimore, The Johns Hopkins University Press, 2002
3. Pesso R, Berkenstadt M, Cuckle H, Gak E, Peleg L, Frydman M, Barkai G: Screening for fragile X syndrome in women of reproductive age. Prenat Diagn 2000, 20:611–614
4. Rousseau F, Rouillard P, Morel ML, Khandjian EW, Morgan K: Prevalence of carriers of premutation-size alleles of the FMR1 gene–and implications for the population genetics of the fragile X syndrome. Am J Hum Genet 1995, 57:1006–1018
5. Crawford DC, Meadows KL, Newman JL, Taft LF, Scott E, Leslie M, Shubek L, Holmgreen P, Yeargin-Allsopp M, Boyle C, and Sherman SL: Prevalence of the fragile X syndrome in African-Americans. Am J Med Genet 2002, 110:226–233
6. Dombrowski C, Lévesque S, Morel ML, Rouillard P, Morgan K, Rousseau F: Premutation and intermediate-size *FMR1* alleles in 10572 males from the general population: loss of an AGG interruption is a late event in the generation of fragile X syndrome alleles. Hum Mol Genet 2002, 11:371–378
7. Toledano-Alhadef H, Basel-Vanagaite L, Magal N, Davidov B, Ehrlich S, Drasinover V, Taub E, Halpern G, Ginott N, and Shohat M: Fragile-X carrier screening and the prevalence of premutation and full-mutation carriers in Israel. Am J Hum Genet 2001, 69:351–360
8. Hagerman PJ: The fragile X prevalence paradox. J Med Genet 2008, 45:498–499
9. Hagerman RJ, Hagerman PJ: The fragile X premutation: into the phenotypic fold. Curr Opin Genet Dev 2002, 12:278–283
10. Tzeng C, Tsai L, Hwu W, Lin S, Chao M, Jong Y, Chu S, Chao W, Lu C: Prevalence of the FMR1 mutation in Taiwan assessed by large-scale screening of newborn boys and analysis of DXS548-FRAXAC1 haplotype. Am J Med Genet A 2005, 133:37–43
11. Hagerman PJ, Hagerman RJ: The fragile-X premutation: a maturing perspective. Am J Hum Genet 2004, 74:805–816
12. Allingham-Hawkins DJ, Babul-Hirji R, Chitayat D, Holden JA, Yang KT, Lee C, Hudson R, Gorwill H, Nolin SL, Glicksman A, Jenkins EC, Brown WT, Howard-Peebles PN, Becchi C, Cummings E, Fallon L, Seitz S, Black SH, Vianna-Morgante AM, Costa SS, Otto PA, Mingroni-Netto RC, Murray A, Webb J, MacSwinney F, Dennis N, Jacobs PA, Syrrou M, Georgiou I, Patsalis PC, Giovannucci Uzielli ML, Guarducci S, Lapi E, Cecconi A, Ricci U, Ricotti G, Biondi C, Scarselli B, Vieri F: Fragile X premutation is a significant risk factor for premature ovarian failure: the international collaborative POF in fragile X study—preliminary data. Am J Med Genet 1999, 83:322–325
13. Amiri K, Hagerman RJ, Hagerman PJ: Fragile X-associated tremor/ataxia syndrome: an aging face of the fragile X gene. Arch Neurol 2008, 65:19–25
14. Hagerman PJ, Hagerman RJ: Fragile X-associated tremor/ataxia syndrome–an older face of the fragile X gene. Nat Clin Pract Neurol 2007, 3:107–112
15. Berry-Kravis E, Potanos K, Weinberg D, Zhou L, and Goetz CG: Fragile X-associated tremor/ataxia syndrome: clinical features, genetics, and testing guidelines. Mov Disord 2007, 22:2018–2030
16. Willemsen R, Mientjes E, Oostra BA: FXTAS: a progressive neurologic syndrome associated with Fragile X premutation. Curr Neurol Neurosci Rep 2005, 5:405–410
17. Hagerman RJ, Leehey M, Heinrichs W, Tassone F, Wilson R, Hills J, Grigsby J, Gage B, and Hagerman PJ: Intention tremor, parkinsonism, and generalized brain atrophy in male carriers of fragile X. Neurology 2001, 57:127–130
18. Tassone F, Pan R, Amiri K, Taylor AK, Hagerman PJ: A rapid polymerase chain reaction-based screening method for identification of all expanded alleles of the fragile X (FMR1) gene in newborn and high-risk populations. J Mol Diagn 2008, 10:43–49
19. Fu YH, Kuhl DPA, Pizzuti A, Pieretti M, Sutcliffe JS, Richards S, Verkert AJMH, Holden JJA, Fenwick RG Jr, Warren ST, Oostra BA, Nelson DL and Caskey CT: Variation of the CGG repeat at the fragile X site results in genetic instability: resolution of the Sherman paradox. Cell 1991, 67:1047–1058
20. Dawson AJ, Chodirker BN, Chudley AE: Frequency of FMR1 premutations in a consecutive newborn population by PCR screening of Guthrie blood spots. Biochem Mol Med 1995, 56:63–69
21. Jacquemont S, Leehey MA, Hagerman RJ, Beckett LA, Hagerman PJ: Size bias of fragile X premutation alleles in late-onset movement disorders. J Med Genet 2006, 43:804–809
22. Farzin F, Perry H, Hessl D, Loesch D, Cohen J, Bacalman S, Gane L, Tassone F, Hagerman P, and Hagerman R: Autism spectrum disorders and attention-deficit/hyperactivity disorder in boys with the fragile X premutation. J Dev Behav Pediatr 2006, 27(Suppl 2):S137–S144
23. Goodlin-Jones BL, Tassone F, Gane LW, Hagerman RJ: Autistic spectrum disorder and the fragile X premutation. J Dev Behav Pediatr 2004, 25:392–398
24. Morton JE, Bundey S, Webb TP, MacDonald F, Rindl PM, Bullock S: Fragile X syndrome is less common than previously estimated. J Med Genet 1997, 34:1–5
25. Turner G, Webb T, Wake S, Robinson H: Prevalence of fragile X syndrome. Am J Med Genet 1996, 64:196–197
26. de Vries BB, van den Ouweland AM, Mohkamsing S, Duivenvoorden HJ, Mol E, Gelsema K, van Rijn M, Halley DJ, Sandkuijl LA, Oostra BA,

Tibben A, and Niermeijer MF: Screening and diagnosis for the fragile X syndrome among the mentally retarded: an epidemiological and psychological survey: Collaborative Fragile X Study Group. Am J Hum Genet 1997, 61:660–667
27. Wang YC, Li C, Lin ML, Lin WH, Li SY: Molecular diagnosis of fragile X syndrome and distribution of CGG repeats in the FMR-1 gene in Taiwanese. J Formos Med Assoc 2000, 99:402–407
28. Snow K, Doud LK, Hagerman R, Pergolizzi RG, Erster SH, Thibodeau SN: Analysis of a CGG sequence at the FMR-1 locus in fragile X families and in the general population. Am J Hum Genet 1993, 53:1217–1228
29. Milà M, Kruyer H, Glover G, Sánchez A, Carbonell P, Castellví-Bel S, Volpini V, Rosell J, Gabarrón J, López I, Villa M, Ballesta F and Estivill X: Molecular analysis of the (CGG)n expansion in the FMR-1 gene in 59 Spanish fragile X syndrome families. Hum Genet 1994, 94:395–400
30. Chen TA, Lu XF, Che PK, Ho WK: Variation of the CGG repeat in FMR-1 gene in normal and fragile X Chinese subjects. Ann Clin Biochem 1997, 34:517–520
31. Rife M, Badenas C, Mallolas J, Jiménez L, Cervera R, Maya A, Glover G, Rivera F, Milà M: Incidence of fragile X in 5,000 consecutive newborn males. Genet Test 2003, 7:339–343
32. Crawford DC, Acuna JM, Sherman SL: FMR1 and the fragile X syndrome: human genome epidemiology review. Genet Med 2001, 3:359–371
33. Patsalis PC, Sismani C, Hettinger JA, Boumba I, Georgiou I, Stylianidou G, Anastasiadou V, Koukoulli R, Pagoulatos G, Syrrou M: Molecular screening of fragile X (FRAXA) and FRAXE mental retardation syndromes in the Hellenic population of Greece and Cyprus: incidence, genetic variation, and stability. Am J Med Genet 1999, 84:184–190
34. Holden JJ, Chalifoux M, Wing M, Julien-Inalsingh C, White BN: A rapid, reliable, and inexpensive method for detection of di- and trinucleotide repeat markers and disease loci from dried blood spots. Am J Med Genet 1996, 64:313–318
35. Saul RA, Friez M, Eaves K, Stapleton GA, Collins JS, Schwartz CE, and Stevenson RE: Fragile X syndrome detection in newborns-pilot study. Genet Med 2008, 10:714–719
36. Bailey DB, Jr.: Newborn screening for fragile X syndrome. Ment Retard Dev Disabil Res Rev 2004, 10:3–10
37. Bailey DB, Jr., Skinner D, Warren SF: Newborn screening for developmental disabilities: reframing presumptive benefit. Am J Public Health 2005, 95:1889–1893
38. Bailey DB, Jr., Beskow LM, Davis AM, Skinner D: Changing perspectives on the benefits of newborn screening. Ment Retard Dev Disabil Res Rev 2006, 12:270–279
39. Bailey DB, Jr., Skinner D, Davis AM, Whitmarsh I, Powell C: Ethical, legal, and social concerns about expanded newborn screening: fragile X syndrome as a prototype for emerging issues. Pediatrics 2008, 121:e693–e704