Fragile X full mutations are more similar in siblings than in unrelated patients: further evidence for a familial factor in CGG repeat dynamics

Robert W. Burman, BA, Kim S. Anoe, BS, and Bradley W. Popovich, PhD, FACMG

Purpose: We sought to compare patterns of full mutation repeat-length variability in the peripheral blood DNA of patients with fragile X syndrome to determine whether siblings possess mutation patterns more similar than those of unrelated patients. **Methods:** Mutation patterns were visualized by Southern blot analysis and captured digitally with a phosphor imager. Novel comparison strategies based on overlapping profile plots and calculation of weighted mean CGG repeat values were used to assess mutation pattern similarity. **Results:** Within the population that we analyzed of 56 patients with full mutation, mutation patterns were found to be more similar in siblings than in unrelated patients. **Conclusion:** These results indicate that repeat-length variability may be generated in a nonrandom manner and that familial factors influence this process. *Genetics in Medicine*, 2000;2(4):242-248.

Key Words: fragile X syndrome, CGG, repeat-length variability, mutation pattern, familial clustering

Fragile X syndrome is a common form of X-linked mental retardation caused by expansion of an unstable CGG trinucleotide repeat in the 5' UTR of the *FMR1* gene.¹⁻⁴ In normal populations the length of the repeat tract is polymorphic and contains approximately 5 to 50 triplets. These normal alleles are stable both in the somatic cells of an individual and when transmitted between generations. Premutation alleles with approximately 50 to 220 repeats are found in unaffected carriers and, like normal alleles, are usually stable somatically.⁵ However, premutation alleles have a propensity to change size intergenerationally and, when transmitted by a female, can expand to form full mutations with >220 repeats.^{2,5,6} Full mutation alleles are almost always associated with hypermethylation of the CGG repeat and a surrounding CpG island.^{2,7-9} Hypermethylation initiates and/or stabilizes transcriptional silencing of the *FMR1* gene, and the resulting deficit of FMR1 protein (FMRP) renders individuals at risk to express the fragile X syndrome phenotype.¹⁰⁻¹²

Full mutation alleles can be highly unstable in somatic cells.^{5,13} As a result, most patients with full mutations possess a high degree of repeat-length variability, which is manifest on Southern blot analysis as a complex smeared and polybanded mutation pattern. These mutation patterns are typically well conserved among multiple tissues within individuals¹⁴⁻²² and can be conserved in monozygous twins.^{15,23,24} Furthermore,

analysis of cultured fibroblasts derived from adult patients with full mutation has demonstrated that methylated full mutation alleles are stable in these cells.^{17,25} Together, this evidence suggests that somatic instability is normally limited to a period during embryogenesis, and thereafter, the repeats are maintained with little or no variation in size.¹⁵ Somatic mutation patterns might then represent a snapshot of the mutational process frozen at the point the cells cease to be permissive to further changes in repeat number.

Although the process that generates variation of repeat length in somatic cells is likely to involve multiple consecutive changes in repeat number, the instant at which repeat sizes become fixed could reveal a degree of familial similarity if the process is more determinate than stochastic. To our knowledge, analysis of familial similarity in full mutation sibships has not been reported. However, similar mutation patterns have been observed in at least one brother pair with full mutation and was theorized to indicate that other gene(s) may influence the pattern of expansion.²⁶ Furthermore, examination of sibships containing only offspring with a premutation has demonstrated familial clustering in which premutation size is significantly more similar within families than between families.²⁷ Thus, a so far unidentified familial factor(s) may influence CGG repeat dynamics.

In the present study we assessed mutation pattern similarity in a population of 56 patients with full mutations to test the hypothesis that mutation patterns are more similar within sibships than between unrelated individuals. By examining full mutations, we sought to determine whether a familial factor(s) may influence repeat dynamics in somatic cells. Our data demonstrate that, within the patient population under study, full mutation patterns seem to be more similar within sibships than in unrelated patients.

From the Department of Molecular and Medical Genetics, DNA Diagnostic Laboratory, Oregon Health Sciences University, Portland, Oregon

Bradley W. Popovich, PhD, FACMG, Oregon Health Sciences University, DNA Diagnostic Lab, Marquam Plaza MP350, 2525 SW 3rd Avenue, Portland, OR 97201.

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MATERIALS AND METHODS

DNA isolation and analysis

DNA was extracted from the peripheral blood leukocytes of 56 patients with fragile X who were referred to the Oregon Health Sciences University DNA Diagnostic Laboratory for diagnostic testing. DNA isolation and Southern blot analysis were performed as described.²⁸

Similarity measurements and statistical analysis

Digital representations of Southern blot membranes were captured with a phosphor-imaging system (PhosphorImager SI; Molecular Dynamics, Sunnyvale, CA) scanning at a resolution of 100 pixels/cm. The portion of a typical Southern blot that contained relevant data were usually restricted to a region approximately 12 to 15 cm in length. Thus each lane was composed of 1200 to 1500 rows of data points. Profile plots for each lane in the Southern blot images were generated with ImageQuant software (Molecular Dynamics), and the raw data were exported to Excel version 8.0 (Microsoft, Redmond, WA) for analysis. Because the profile plots are not smooth curves but rather a series of interconnected points, the area under a plot can be estimated simply by summing signal intensity values. For each sample pair used in this study, an Excel spreadsheet was used to calculate total area and area in common, while excluding from analysis that portion of the data that fell below an adjustable baseline. The weighted mean number of CGG repeats in each of the 56 sibling samples also was calculated in Excel as follows: weighted mean lane position = $\sum ((\text{lane position})(\text{signal intensity}))/\sum (\text{signal intensity})$. Signal intensity values were obtained with the baseline set at the level of the system background (normal alleles in samples from females were excluded from analysis). Weighted mean lane positions were converted to CGG repeat units by reference to the molecular weight markers (DNA Analysis Marker System; Gibco BRL, Rockville, MD). All statistical analyses, including analysis of variance (ANOVA) and *t*-tests, were performed with SPSS software version 6.1 (SPSS Inc., Chicago, IL). Significance levels are presented without consideration of multiple comparisons.

RESULTS

To determine whether a familial component influences somatic instability, we studied the genomic DNA of 56 patients with full mutations by Southern blot analysis. The patients were grouped into 16 sibling pairs in 12 families (including 2 families with 3 affected siblings) and 15 pairs of randomly matched unrelated patients. DNA samples derived from peripheral blood were digested with *Pst*I, and each pair was run in adjacent lanes for analysis. *Pst*I was used as the restriction endonuclease, because digestion produces CGG repeat containing fragments that are significantly smaller than those obtained with the more common *Eco*RI/*Eag*I assay and thus yield much better resolution of the peaks and smears that comprise the mutation pattern. Figure 1 shows representative Southern blot

results for seven patients within three sibships that are suggestive of familial mutation pattern similarity.

Numerical descriptions of full mutations are complicated by the often highly heterogeneous mix of allele sizes present within an individual. Consequently, we sought a system in which comparison of mutation patterns could be made directly. One means of representing mutation patterns is the creation of profile plots from phosphor imager-derived Southern blot images (Fig. 2). Using the profile plots, we compared mutation pattern similarity by overlapping two plots and determining the amount of area that the plots shared in common. By measuring similarity as the proportion of the total area that was shared, the data were normalized for gel-to-gel variation in signal strength, allowing integral comparisons to be made. One variable that affects the measurement of area under the profile plots is the selection of the baseline level, below which area is excluded from analysis. When the baseline is set at the level of the system background, the area under the plots is derived entirely from the sample signals, with no contribution from system noise (Fig. 3, panels with "1x baseline level"). To distinguish between specific similarities, such as coincident peaks and less specific similarities that occur near the level of the system background, the baseline was systematically varied by fractional multiples of the system background level (Fig. 3). Using this pattern overlap approach, we obtained similarity measures for each of the full mutation sample pairs at each baseline setting to test the hypothesis that mutation patterns, as measured by shared area, are more similar in the sibling pairs than the unrelated pairs. Comparison of the two groups demonstrated that similarity was not significantly greater in the sibling pairs until the baseline was raised to a level 1.5 times greater than the system background (Table 1). Thereafter, the difference between the sibling and unrelated pairs increased in significance as the level of the baseline was raised.

As an alternative to the profile plot overlap strategy, we used the phosphor imager-derived data to compute the weighted mean number of CGG repeats (wMean-CGG) in each full mutation sample as presented in MATERIAL AND METHODS. In the population of 56 patients with full mutation used in this study, the arithmetic mean of the wMean-CGG values was 575 repeats (range: 318–967 repeats). We used the wMean-CGG values of the sibling samples to test the hypothesis that variation in the wMean-CGG number is greater between families than within families. Nolin and colleagues²⁷ previously used ANOVA to demonstrate highly significant clustering of CGG repeat size in sibships containing only offspring with a premutation. We applied the ANOVA strategy to the 12 full mutation sibships and found that the wMean-CGG repeat sizes were significantly more similar within families than between families ($P = 0.004$). To verify the suitability of the randomly matched unrelated samples as controls in the previous comparison strategy, we applied the ANOVA analysis to the 15 unrelated pairs. As expected, we found no significant difference within and between groups ($P = 0.992$).

Fig. 1 Repeat-length variability in three fragile X families. (A, B, C) Southern blot analysis of genomic DNA samples digested with *Pst*I and hybridized with probe *pfxa3*. Each panel contains DNA samples from a premutation carrier and her full mutation offspring. The patterns of repeat-length variability in the sibships show some degree of similarity. In each of the panels, the samples are flanked by blank lanes and the outermost lanes contain a molecular weight marker.

DISCUSSION

The molecular basis of repeat-length variability in patients with fragile X full mutations is largely unknown. Because the mutational status of the repeat tract in oocytes of premutation females has yet to be described, it is unknown whether the transition from premutation to full mutation occurs prezygotically or postzygotically. Thus, repeat-length variability may be the product of either repeat expansion from an inherited premutation or retraction from an inherited full mutation. Some experimental evidence indicates that full mutations can be tolerated in the oocytes of patients with full mutation²⁹ and that premutation alleles in individuals who possess both a full and premutation are better described as retraction events rather than remnants of an inherited premutation that has failed to expand in a subset of cells.^{20,30} Together, this evidence favors a model in which an inherited full mutation undergoes repeat retraction in the cells of the early embryo to produce variation in the length of the repeat tract.

Most models proposed to explain repeat-length variability are based on DNA replication errors involving polymerase slippage³¹ and the formation of secondary structures, such as hairpins, that stabilize slippage structures by minimizing the energy difference between the duplex and slippage states.^{32,33} When CTG repeats are replicated in *Escherichia coli*, the products of repeat contraction have shown a regularity in size that

implies that defined DNA structures may be involved in the contraction process.³⁴ Some evidence suggests that defined DNA structures may likewise play a role in CGG repeat variability. In particular, a hotspot for deletions¹⁹ has been described in a number of unrelated patients with fragile X syndrome^{19,35–38} and in cloned *FMR1* sequence replicated in *E. coli*³⁹ and *Saccharomyces cerevisiae*.⁴⁰ One explanation for the existence of this hotspot is that the sequence in the vicinity may be involved in the formation of a secondary structure(s) that mediates the deletion process.¹⁹ Because many of the deletions in these patients with fragile X syndrome have unmethylated flanking sequence and occur in mosaic form in conjunction with full mutation alleles, the deletions must be the product of somatic events and may be a consequence of early embryonic repeat contraction extending into flanking sequences before de novo methylation. The identification of similar deletion breakpoints in multiple unrelated patients and other organisms implies that the process may involve conserved DNA structures.

In the current study we applied a strategy of overlapping plots that represent patterns of repeat-length variability to determine whether a familial component may influence the generation of variation in repeat length and whether, by extension, somatic variability may occur in a nonrandom manner, as suggested by the deletion hotspot. One advantage of the profile plot overlap method is that description and comparison of

Fig. 2 Profile plot representations of mutation patterns. (A) Southern blot analysis of genomic DNA samples digested with *Pst*I and hybridized with probe *pfxa3*. Lanes 3 to 6 are unrelated full mutation samples that were randomly paired before gel loading. The four samples are flanked by blank lanes to avoid signal interference from the molecular weight markers loaded in the outermost lanes. (B) Representative profile plots of the samples in lanes 4 and 6. Digestion with *Pst*I and long electrophoresis run times allow resolution of mutation pattern features that might not otherwise be visible. Only two of the four possible profile plots are shown in order to conserve space.

patterns can be made directly, with little manipulation of the underlying data. This somewhat mechanical approach has shortcomings as well, including allowing specific similarities, such as two overlapping peaks, to be obscured by less intense but more numerous overlaps that occur as a consequence of most full mutation alleles falling within a common size range. To overcome this weakness, we employed a comparison strategy that uses an adjustable baseline level. Comparison of the sibling pairs and randomly matched unrelated pairs shows that shared area in both groups decreases as the baseline is raised, but in the unrelated group this decrease occurs at a faster rate than in the sibling group. As a result, the difference in mean similarity between the groups grows larger and eventually reaches marginal significance when the baseline is set to a level 1.5 times greater than the system background. Further increases in the baseline result in an increasingly significant difference between the sibling and unrelated pairs. It should be noted that we used a one-tailed *t*-test to assess the *a priori* hypothesis that similarity is greater in the sibling pairs than in the unrelated pairs. Consequently, the *P*-values we obtained, when considered in conjunction with the relatively small sample size, are of somewhat questionable significance. Nonetheless, the inverse relation between baseline level and *P*-value does indicate a trend in which similarities among the sibling cohort are driven by overlapping peaks, whereas similarities in the unrelated group are the product of low-intensity overlaps that are more readily obscured as the baseline is raised.

In a study addressing familial transmission of the fragile X repeat, Nolin and colleagues²⁷ evaluated familial clustering in sibships containing only offspring with a premutation. In that study ANOVA was used to test for statistical significance and demonstrated that repeat size in the offspring of both male and female carriers was significantly more similar within families than among families.²⁷ The analysis of familial clustering in premutation sibships was facilitated by the somatic stability of premutation alleles that results in an absence of length variation and allows a simple numerical description of repeat size. In an effort to describe full mutations numerically in a systematic manner, we used signal intensity to weight repeat size and generate a weighted mean CGG repeat value. These weighted mean values have the advantage that all of the numerous alleles present in a Southern blot representation of a complex mutation pattern contribute to calculation of the weighted mean. In addition, weighted mean estimates are largely free of observer bias, and the simplicity of the description (e.g., one value per full mutation sample) facilitates statistical analysis. When the wMean-CGG repeat values were used in an ANOVA analysis, we found that significantly less variation occurs within families than between families.

Our results suggest that repeat-length variability is generated in a nonrandom process that may be influenced by familial factors. The identity of these factors and the basis of the apparent similarity are not known. One possibility is that repeat variability "pathways" depend on defined DNA secondary

Fig. 3 Overlap of profile plots to measure pattern similarity. (A) Two overlapping profile plots from a sibling pair with a high degree of pattern similarity (the Southern blot for this pair is shown in Fig. 1, panel A). (B) Two overlapping plots from unrelated patients with full mutation with a low degree of pattern similarity composed of low-intensity overlaps. In each panel solid and dashed lines distinguish separate samples. Females' samples are arbitrarily represented with dashed lines (note the normal allele on the right side of each panel). The area that the plots share in common was used as a measure of similarity and is shaded in gray. The abscissa of each plot corresponds to the baseline, below which area is excluded from analysis. In the panels designated "1× background," the baseline is set to the level of the system background. The panels designated "1.5× background" and "2× background" illustrate the effect on area under the plots when the baseline is set to a level 1.5 and 2 times greater than the system background level, respectively. As the baseline is raised, low intensity overlaps as shown in B fall below the baseline level and are not included in the analysis. In contrast, the coincident peaks in A persist and continue to share area.

Table 1
Full mutation similarity as measured by area in common

Baseline level	Mean similarity (%)		t-Test <i>P</i> (one-tailed)
	Sibling pairs (<i>n</i> = 16)	Unrelated pairs (<i>n</i> = 15)	
1x	30.0	22.4	.09
1.5x	20.3	11.2	.05
2x	15.2	6.2	.04
2.5x	12.3	3.6	.03
3x	10.0	1.5	.02

structures that are in turn determined by the sequence composition of the repeat and adjacent genomic regions. This possibility is supported by the observation of ordered deletions of CTG repeats in *E. coli* and the fragile X deletion hotspot as mentioned previously. Support for a potential role of nonrepeated DNA sequence in triplet repeat stability is provided by a study that showed that intergenerational stability of the Machado-Joseph disease CAG repeat is influenced by a C/G polymorphism adjacent to the repeat tract.⁴¹ Some evidence indicates that familial factors may also influence the inheritance of the Huntington disease (HD) triplet repeat. In a study of juvenile HD, Telenius and colleagues⁴² found a significant correlation between repeat length in sibling pairs. Potential familial influences were further suggested in a study of germ-

line mutation at the HD locus, which identified a father and son that shared an unusual mutation spectrum.⁴³

Although it is not known whether individuals with fragile X full mutations inherit a full or premutation, mutation pattern similarity in siblings is more easily accounted for if both siblings inherit the same or similar allele size. In the case of postzygotic expansion, both siblings might inherit the premutation allele size present in the soma and germline of their mother. Prezygotic expansion is more difficult to reconcile and would require either that the premutation to full mutation transition occurs similarly in two or more independent events in the mother's germline or that the expansion occurs in the primordial germ cells, and as a consequence, the same expansion allele is present in multiple oocytes. In either case it is worth noting that the unrelated patients with full mutations used as controls in the pattern overlap strategy were randomly matched without regard to maternal premutation size. Thus, the pattern overlap comparison we made is one between siblings with identical maternal premutation and nonsiblings with random maternal premutation sizes. Although it might have been instructive to pair the nonsiblings according to maternal premutation size, we were unable to do so in this study because of limited maternal information for most of the isolated unrelated full mutation cases. Our results contradict, to a very limited extent, a report that found size mosaicism (coexistence of full and premutation in an individual) does not have a familial basis.⁴⁴ However, because size mosaicism was addressed as a

binary variable (present or absent), the methodology used was quite different from that of the current study, making comparison of the findings difficult.

In conclusion, our study indicates that patterns of full mutation repeat-length variability are more similar within sibships than in unrelated individuals. This finding suggests that familial factors may influence somatic repeat variation and that careful examination of mutation patterns could provide mechanistically relevant information.

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