Unusual mutations in high functioning fragile X males: apparent instability of expanded unmethylated CGG repeats

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Abstract
We report on further cases of high functioning fragile X males showing decreased expression of FMR1 protein, absence of detectable methylation at the Eagl site in the FMR1 gene promoter, and highly unusual patterns of fragile X mutations defined as smears of expansions extending from premutation to full mutation range. Very diffuse and therefore not easily detectable patterns of full mutations were also observed on prenatal testing using DNA from chorionic villi sampled at a time of development when full mutations were still unmethylated in this particular tissue. In the search for possible determinants of such unusual patterns, repeat expansions in the premutation and in the lower full mutation range were identified on genomic PstI blots previously prepared for fragile X DNA testing. Cases with 130 or more triplets, and a number of shorter repeats, were reinvestigated on EcoRI plus Eagl digests. Among the 119 expansions, there were 22 in our sample showing either blurred bands or smears on PstI blots. This particular characteristic was strongly associated with the coincidence of a repeat size of more than 130 triplets and absence of Eagl site methylation. Our data set also includes cases of mosaic patterns consisting of smears of unmethylated expansions to more than 130 CGGs and of clear bands of methylated expansions. We therefore suggest that in fragile X syndrome unusual smeared patterns of mutations result from somatic instability of larger repeats under circumstantial absence of repeat methylation. (J Med Genet 1998;35:103-111)

Keywords: fragile X syndrome; repeat instability; DNA methylation

Fragile X syndrome is a frequent diagnosis in mental retardation. Inherited in an X linked fashion, fragile X syndrome affects both males and females. Affected males usually show intellectual deficits, specific behavioural characteristics such as hyperactivity and autistic-like behaviour, and physical features such as large, protruding ears, long and narrow face, and macro-orchidism. Nearly all cases of fragile X syndrome result from large expansions of the CGG trinucleotide repeat found in the 5' untranslated region of the FMR1 gene. Large increases of repeat size are called full fragile X mutations and usually coincide with hypermethylation of the expanded repeat and of upstream promoter elements. This results in downregulation of transcription and absence of FMR1 protein (FMRP) in cells normally expressing the FMR1 gene. Most patients with full mutations have somatic mosaicism of the repeat expansion, and some show mosaicism of methylation in that a proportion of cells have an unmethylated promoter allowing for apparently normal transcription of the FMR1 gene despite the repeat expansion. In the latter cases, however, expression of FMRP is markedly reduced in the presence of more than 200 CGGs, probably because of an impediment of the linear migration of the 40S ribosomal subunit along the 5' untranslated mRNA sequence by trinucleotide expansion.

Another mutational class identified in fragile X families is premutations which are smaller repeat expansions to CGG copy numbers between 45 and 200. They are usually not associated with methylation. Premutations are carried by transmitting males and by some of the transmitting females. Carriers of premutations are generally unaffected and show normal expression of the premutated gene both at the RNA and the protein level.

The full mutation may occur at germ cell proliferation or in an early transitional stage of embryogenesis. The substrate is a maternally inherited premutation and the product is usually a mosaic pattern of full mutations detectable in early fetal life. The patterns of methylated full mutations have been shown to be mitotically stable as different somatic tissues of fetuses with full mutation show identical patterns of mutations and the length of expanded repeats is clonally maintained. This high mitotic stability has been assumed to result from hypermethylation. As this hypothesis predicts that unmethylated repeat expansions will be unstable, cases of unmethylated full fragile X mutations are of particular interest.

Recently, transmitting males have been found with normal or borderline IQs, without or with a few characteristics of fragile X syndrome, and with molecular genetic evidence of full mutations. In the majority of these non-retarded or "high functioning fragile X males," full mutation was not
associated with 100% methylation and most of them were mosaic for expansions in the premutation and full mutation range. Based on only a small number of cases observed so far, it has been suggested that full mutation males with less than 10% methylation would produce FMR protein at quantities sufficient to prevent intellectual deficits.15

We report on two further cases of non-retarded males with full mutations who were by segregation analysis classified as normal transmitting males and were the founders of large fragile X families. Both were expected to carry premutations but showed highly unusual patterns of mutations which may not always be recognised by inexperienced investigators. We also show the presence of similar patterns of full mutations in DNA from chorionic villi sampled for prenatal diagnosis at an early stage of development. Analysis of a large number of repeat expansions in the premutation and full mutation range, identified on Southern blots prepared for fragile X DNA testing, led us to suggest that unusual smeared patterns of CGG repeat expansions result from somatic instability of larger repeats under circumstantial absence of methylation.

Case reports
Routine molecular testing for fragile X mutations led to the identification of two particular
Unusual mutations in high functioning fragile X males

Families (A and B) which both included non-retarded full mutation transmitting males. The pedigrees are shown in figs 1 and 2. An unusual pattern of full mutations was also found in the DNA isolated from chorionic villi sampled for prenatal analysis in the 10th week of pregnancy of a full mutation female.

FAMILY A

In this family, fragile X mutations were identified by molecular analysis in four generations (fig 1). The proband (IV.3) was the only affected male in this family. He was diagnosed at the age of 3 years when he presented a typical infantile phenotype of fragile X syndrome (fig 3A). His two sisters (IV.4, IV.5) also had full mutations and were reported to be affected to some degree. They were born to a normal mother (III.5) carrying a premutation. Her two sisters (III.9, III.11) were also identified as carriers of premutations but they had normal children who had received their mother's wild type allele. A cousin of the proband's mother (III.3), who is also a carrier of a premutation, has two affected full mutation daughters (IV.1, IV.2).

Three normal carriers were identified in the first two generations. The proband's great grandmother (I.1) and one of her sons (II.2) carried premutations. Another son (GZ, II.3), however, surprisingly had expansions of the FMR1 CGG repeat in the full mutation range.

The non-retarded full mutation male GZ was seen at the age of 63 years and has a normal appearance (fig 3B, C). He has led a productive life. He attended regular school, completed all classes without repetition, and got a school leaving qualification. Then he successfully served his apprenticeship as a bricklayer. He got married, raised five children, built two houses for his large family, and was gainfully employed throughout his life. He finished his working life in the luggage office of a railway station.

FAMILY B

The pedigree of this family is shown in fig 2. The family was ascertained through two male probands (III.8, IV.6) who both showed the typical phenotype of fragile X syndrome, expressed the fragile site on the X chromosome cytotogenetically in a high percentage of cells, and showed full mutations on molecular genetic testing. In two sisters of proband III.8 (III.7 and III.9), including the mother of proband IV.6, full mutations were found. The normal grandmother of proband IV.6 (II.5) carried a premutation. She had a normal sister (II.3), who was not tested, and a normal brother (KK, II.2). The latter transmitted the trait to his normal daughters (II.4 and III.4). One of his daughters (III.2), who has a normal son, was tested and found to carry a premutation.

Table 1. Repeat sizes (given by CGG index), methylation status (EagI site), and patterns of expansions (PstI blot)

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Note that patterns of fuzzy bands or smears were observed on expansions with 130 or more triplet units but only in the absence of EagI site methylation.

II.2); 10: control male; 13: KK's daughter (III.4) carrying a premutation; 14, 15: KK's affected grandson (IV4) carrying a (methylated) full mutation.

Figure 4 Southern blot analysis of DNA isolated from blood leucocytes (1, 3, 5, 7-15) and cultured fibroblasts (2, 6) of GZ, KK, and family members and controls. DNA was digested with HindIII (1-4, 10-11, 14), EcoRI + Eagl (5-9), or PstI (12, 13, 15) and hybridised to probe Ox0.55. The lanes are as follows: 1, 2, 5, 6: GZ (family A, II.3); 3, 7: fragile X male with methylation mosaicism of full mutations; 4, 8: control female; 9, 11, 12: KK (family B, II.2); 10: control male; 13: KK's daughter (III.4) carrying a premutation; 14, 15: KK's affected grandson (IV4) carrying a (methylated) full mutation.

The other (III.4) was not tested but is a carrier of a premutation since she has an affected son with a full mutation. She also gave birth to a normal son and two daughters who were reported to have some learning problems and to show hyperactive behaviour but have not been tested for fragile X mutations.

The non-retarded male KK, who has two carrier daughters and an affected grandson, unexpectedly presented CGG repeats in the full mutation size range. He was seen at the age of 60 years and was reported undoubtedly to be intelligent. He attended regular school, successfully served his apprenticeship as a skilled worker, got married, raised children, and has been gainfully employed.

Material and methods

CYTOGENETIC ANALYSIS

Cytogenetic analysis was performed on peripheral blood cultured in medium 1A (Gibco). Fragile X expression was induced by adding 0.3 μmol/l FUdR or 10 μg/ml methotrexate 24 hours before harvest.

GENOMIC DNA

Genomic DNA was isolated from white blood cells, purified after isotonic lysis of erythrocytes, from cultured fibroblasts and from freshly prepared chorionic villi using standard procedures. Aliquots (10 μg) were digested with restriction enzymes PstI, HindIII, or EcoRI plus Eagl. The double digest (EcoRI plus Eagl) permitted evaluation of the methylation status of the FMR1 promoter as the status of the single methylation sensitive restriction site of Eagl has been shown to correlate to a great extent with the presence or absence of methylation at numerous other Cpg dinucleotides in this gene region, which includes the CGG repeat, and with the presence or absence of binding of transcription factors to FMR1 promoter sequences.

Restriction fragments were separated by 0.8% agarose gel electrophoresis and transferred to positively charged nylon membranes by alkali blotting. The membranes were hybridised to 32P labelled probes Ox0.55 or Ox1.9, and subsequently washed to a stringency of 0.1 x SSCP. Labelling was performed using a random primer DNA labelling system (Boehringer). Exposure to x ray films was done at ~70°C for three days using intensifying screens.

IMMUNOCYTOCHEMICAL DETECTION OF FMR1 PROTEIN (FMRP)

Immunocytochemical detection of FMRP was done on blood smears and on fibroblasts of the grandfather of family A. Cells from an established culture of skin fibroblasts were allowed to grow for three days on microscopy slides. Fibroblasts from an affected fragile X male with methylated full mutations and from a male with a normal FMR1 gene were used as negative and positive controls and were cultured in separate areas on the same slides. The cells were fixed in 3% paraformaldehyde for 10 minutes followed by 20 minutes permeabilisation in 100% methanol. Immunolabelling was performed with monoclonal antibodies 1C3-1a (Euromedex), directed against an epitope in the N-terminal half of FMRP, following the procedure previously described for blood smears. Antibodies were used in a dilution of 1:2000.

ASSOCIATION STUDY

On PstI blots prepared for routine fragile X DNA testing in our laboratories in a continuous period of two years, all signals of repeat expansions in the premutation and the lower full mutation range were analysed (table 1). Signals from complex mutational patterns consisting of multiple clear bands, associated with somatic mosaicism of full mutations, that is, the typical pattern of fragile X full mutations, were not considered. Only six to more than 300 CGG expansions were included in our test sample of 119 signals of repeat
expansions. Owing to complete selection of all available signals of repeats between 45 and 300, this sample is probably representative of premutations and full mutations in the given size interval. Expansion size was measured as CGG repeat index given by the difference in size (base pairs) of normal and mutant bands, dividing by 3, and adding 30, the most common CGG repeat number of normal alleles in the German population. All measurements were done on Pfbl blots in order to get results which were comparable to each other. A large number of repeats from this sample, including all cases with repeat indices of 130 or larger, was re-investigated on EcoRI plus Eagl digests. We measured the strengths of association (1) of the unusual mutation pattern (blurred band or smear) and the repeat size (CGG index), (2) of the unusual mutation pattern and DNA methylation (determined at the Eagl restriction site), and (3) of the unusual mutation pattern and the combined parameter of repeat size and DNA methylation. The measures of associations were the odds ratios and the differences of incidences. For further details see Khoury et al.

**Results**

The results of fragile X mutation analysis in families A and B are illustrated in figs 1 and 4. In both families, patterns of fragile X mutations were recognised in a number of subjects. The full mutation patterns in the affected males and their female relatives were typical in that they were characterised by multiple distinct bands of large repeat expansions (fig 1) and methylation of the Eagl site in the FMR1 gene promoter (fig 4). In contrast to these clear bands of mutations, the two non-retarded transmitting males showed very unusual patterns of full mutations.

**FAMILY A, GZ**

The proband's grandfather in family A (GZ, II.3) showed a very broad smear of expansions ranging continuously from 48 to 1600 CGGs (figs 1 and 4) in the DNA extracted from white blood cells. Within this smear two particular segments with higher signal intensity were identified; one was between 100 and 160 with a midpoint at 130, the other extended between 200 and 245 around a midpoint at 220 triplets. In contrast to the findings in affected full mutation males, the smear of expansions was still continuous on EcoRI plus Eagl double digests and not divided into separate proportions, one of methylated expansions to fragments larger than 5.2 kb and another consisting of unmethylated mutations on smaller fragments cut at the Eagl sites (fig 4). Since the 5.2 kb band is, however, exceeded by the continuous smear, methylation of a small proportion of expansions is not excluded.

Analysis of cultured skin fibroblasts of GZ gave similar results. As the establishment of a cell culture leads to selection of cells with the highest potency to proliferate in vitro, changes in the mutation patterns are expected compared to DNA analysis of solid tissues of the same subject. The modified pattern of mutations in the fibroblast culture consisted of a number of very diffuse bands embedded in a smear of expansions and did not show any significant difference when analysed on Pfbl and EcoRI plus Eagl digests. All the expansions of the separated diffuse bands were unmethylated (fig 4).

Immunocytochemical analysis of FMR1 protein (FMRP) showed a mosaic pattern of gene expression (fig 5). On microscopic examination of 130 fibroblasts, 33 cells (25%) showed a clear positive FMRP staining at levels corresponding to normal control fibroblasts on the same slide. In 27 cells (21%), the FMRP
staining was also positive but the level was significantly reduced, and in 70 cells (54%) there was complete absence of FMRP staining. Among the fibroblasts of the normal control male, 81 cells (99%) were positively stained whereas one cell was found to be negative. The fibroblasts of the affected male with a methylated full mutation, used as negative control, did not show any staining for FMRP in 115 of 116 cells examined (99%). Smears prepared from blood of GZ showed a similar reduction of FMRP expression in lymphocytes (results not shown).

**FAMILY B, KK**

The mutation pattern of the grandfather of family B (KK, II.2) was characterised by a smear of expansions between 230 and 850 units with an average size of 500 (fig 4). On EcoRI plus EagI double digest (fig 4), a clump of about 99% of expanded fragments was cut at both restriction sites indicating that their EagI sites are not methylated. Only a very faint band was obtained which could represent a signal of methylated expansions of about 1 kb (350-350 CGGs).

**OTHER CASES OF UNUSUAL SMEARS OF FRAGILE X MUTATIONS**

Fragile X mutation patterns very similar to those found in the grandfathers of families A and B were also recognised on other occasions (fig 6). DNA analysis of chorionic villi sampled at a developmental age of 8 weeks resulted in detection of an unmethylated full mutation with a pattern of expansions consisting of numerous diffuse bands and smears (fig 6C). Re-examination of PstI blots, prepared for routine fragile X DNA testing, showed that blurred bands or smears of expansions do not
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infrequently occur in the premutation and the lower full mutation range (table 1). A number of examples are illustrated in fig 6A and B. As also illustrated in this figure there is a striking resemblance to the mutation patterns of myotonic dystrophy patients (fig 6D), which are known to result from mitotic instability.22

An association study was performed on a sample of 119 expansions, analysed on genomic PstI blots (table 1), which includes 22 cases of blurred bands or smears. This characteristic was most frequently seen in the range between 130 and 300 triplets (16/32, 50%) but also occurred in the range of larger expansions. The actual incidence remains unknown, however, owing to incomplete ascertainment of larger expansions in our sample. The characteristic of interest, a blurred band or diffuse smear, was only observed in the absence of Eagl site methylation and only on expansions of at least 130 CGGs. This indicates strong associations of this characteristic with both methylation status and repeat size.

The strengths of association were measured by odds ratios and by the differences in incidences of the characteristic associated with various repeat sizes, with particular states of methylation, or with both factors. The odds ratios, separately calculated for repeat sizes larger than 130 and for unmethylated expansions, are indefinitely large indicating that both factors could be necessary for the outcome of the characteristic. Within the representative part of our sample, the incidence of blurred bands or smears increased with increasing repeat size and reached a value of 0.641 for expansions of at least 130 CGG triplets. In the absence of methylation, the incidence of the characteristic among expansions larger than 130 was 1.0, but was close to zero in the presence of methylation.

Discussion

High functioning fragile X males were originally defined as non-retarded males who yielded standardised IQ scores of 70 or higher, thus representing an intermediate state between the affected and unaffected males.27 There have been several additional reports on non-retarded fragile X males who obviously belong to this particular subgroup.34-32 Not all of these probands actually had IQ tests but were believed to function at sufficiently high levels as they had different grades of school or college leaving qualifications and were all gainfully employed.

A proportion of high functioning males showed some physical manifestations of fragile X syndrome, including slight facial dysmorphism and enlarged testes. On cytogenetic testing for fragile X expression some had positive results in 4-13% of cells,27 29 31 whereas others did not express the fragile X site.4 30 The most striking features, common to all high functioning males, are the patterns of mutations. These are highly unusual in fragile X syndrome and emphasise the intermediate state between the affected male with methylated full mutation and the normal transmitting male with unmethylated premutation. On Southern blot analysis, all high functioning fragile X males presented nearly continuous and diffuse smears of repeat expansions between 100 and 1500 triplets, that is, in both the premutation and the full mutation range, and, additionally, showed a substantial lack of methylation despite repeat expansion to full mutation sizes.

In this report we describe two non-retarded male probands who both showed the unusual mutation patterns classifying them as high functioning fragile X males. Before molecular analysis, both were considered to be normal transmitting males as they were believed to be intellectually normal by family members and by physicians as well. They attended regular schools, had school leaving qualifications, successfully served apprenticeships as skilled workers, were gainfully employed, married, raised children, and supported large families.

In the affected full mutation fragile X males, retardation is generally assumed to result from absence of FMR1 protein owing to silencing of gene transcription in the presence of methylation. We have recently shown that transcription factors are unable to interact with upstream promoter elements and do not initiate transcription when these sequences are methylated.7 Consequently, the relatively normal intellectual levels of high functioning males can be attributed to lack of methylation allowing for apparently normal transcriptional activity of the FMR1 gene independent of large repeat expansion.33 Despite normal mRNA levels, however, reduced quantities of FMR1 protein were found in EBV transformed lymphoblasts with unmethylated full mutations suggesting translational inhibition by CGG repeats expanded to more than 200 copies.34

In one of our probands immunocytochemistry for FMR1 protein (FMRP) was done. There was a significant overall reduction of this protein since only about 25% of the cells showed normal FMRP staining while more than 50% of the cells stained negative and 22% showed only very low levels of FMRP. These findings are consistent with the molecular finding of a broad range of unmethylated repeat expansions and clearly support previous results suggesting that premutations do not significantly reduce FMRP production, but expansions to larger repeat sizes do inhibit translation of gene transcripts.35 Similar reductions of FMRP have been reported in five other high functioning fragile X males.36 Measurements of FMRP on western blot analysis showed residual levels of 35% and 12%,37 38 and suggested that such low levels might account for some minor deficits in performance and for a partial physical phenotype of fragile X syndrome in this particular subgroup of fragile X males.

The main characteristic of the unusual mutation patterns of high functioning fragile X males, that is, smears of expansions rather than clear bands, is not specific to these subjects but is also seen in other cases. Blurred large and small smears of expansions have been found on Southern blot analysis of DNA isolated from...
chorionic villi of full mutation fragile X fetuses. Examples were reported in this study and shown to be associated with lack of EagI site methylation on the expanded fragments at these early stages of development. Blurred bands and smears of expansions are not infrequently recognised on routine DNA testing. In the size range between 130 and 300 triplets, the incidence of this characteristic was found to be as high as 0.64 and to increase with increasing expansion size in the interval between 80 and 130 CGGs. The strongest association was measured with absence of methylation. Blurred bands or smears were not seen in combination with methylated expansions. Therefore, absence of methylation could be necessary and could, in combination with repeat sizes larger than 130 triplets, represent a sufficient cause of this particular pattern of fragile X repeat expansion. As shown only recently by genomic sequencing, the methylation status of the EagI restriction site correlates to a great extent with the presence or absence of methylation at numerous other CpG dinucleotides in this gene region including the CGG repeat.

Further evidence for the association of blurred bands and smears of expansions with repeat size and methylation comes from its biological plausibility. This characteristic is very frequently seen in patients with myotonic dystrophy where the basic mutation is expansion of a CTG trinucleotide repeat situated in the 3′ untranslated region of the DMPK gene. As the repetitive sequence of this trinucleotide does not contain CpG dinucleotides, it will most probably remain unmethylated and represent another example of absence of methylation on expanded repeats recognised as blurred bands or smears on Southern blot analysis. Extreme manifestations of this characteristic may be found in patients of older ages (fig 6). We and others have recently shown that this feature in myotonic dystrophy patients reflects mitotic instability associated with a further increase of repeat size, heterogeneity of expansions between tissues, and large increases of the size variation of expansions among different cells, so that expansions appear as smears on Southern blot analysis of DNA from uncloned cells or solid tissues.

Our data suggest that the instability of CGG repeats is significantly increased beyond a threshold of repeat size and that this increased instability results in large changes of repeat size detectable on genomic PstI blots. In view of the scope of our study, another finding is, however, more important. The instability of CGG repeats larger than about 130 triplets is restricted to unmethylated sequences.

We have recently proposed that absence of methylation from expanded trinucleotide repeat sequences leads to mitotic instability and could be caused by misdirection of postreplicative strand specific DNA mismatch repair. With increasing size, these repetitive sequences become more and more prone to slipped strand misalignment at DNA replication. The stability of such sequences, therefore, requires effective repair by the DNA mismatch repair system. Replication results in an intermedi-
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