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ORIGINAL INVESTIGATION

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A fragile X case with an amplification/deletion mosaic pattern

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Abstract Fragile X syndrome is the most common cause of hereditary mental retardation. The FMR1 gene, which is involved in fragile X syndrome, contains a polymorphic CGG repeat, which expands in affected patients. Expanding triplet repeats have been shown to be a new type of mutation, termed "dynamic mutation", responsible for more than 12 genetic diseases. These mutations occur as multiple steps rather than as a single event. The first step leads to an unstable allele that then becomes increasingly unstable generally achieving further increases in copy or occasionally contraction. In this report, we describe a fragile X boy with both a hypermethylated full mutation and a deletion of 905 bp encompassing the CGG repeat. The upstream breakpoint is 438 bp 5' to the CGG repeat and the downstream breakpoint is 420 bp 3' of the triplet repeats. The deletion includes the ATG starting codon for translation of the FMR1 gene. This was confirmed by using FMRP immunocytochemistry both on blood smears and hair roots. The deleted region is flanked by a ccgg direct repeat next to the breakpoints; this may have had a critical role in the formation of a secondary DNA structure leading to the deletion.

Introduction

Fragile X syndrome is the most common cause of hereditary mental retardation, with an incidence of 1 in 4000 males (Turner et al. 1996). The phenotype is characterized by mental retardation, facial dysmorphism, and postpuberal macroorchidism (Hagerman and Cronister 1996). The FMR1 gene, which is involved in fragile X syndrome, contains a polymorphic CGG repeat within the first exon. In normal subjects, the number of CGG repeats is on average 30. Non-penetrant carriers of fragile X syndrome have between 50 and 200 repeats. The length of the repeat section correlates with the repeat instability in this syndrome. In patients with fragile X syndrome, the number of repeats is more than 200. This is known as the full mutation and is generally associated with the methylation of a CpG island upstream of the FMR1 gene and down-regulation of the transcription of the gene resulting in the absence of the FMR1 protein.

The variation in the degree of clinical impairment is known to be related to the variation in the characteristics of the mutation responsible for the fragile X syndrome. Approximately 40% of affected patients are mosaics: methylated/non-methylated full mutation, full/normal alleles, full/premutated alleles, full/deleted alleles (Nolin et al. 1994). In this report, we describe a fragile X boy with both a hypermethylated full mutation and a deletion of 905 bp flanked by a short direct repeat.

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Subject and methods

Subject

The index subject is an 11-year-old male, who was seen at the Genetic Clinic at the age of 8; he was referred for diagnostic evaluation, because he was severely mentally retarded and had a specific physical and behavioral phenotype. Our study of this patient was performed in accordance with ethical standards after his family gave their informed consent.

Southern blot analysis

Genomic DNA was isolated from blood leukocytes according to standard procedures: 10 μg genomic DNA was digested with *Eco*RI and *Eag*I and separated by electrophoresis in a 0.8% agarose gel. The gel was blotted onto a Hybond+ membrane, and the filters were hybridized with probe pP2 overnight at 65°C in SSPE buffer (360 mM NaCl, 20 mM Na_2HPO_4, 2 mM EDTA). Membranes were washed to a final stringency of 0.1% SDS, 0.1 × SSPE and exposed for 4 days to X omat biomax films (Kodak).

Polymerase chain reaction analysis and sequencing

Radioactive polymerase chain reaction (PCR) analysis of the CGG repeat numbers was carried out by using primers c and f as described by Fu et al. (1991). The PCR products were run on a 6% denaturing acrylamide/bisacrylamide gel. In order to determine the deleted sequence, we employed different pairs of primers encompassing the CGG sequence and genomic DNA from controls and the patient. The index sample and control sample were amplified with pfu polymerase and visualized in 2% agarose with ethidium bromide. The deleted PCR products were purified by using the Concert Rapid PCR Purification System (Life Technologies). The PCR products, after the sequencing reaction, were run on a 6% denaturing gel on an ABI 100 automated DNA sequencer, together with the controls.

Immunocytochemistry

The presence of the FMRP protein both in hair roots and in blood smears was evaluated by using the rapid antibody test as described (Willemsen et al. 1995, 1999; for detailed information see: www.eur.nl/fgg/ch1/fragx). In total, 100 lymphocytes were scored for FMRP-positive labeling; labeled cells were expressed as the percentage of the total number of examined lymphocytes. Immunolabeled hair roots were examined with a stereo zoom microscope at a final magnification of 70× and scored for the presence or absence of FMRP. The number of FMRP-positive hair roots showing a red color was expressed as the percentage of the total number of examined hair roots.

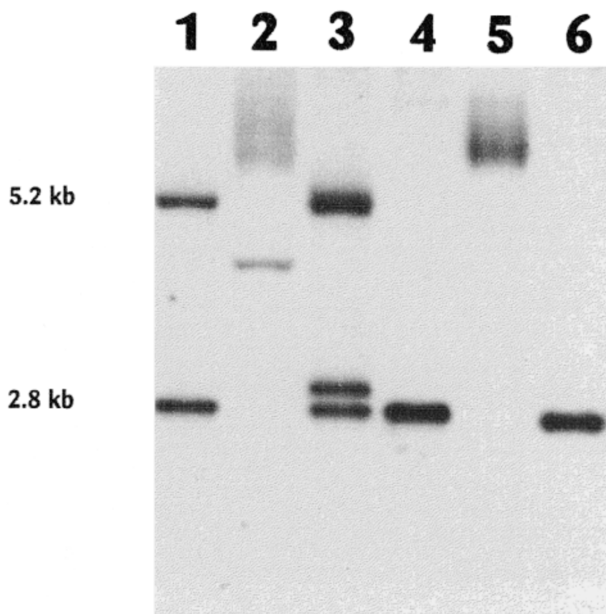

Fig. 1 Southern blot analysis of the index case (*lane 2*), his mother (*lane 3*), and his father (*lane 4*) with probe pP2. Eight micrograms of genomic DNA, isolated from peripheral blood samples were digested with *Eco*RI and *Eag*I (*lane 1* normal female control, *lane 5* full mutated male control, *lane 6* normal male control)

Results

The index case has a typical facial appearance, including long face and long prominent ears (*P*>97), hyperextensible fingers, flat feet, scoliosis, and macroorchidism (*P*>97). His weight, height, and head circumference are around the fiftieth percentile. He is the only child of healthy normal parents. At his birth, his mother was 33 years old. The pregnancy was normal, and vaginal delivery took place at the 38th week with an umbilical cord double circular, which caused cyanosis. Medical problems were noticed during the first year of life: he sat up when he was 11 months and started walking without support at the age of 20 months. When he was 3 years old, several behavioral features were noticed: avoidance of eye contact, hand flapping and other preservative movements, and irritability and aggression. Psychological evaluations were made every two years, and these showed hyperactivity, attention deficit, tactile defensiveness, and several problems in language management.

Southern blot analysis with probe pP2 showed a mosaicism consisting of a full mutation and a deletion in the proband. The mother is not a carrier of the deletion (Fig. 1, lanes 2, 3) but is a carrier of a premutation. The same pattern was observed when the proband's genomic DNA was digested with *Eco*RI (data not shown). The full mutation is illustrated by a smear with a length of approximately 6.0 kb (300–350 CGG) and is fully methylated. The deletion is approximately 900 bp smaller than the normal 5.2-kb methylated allele). The unmethylated premutation of his mother is about 100 CGGs in length (Fig. 1, lane 3). PCR with primers c and f showed no product with genomic DNA of the proband (data not shown).

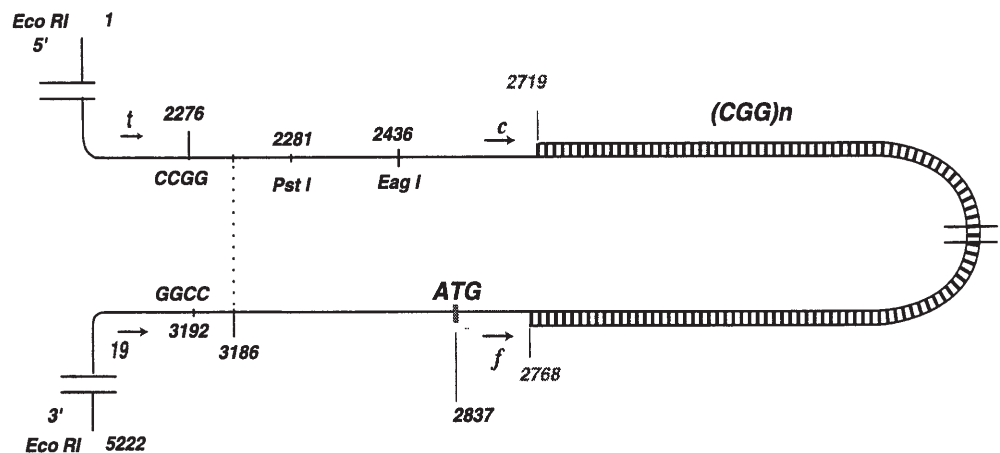

Fig. 2 Schematic model of the 5.2-kb *Eco*RI fragment pE5.1 forming a secondary structure leading to the deletion junction observed in the index case. *Horizontal arrows* Location of the primers used for amplification and sequencing (not on scale), *ATG* start codon for translation, *CCGG*, *GGCC* direct repeats encompassing the cleavage site indicated by the *dotted line* at positions 2281–3186

To study the location of the deletion, the region surrounding the repeat was amplified by primers t and 19 (Fig. 2); a PCR product of 300 bp, approximately 900 bp shorter than the expected 1250 bp was observed (data not shown). The exact breakpoint of the deletion was determined by sequencing the PCR product obtained by amplification with primers t and 19. The deletion of 905 bp, from positions 2281–3186 of pE5.1 (Fu et al. 1991), was found to encompass the CGG repeat, in addition to 438 bp 5' to the CGG repeat and 420 bp downstream of the triplet repeats. This deletion includes the ATG starting codon for translation of the FMR1 gene.

FMRP detection both in blood and in hair roots was negative. Indeed, the patient was found to express 0% of protein compared with normal controls. This confirmed that neither the fully mutated allele nor the deleted allele were able to be transcribed or translated efficiently into FMR1 protein. His mother had a normal FMRP expression of 90% in blood and 80% of her hair roots as might be expected from a carrier of a premutation.

Discussion

Repeats of nucleotides occur throughout the DNA of the human genome. The triplet repeat has been found in almost every animal species. Some of these triple-repeated DNA sequences are unstable and are amplified to a higher than normal number of copies. In the past eight years, these expanding triplet repeats have been shown to be a new type of mutation, termed a "dynamic mutation", responsible for more than 12 genetic diseases, including fragile X syndrome. Dynamic mutations occur in multiple steps rather than as a single event. The first step leads to an unstable allele, which then becomes increasingly unstable, generally achieving further increases in copy number or occasionally contraction.

Adult fragile X males who exhibit the fully expanded FMR1 repeat in all tested somatic cells show only premutation length repeats in their sperm (Reyniers et al. 1993), although the analysis of intact gonadal tissue has demonstrated that germline cells of full mutation fetuses of either sex show evidence of full mutations. Thus, the presence of premutation sperm in full mutation males reflects a selective advantage for spermatogonia that have undergone mitotic contraction of the length repeat (Malter et al. 1997). The somatic variation of the CGG repeat length, which is observed in cells of patients with a full mutation, is a result of mitotic instability of the allele, as only a single FMR1 allele is passed on via the oocyte.

The existence of deletion mosaics indicates that the instability of the repeat is not restricted to the CGG repeat itself but can also extend to the flanking sequences. The reduction of the CGG repeat is a more frequent event than expected, as the regression of a full mutation may give rise to mosaics: full mutation/premutation, full mutation/normal allele, full mutation/deletion. Nolin et al. (1994) have reported that 41% of fragile X males are mosaic for a premutation and a full mutation, based on Southern blot analysis. Results from FMRP immunocytochemistry on blood smears, which is a more sensitive method, suggests that a higher percentage are mosaic to a small degree (Willemsen et al. 1997; Tassone et al. 1999).

Several studies of the mechanism of deletions have shown that approximately 40% of the large deletions in human disorders are flanked by very short direct repeats of 2–6 bp. These short repeats slip and mispair during replication, resulting in the formation of a loop. Subsequent excision of this loop removes the sequences between the repeats, resulting in a deletion. De Graaff et al. (1995) have identified a hotspot for deletions approximately 70 bp upstream of the triplet repeat region of the FMR1 gene, although, after analyzing more than 22 published deletion breakpoints, we find that most of them occur in different regions upstream of the mentioned hotspot (85 bp to 100 kb at 5' of the CGG repeat sequence).

In the present case, the deletion of 905 bp, from positions 2281–3186 of pE5.1 (Fu et al. 1991), encompasses the CGG repeat; the upstream breakpoint is 438 bp 5' to the CGG repeat and the downstream breakpoint is 420 bp 3' of the triplet repeats. The deletion includes the ATG starting codon for translation of the FMR1 gene. This was confirmed by using FMRP immunocytochemistry both on blood smears and in hair roots. The deleted region is flanked by a ccgg direct repeat (Fig. 2) next to the breakpoints; this may have had a critical role in the formation of a secondary DNA structure leading to the deletion.

The molecular mechanisms responsible for the genetic instability observed in fragile X syndrome involve both the loss of AGG trinucleotides interrupting the CGG repeats and structural properties associated with simple-repeat tracts. However, at the present time, no information is available about which events trigger the reversion of the mutation in somatic and germ cells.

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References

Fu YH, Kuhl DPA, Pizzutti A, Pieretti M, Sutcliffe JS, Richards S, Verkerk AJMH, Holden JJA, Fenwick RG, Warren ST, Oostra BA, Nelson DL, Caskey CT (1991) Fragile X site: a polymorphic and highly unstable CGG repeat in the FMR1 gene. Cell 67:1047–1058

Graaff E de, Rouillard P, Willems PJ, Smits APT, Rousseau F, Oostra BA (1995) Hotspot for deletions in the CGG repeat region of FMR1 in fragile X patients. Hum Mol Genet 4:45–49

Hagerman RJ, Cronister A (1996) Fragile X syndrome: diagnosis, treatment and research, 2nd edn. Johns Hopkins University Press, Baltimore London, pp 1–68

Hammond Lyn S, Macias MM, Tarleton JC, Pai GS (1997) Fragile X syndrome and deletions in FMR1: new case and review of the literature. Am J Med Genet 72:430–434

Malter HE, Iber JC, Willemsen R, Graaff E de, Tarleton JC, Leistri J, Warren ST, Oostra BA (1997) Characterization of the full fragile X syndrome mutation in fetal gametes. Nat Genet 15:165–169

Nolin SL, Glicksman A, Houck GE Jr, Brown TW, Dobkin CS (1994) Mosaicism in fragile X affected males. Am J Med Genet 51:509–512

Reyniers E, De Boulle K, Van Roy B, Van Velzen D, Graaff E de, Verkerk AJMH, Jorens HZ, Darby JK, Oostra BA, Willems PJ (1993) The full mutation in the FMR1 gene of male fragile X patients is absent in their sperm. Nat Genet 4:143–146

Tassone F, Hagerman RJ, Ikle DN, Dyer PN, Lampe M, Willemsen R, Oostra BA, Taylor AK (1999) FMRP expression as a potential prognostic indicator in fragile X syndrome. Am J Med Genet 84:250–261

Turner G, Webb T, Wake S, Robinson H (1996 The prevalence of the fragile X syndrome. Am J Med Genet 64:196–197

Willemsen R, Mohkamsing S, De Vries B, Devys D, Van den Ouweland A, Mandel JL, Galjaard H, Oostra B (1995) Rapid antibody test for fragile X syndrome. Lancet 345:1147–1148

Willemsen R, Smits A, Mohkamsing S, Beerendonk H van, Haan A de, Vries B de, Ouweland A van der, Sistermans E, Galjaard H, Oostra BA (1997) Rapid antibody test for diagnosing fragile X syndrome: a validation of the technique. Hum Genet 99:308–311

Willemsen R, Anar B, Diego Y de, Vries BBA de, Hilhorst-Hofstee Y, Smits A, Van Looveren E, Willems PJ, Galjaard H, Oostra BA (1999) Hair root test for fragile X syndrome. Am J Hum Genet 65:98–101