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FRAXE mutation in a mentally retarded subject and in his phenotypically normal twin brother

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The FRAXE fragile site, 600 kb distal to the more common FRAXA, has been reported to be expressed in subjects with mild non-syndromal mental retardation (MR). Amplification of more than 200 GCC repeats, associated with methylation of the adjacent CpG island at Xq28, leads to the expression of the fragile site. In 1996 a large gene, *FMR2*, transcribed distally from the CpG island and downregulated by repeat expansion and methylation, was identified. Among 232 mentally retarded patients, tested FRAXA negative, we identified an Italian family segregating a hypermethylated expansion at the FRAXE locus in two dizygotic twin brothers, their sister and their mother. The index case was referred at 23 years of age with severe MR, epilepsy, a dysmorphic face with a high arched palate, marfanoid habitus and hyperreflexia of the lower limbs. His brother was referred to as normal and psychometric tests confirmed he is not mentally retarded. All members of the family underwent FRAXE molecular analysis, after cytogenetic expression of the fraX site and negative FRAXA test. Interestingly, an expansion and a hypermethylation at the FRAXE locus were found in all of them. Fibroblasts from the clinically normal brother were assayed for *FMR2* expression and the transcription of the gene was found to be silenced. The presence of a phenotypically normal male with absent *FMR2* expression in fibroblasts suggests that the relationship between the FRAXE mutation, *FMR2* expression and MR needs to be further investigated. *European Journal of Human Genetics* (2000) 8, 157–162.

Keywords: FRAXE; mental retardation; genotype/phenotype correlation; genetic counselling

Introduction

Approximately 3% of the population suffers from mental impairment; 25–50% of this is X-linked.[1]

FRAXE is a folate sensitive fragile site in Xq28, 600 Kb distal to the FRAXA.[2] Molecular characterization revealed that individuals expressing FRAXE had amplification of a GCC repeat adjacent to a CpG island. Normal subjects show 6–25 copies of the repeat, while individuals expressing the fragile site have > 200 copies and their CpG island is fully methylated.[3]

Several families have been described, in whom expression of the FRAXE fragile site and mental retardation (MR) segregate.[4–7]

Recently, a gene associated to FRAXE and designed as *FMR2* has been cloned and shown to be expressed in brain and placenta.[8,9] Loss of *FMR2* expression seems to correlate with $(GCC)_n$ expansion at FRAXE, suggesting the involvement of this gene in FRAXE-associated mild MR.[8–10] However, a few phenotypically normal males have been reported with complete methylation at the FRAXE locus and no *FMR2* expression.[6–8]

Materials and methods
Molecular analyses
The FRAXE locus was studied with conventional Southern blotting of genomic DNA digested with the restriction enzymes *Hin*dIII + *Not*I and hybridised with the probe OxE.20, as described by Knight *et al.*[3] *Bam*HI digestion was performed to confirm the presence of the mutation and to

exclude the possibility of a rare, previously reported, HindIII polymorphism.[11]

Expression studies by standard RT-PCR were performed on RNA extracted from cultured fibroblast cells using *FMR2* specific primer pairs 10–37 and 38–7, as described by Gecz.[10] As control, connexin26 primers F5 and R8[12] were used.

Neuropsychological tests

The phenotypically normal brother was examined by neuropsychological tests to evaluate his general intellectual ability, immediate and short-term memory, visuo-spatial memory, language functions, gnostic and prassic functions.

General intellectual ability was assessed using the Wechsler–Bellevue Adult Intelligence Scale (WB-1).[13] The memory functions were evaluated using the Wechsler Memory Scale (WMS).[14] The WMS includes numeric spans, Babcock tales, memory for designs, associations. The visuo-spatial functions were assessed using a visuo-spatial memory test, the Benton Visual Retention Test (VRT).[15] The language functions were assessed using the Aachen Aphasie Test (AAT).[16] The test includes items for repetition, denomination of objects,

comprehension and spontaneous language. The prassic function was assessed from copying 10 designs. The gnostic function was assessed using the Judgement of Line Orientation (JLO)[17] and Facial Recognition (FR).[18]

Results

The 23-year-old propositus was referred with severe mental retardation (MR) and epilepsy. The parents were unrelated. He had a phenotypically normal dizygotic twin brother and a sister. He had a long face with a high forehead and a prominent jaw, a mild degree of hypertelorism and strabismus, low set ears, hypoplastic alae nasi, a prominent lower lip, a high arched palate and crowded teeth (Figure 1A). His height was 192 cm and weight 45 kg. He had narrow sloping shoulders, long slender hands and feet with long fingers. Testicular size was normal. On neurological examination, he presented with hyperreflexia of the lower limbs. No instrumental investigation could be performed and IQ could not be evaluated. Differential diagnosis included Lujan-Fryns syndrome and fragile X syndrome.

Figure 1 Picture of the proband at the age of 36, **A** and of the phenotypically normal twin brother at the age of 38, **B**.

Cytogenetic analysis revealed a fragile site at Xq27–28 in 18% of metaphases of the propositus. Similar cytogenetic results were obtained in his phenotypically normal brother, his sister and his mother (6%, 14% and 4%, respectively). No expansion was detected in the *FMR1* gene (data not shown), thus excluding fragile X syndrome as the cause of mental retardation.

Molecular analysis at the FRAXE locus was therefore performed in the propositus, his siblings and his mother. The results are shown in Figure 2. A smear corresponding to a $\Delta > 400$ bp demonstrated expansion and methylation in the peripheral blood of the proband (lane 4). The hybridisation pattern in the n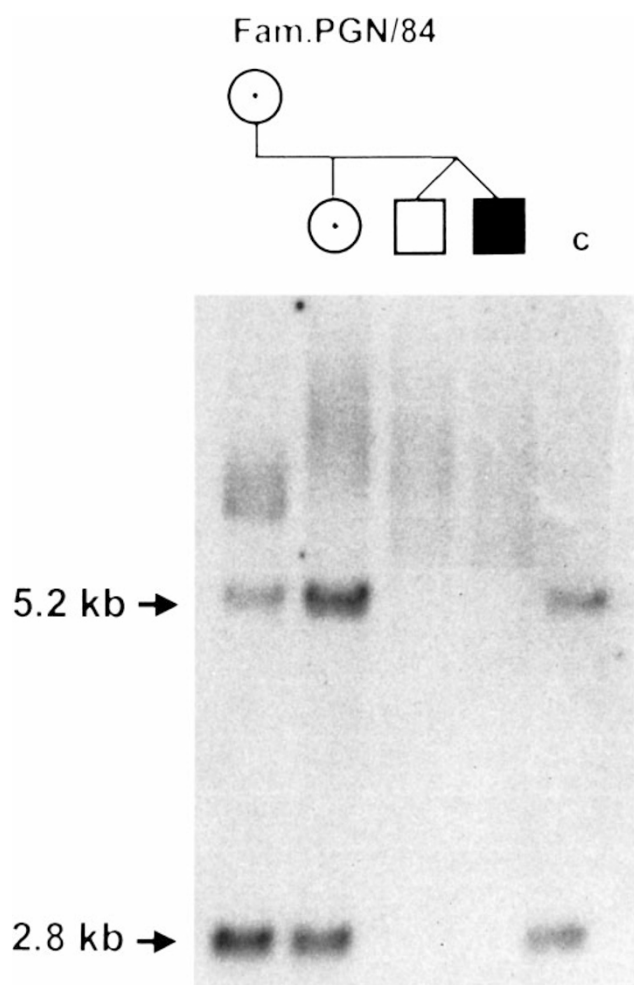ormal brother, sister and mother indicated a fully methylated genotype, corresponding to a $\Delta > 400$ bp, $\Delta > 1000$ bp and $\Delta > 800$ bp respectively (lanes 3, 2, 1). The previously identified *Hin*dIII polymorphism at position 14396–97[11] was excluded by Southern blot analysis (data not shown).

A further evaluation of the brother (Figure 1B), who is married, has a high school certificate and works as an employee in a school, revealed that none of the clinical features of the proband was present and all the neuropsychological tests performed resulted in the normal range (Table 1).

In order to investigate the possibility of a tissutal mosaicism in the normal brother, Southern blot analysis was performed on DNA extracted from fibroblasts and sperm. In fibroblasts a methylated expansion was found ($\Delta > 1500$ bp), whereas in sperm the expansion is lower ($\Delta = 200$ bp) and unmethylated (Figure 3).

Cultured skin fibroblast cells from the phenotypically normal brother were analysed by standard RT-PCR (Figure 4). Control fibroblasts expressed a high level of *FMR2*, while the methylated gene of the normal brother showed no expression, as expected since the strong correlation between full methylation and silencing of the transcription of the *FMR2* gene in all individuals previously tested.[10]

After genetic counselling, the normal brother and his wife chose to undergo cytogenetic prenatal diagnosis because of advanced maternal age and no FRAXE molecular analysis was performed. Cytogenetic analysis on amniotic fluid revealed a normal female karyotype. At birth FRAXE molecular analysis on cord blood and skin of the daughter revealed a non-methylated expansion ($\Delta = 200$ bp) of the same size as the father's sperm (Figure 5).

Figure 2 Southern hybridisation of probe OxE.20 to HindIII + NotI digested DNAs from family members is reported below the pedigree. Lane 5: control female, in which the 2.8 kb and the 5.2 kb bands represent the normal active and inactive allele, respectively. The smears in the other lanes represent the characteristic pattern due to the somatic mosaicism and expansion of the mutated allele in carrier females (lanes 1 and 2) and in FRAXE males (lanes 3 and 4). Open circles and square represent normal clinical phenotype.

Table 1 Results of the neuropsychological tests performed on the phenotypically normal brother

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Overall intelligence (WB-1)	
– Verbal I.Q.	128
– Performance I.Q.	124
– Total I.Q.	128
Memory	
– Wechsler Memory Scale (WMS)	80
– Benton Visual Retention Test (VRT) (no. of errors <16)	6
Aachen aphasie test (AAT)	
– Spontaneous language	No deficit
– Repetition	No deficit
– Denomination of objects	No deficit
– Comprehension	No deficit
– Script language	No deficit
Prassic function	
– Copy of design (n.r. >10)	13
Gnostic function	
– Judgement of line orientation (JLO) (n.r. 25–30)	28
– Facial recognition (FR) (n.r. 41–54)	50
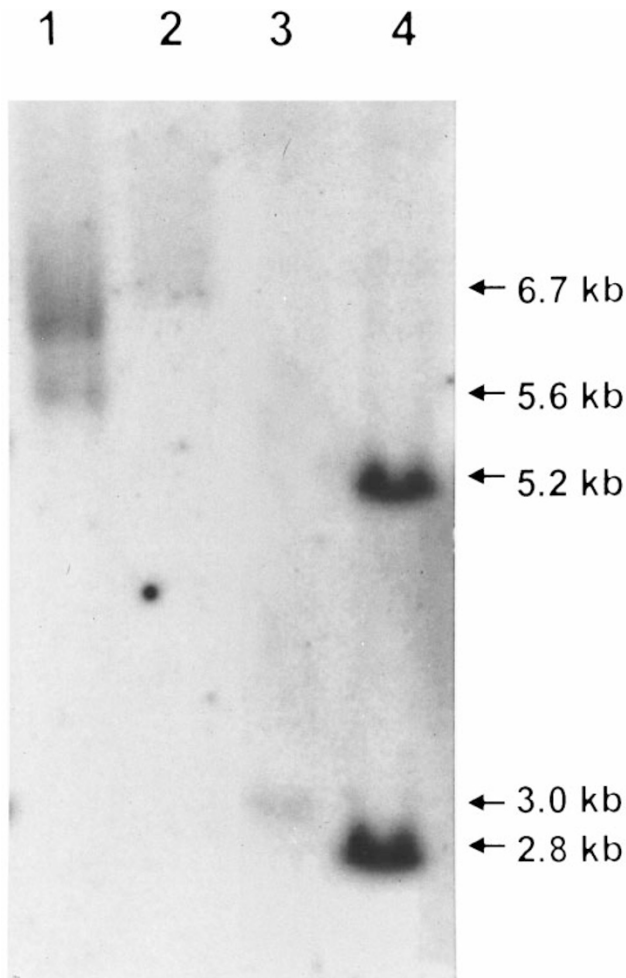
Figure 3 Southern blot analysis on DNAs extracted from whole blood (lane 1), skin fibroblasts (lane 2) and sperm (lane 3) of the phenotypically normal twin brother. Lane 4: control female. In lane 3, a larger and unmethylated allele was detected.

Discussion

In the first family reported with FRAXE[2] the fragile site did not clearly segregate with mental retardation (MR). Further studies on additional families co-segregating MR and the FRAXE fragile site suggested that an etiological relationship may exist between FRAXE and mild non-specific X-linked MR.[4–7] The relationship was difficult to establish in the absence of a phenotype other than mild (or borderline) non-specific MR.

Screening programs in different candidate populations of mentally impaired boys detected no[19–21] or only a few cases of FRAXE MR.[22–25] Based on these studies, the prevalence of FRAXE was estimated to be approximately 1:50000 males.[26]

Here we describe a family segregating a hypermethylated expansion at the FRAXE locus in two dizygotic twin brothers, their sister and their mother. Despite molecular identity, compatible with inactivation of the *FMR2* gene and no
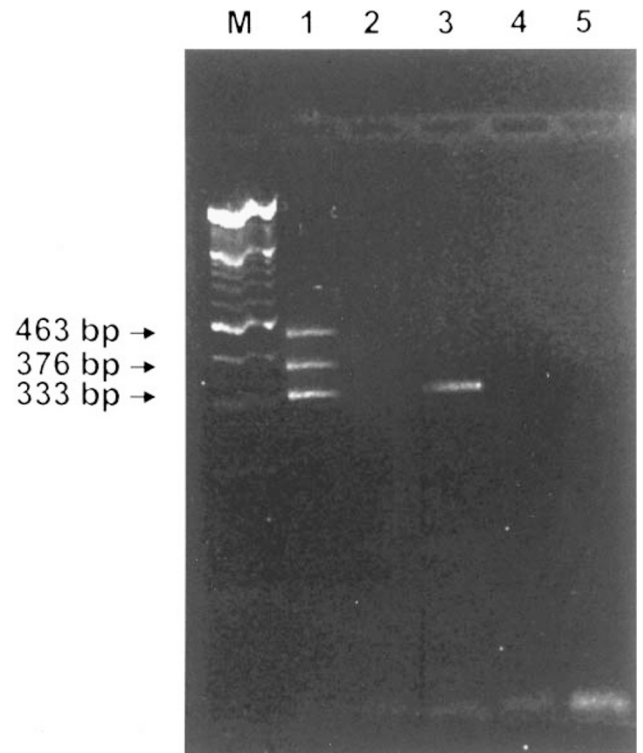
Figure 4 RT-PCR analysis on skin fibroblasts from the phenotypically normal twin brother (lanes 3 and 4) and from a control male (lanes 1 and 2); lanes 1 and 3 are with the presence of reverse transcriptase in the cDNA synthesis reaction (RT⁺), lanes 2 and 4 are the RT⁻ reactions. In lane 5 is the negative control. Marker is the 100 bp ladder. Amplification products of 463 and 376 bp are obtained from the alternative splicing of FMR2, exon 5. As control, connexin26 primers F5 and R8 were used (amplicon 333 bp).

protein production, the two brothers present a very different phenotype: one suffers from severe MR, whereas the phenotype and the intellectual performance of the other appear completely normal.

The hypothesis of a somatic mosaicism of the molecular defect was tested, since specific damage or sparing of a target tissue (especially neuronal) might explain the extreme phenotypic variability observed. The expansion and methylation status were investigated on DNA extracted from fibroblasts and sperm of the clinically normal brother; in fibroblasts a methylathed expansion was revealed ($\Delta > 1500$ bp), whereas an unmethylated fragment of large size ($\Delta = 200$ bp) was observed in sperm. Moreover, expression studies on cultured fibroblast cells from the phenotypically normal brother are consistent with complete inactivation of the FMR2 gene. Other two clinically normal individuals were reported to have the FRAXE CpG island 100% methylated and, as a consequence, a transcriptionally silent *FRM2* gene.[6–8,10] Similar cytogenetically positive, fully methylated but unaffected males have been described by Knight[4] and Murgia;[27] although *FMR2* expression was not
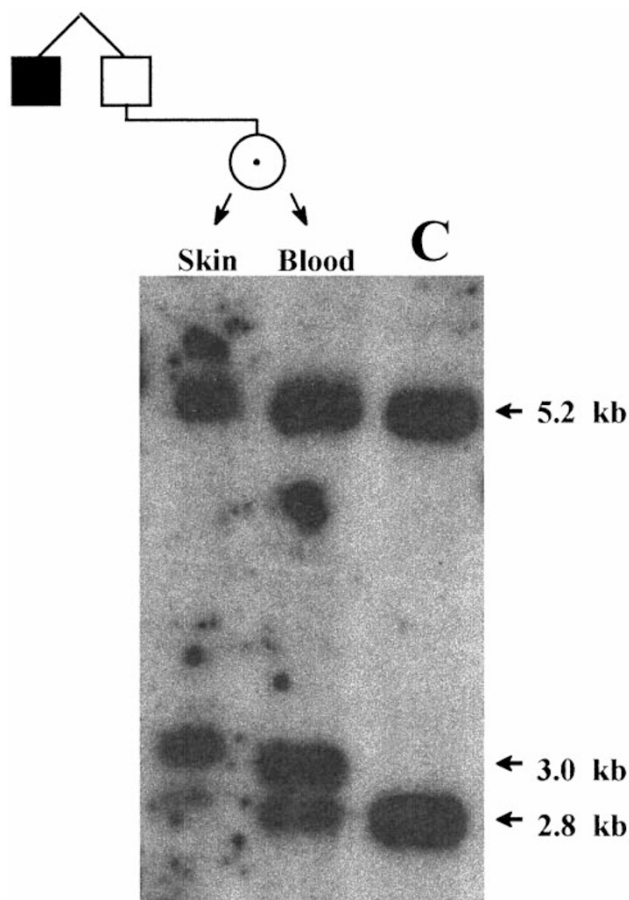
Figure 5 Southern blot analysis on DNAs extracted from cord biopsy (lane 1) and cord blood (lane 2) of the clinically unaffected brother's daughter. Lane 3: control female. In lanes 1 and 2 the presence of a larger and unmethylated allele, transmitted through the father's sperm, was revealed. Open square represents normal clinical phenotype.

tested. That could be explained considering that clinical variability may arise if the severity of the FRAXE MR phenotype is compensated by modifying factors present in the genetic background or if the effect of the FRAXE mutation is so mild that the phenotype can remain within the normal range in some subjects. Finally, a genotype–phenotype correlation may be confused by the occurrence of mentally retarded subjects unrelated to the fragile site. Considering this, the phenotype of our patient seems to be unusual since mental retardation is severe and dysmorphic features are striking, although Marfan-like habitus, long narrow face, high arched palate and midface hypoplasia have been previously reported in FRAXE patients.[28,29]

Uncertainty about genotype–phenotype correlation in FRAXE-associated MR, resulting from the mild phenotypic effect, the low incidence and the low rate of detection of new cases from large candidate population studies, the variable phenotype of the carrier females and the different size of the

expansions transmitted from fully mutated fathers to daughters[5–7,26] greatly limits the actual possibility of predicting phenotypes in males and females with the mutation and offering prenatal diagnosis to FRAXE families.

In our case, the issue of recurrent risk is raised for the normal brother. The result we obtained on sperm indicates a similarity to FRAXA since males with somatic large methylated expansions are carriers of small unmethylated expansions in germinal cells.[30] In our family, a small and unmethylated allele was transmitted unmodified from the father to his daughter, at least as far as we can infer from the molecular analysis performed on cord blood and skin.

In conclusion, we think that further studies are needed to evaluate the frequency of the FRAXE mutation both among large samples of mentally retarded subjects and in the general population. Moreover, extensive psychometric testing with expression studies in FRAXE-carrying individuals and in related normal controls would be helpful to evaluate the extent of this mental retardation phenotype.

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