The fragile X syndrome

Clinical, genetic and large scale diagnostic studies among mentally retarded individuals

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Cover illustration: Jan Vermeulen

ISBN 90-9010477-1

The edition of this thesis was supported by grants from the Rotterdam Foundation of Clinical Genetics, Stichting Het Remmert Adriaan Laan Fonds, Yamanouchi Pharma and the Nationale Collecte Verstandelijk Gehandicapten.

Print: Offsetdrukkerij Ridderprint B.V., Ridderkerk

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Het fragiele X syndroom

Klinisch, genetisch en grootschalig diagnostisch onderzoek onder verstandelijk gehandicapte mensen

Proefschrift

Ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
Prof. Dr. P.W.C. Akkermans M.A.
en volgens het besluit van het College voor Promoties

De openbare verdediging zal plaatsvinden op
woensdag 23 april 1997 om 15.45 uur

door

Lambertus Bastiaan Arie de Vries

geboren te Puttershoek

Promotiecommissie

Promotor:	Prof. Dr. M.F. Niermeijer
Overige leden:	Prof. Dr. H. Galjaard Prof. Dr. P.J. Willems Dr. B.A. Oostra

The studies described in this thesis were performed at the department of Clinical Genetics, Erasmus University Rotterdam. This project was financially supported by the Rotterdam Foundation of Clinical Genetics.

Ter nagedachtenis van David
Voor Jolijn en Leonie

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1.1 Historical introduction: from the Martin-Bell family, through a cytogenetic marker to the cloning of the fragile X gene

In 1943, Purdon Martin and Julia Bell described sex-linked mental retardation without dysmorphic features in a family in which both affected males and females were observed.[1] The observation of two unaffected brothers apparently transmitting the sex-linked gene to their normal daughters (who had affected sons) was attributed to an unknown controlling factor.[1] Thirty-six years later, Lubs[2] reported a marker X chromosome (later to be known as the fragile X chromosome) as an inconsistent finding in cytogenetic studies in leucocytes of some mentally retarded males (fig. 1). In the same period, in the cell culture laboratories, folic acid enriched culture medium (such as Ham's F10 and F12) replaced the old 'standard' folic acid deficient medium TC199. Although the observation of an abnormal X chromosome was confirmed by others who were still using the 'old' TC199 medium,[3,4] it did not receive much attention.

During the seventies, the combination of X-linked mental retardation with macro-orchidism was recognized.[5-7] In the same period, interest in the fragile X chromosome and the associated mental retardation revived after Sutherland's fortuitous observation of a fragile site on Xq in a percentage of blood cells which had been cultured in medium TC199.[8]

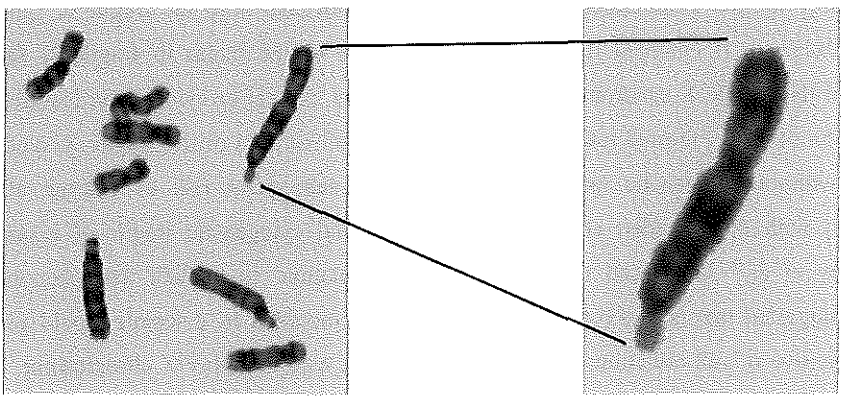

Figure 1 The fragile X chromosome as observed in a chromosome preparation (courtesy Dr. J.O. van Hemel, Dept. Clinical Genetics, Rotterdam)

Subsequently, the relative depletion of folic acid and thymidine in the cell culture medium was identified as the essential factor for the induction of this heritable fragile site at Xq27.[9] Several names were given to the syndrome - examples being macro-orchidism-marker X syndrome (MOMX), Martin-Bell syndrome and Gillian Turner-type X-linked mental deficiency syndrome - before the name 'fragile X syndrome' became generally used.[10-13] In addition to mental retardation, other clinical characteristics were established, e.g. facial features such as a long face with large protruding ears, macro-orchidism and behavioral features, including eye gaze avoidance, hyperactivity, hand-flapping, perseverative speech (reviewed by Fryns[14] and by Hagerman[15], and section 1.5). The folic acid deficiency-dependent expression of the fragile X was intensively studied to obtain an understanding of the causative mechanism (and its potential treatment) without apparent success.

For an X-linked disorder, the fragile X syndrome, as diagnosed at that time by cytogenetic detection of the fragile site at Xq27.3, had some special features:

- Approximately 30% of the obligate carrier females were mildly or moderately mentally retarded, therefore this X-linked disorder was neither recessive nor dominant. A large number of obligate carrier females (≈ 50%) showed no fragile X expression in cytogenetic studies. Clearly, cytogenetic studies were insufficient for carrier detection.
- Mentally normal grandfathers linking two family branches with the fragile X syndrome were observed. It emerged that approximately 20% of the males in fragile X families transmit the fragile X syndrome, without evincing any clinical or cytogenetic features. The daughters of these 'normal transmitting males' were all carriers of the fragile X syndrome, again without cytogenetic expression, but with a subsequent risk of bearing affected children with the disorder, combining retardation and cytogenetic fragile X expression. Pembrey et al.[16] introduced the designation 'premutation' to explain that phenomenon. They suggested that only the transmission of this premutation by a female would generate the definitive or full mutation, through recombination with the other X chromosome.

Sherman[17] found a higher proportion of normal males among the grand-parent generation

of fragile X families than among the grandson generation. This apparent difference of 'penetrance' of mental impairment in males was dependent on their position in the pedigree: the 'penetrance' of mental impairment was only 0.18 in brothers of normal transmitting males but it increased to 0.74 in their grandsons. Mendelian inheritance would dictate equal penetrance in both groups. This paradox became known as the 'Sherman paradox'.[18]

The gene involved in the fragile X syndrome, Fragile X Mental Retardation (*FMR1*) gene, was identified in 1991[19-21] through linkage studies and positional cloning (reviewed by Verkerk[22]). The gene defect was the first expansion of a trinucleotide repeat to be discovered (section 1.2). A whole class of disorders is now known to be associated with this type of mutation. These diseases are therefore known as 'trinucleotide repeat disorders' (section 1.4). In the 48 years between the original report of Martin and Bell and the genetic breakthrough of cloning the *FMR1* gene, there have been more than 1000 scientific publications about the fragile X syndrome.

1.2 The *FMR1* gene: a growing gene causing familial intellectual disability

The identification of the *FMR1* gene[19-21] elucidated the special characteristics of the transmission of the genetic defect in fragile X families. The *FMR1* gene has a size of 38 kb with 17 exons[23] and a polymorphic CGG repeat in the first 5', exon.[24] In the normal population, the CGG repeat varies from 6 to 55 units[24] (fig. 2) with an average of 30 units. The mutations in the *FMR1* gene can be divided into a major and a minor class: mutations within or outside the CGG repeat (table 1).

1.2.1 Mutations within the CGG repeat

On the basis of the size of the CGG repeat and its effect on the phenotype, two types of mutations in the CGG repeat can be distinguished: the premutation (size: 43 to 200 units) without a clinical effect, and the full mutation (size: >200 units) which causes mental

retardation in nearly all male patients and in 50%-70% of female patients.

The mutations in the CGG repeat are dynamic. They may change from generation to generation as well as within a single individual during early embryogenesis. Stable alleles with a CGG repeat < 55 units are defined as normal, and instable alleles ranging from 43 to 200 repeat units are regarded as premutation alleles.

The premutation and its intergenerational instability

The premutation with repeats in the range of 43 to 200 units occurs in phenotypically normal carriers (male and female) of the fragile X syndrome[24] (fig. 2). The (in)stability of the premutation is dependent on the sex of the transmitting parent and on the size of the repeat. Transmission of the premutation through female meiosis may lead to enlargement of the repeat to above 200 units (the full mutation) in the offspring. However, males with a premutation ('normal transmitting males') transmit their premutated gene as a premutation (sometimes differing slightly in repeat size) to their phenotypically normal daughters.[17,25]

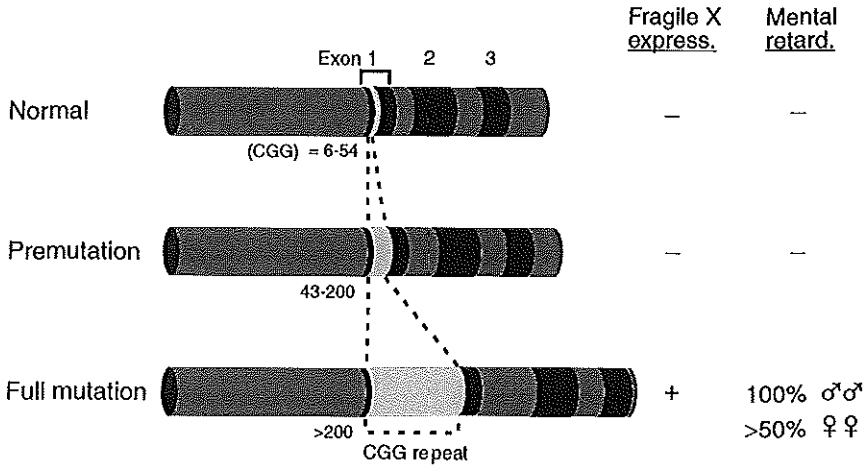

Figure 2 Part of the FMR1 gene and its CGG related mutations in the first exon of the FMR1 gene that contains the CGG repeat (grey) (see text for further details).

Table 1 ***Mutations in the FMR1 gene and their effect***

Mutation	No. of cases	FMRP	MR	Physical features	References[1]
in CGG repeat region					
premutation (43 - 200 CGGs)	innumerable	normal	-	-	24,25,46
full mutation (>200 CGGs)					
>60% methylated	innumerable	reduced/absent	+	+	42-44,46
+ premutation	innumerable	reduced	+	+	47,53-57
+ deletion	7	absent	+	+	57-60
< 40% methylated	16	>60%	-	absent or minor	56,61-67
outside CGG repeat region					
2 base pair change (exon 2)	1	absent	+	+	234
1 base pair deletion (exon 5)	1	absent	+	+	234
1 base pair change (exon 11)	1	present	+	+	78
partial or complete deletion *FMR1* gene	8	absent	+	+	68-77

[1] for additional references see section 1.2

Women with a premutation have a risk of having affected offspring with a full mutation. This risk depends on the size of their premutation.[24] The risk of expansion to a full mutation is therefore less than 20% for smaller premutation alleles (< 70 CGG repeats), but more than 80% for larger premutation alleles (> 80 CGG repeats).[24,26,27] The CGG repeat is generally interspersed with two AGG triplets.[28-33] In females with a premutation, the length of the uninterrupted CGGs at the 3′ end is an additional factor involved in the repeat's stability, with an instability threshold of 34-38 uninterrupted CGG repeats.[28,32] Although the size of the uninterrupted CGG repeat might be helpful in the future for an accurate risk prediction for those female carriers, no adequate data is available at present for this purpose.

In the 43 to 55 repeat range, there is an overlap between normal and premutated alleles, often referred to as the 'grey zone' in the fragile X literature. Only the finding of intra-familial instability in these 'intermediate' alleles can identify them as premutation alleles.[28,29,31] However, such premutation alleles can be stably inherited through many generations.[34,35] No transitions from alleles with < 43 repeats to larger alleles (> 43 repeats) have been observed, suggesting that this transition is very gradual (section 1.7). However, regressions from premutation-size alleles to an allele with < 43 repeats have been observed within one generation.[36-38]

Whether the transition from premutation to full mutation occurs before or after conception is not fully understood (reviewed by De Graaff[39]). The presence of full mutations in the ovary of female fetuses, without evidence of a premutation, supports the hypothesis of pre-conceptual enlargement.[40] This seems to conflict with the finding of only premutations in the sperm of four males with a full mutation in somatic cells.[41] The latter observation might be explained by regression of a full mutation to a premutation in a limited number of cell lineages. In the germline we have to assume a selection for those cells with a premutation.

The full mutation

Individuals with the fragile X syndrome have CGG repeats above 200 units, the 'full

mutation' (fig. 2). The expansion into full mutation is accompanied by hypermethylation of the repeat and its flanking regions, resulting in a shutdown of transcription and the absence of the FMR1 protein.[42-45] The absence or considerable reduction in FMR1 protein production is the cause of mental retardation. All males and a majority of the females with a hypermethylated full mutation are mentally retarded (chapter 4).[46-50]

In somatic cells of fragile X patients, a mosaicism pattern is observed of longer and shorter expansions. As the repeat size in the full mutation (above 200 repeats) varies from cell to cell, all fragile X patients are somatic mosaics. The instability occurs during early embryogenesis and results in this somatic heterogeneity.[51,52]

Mosaic states of the full mutation for its CGG repeat size and its methylation

In fragile X patients, two special subclasses of mosaicism can be distinguished on basis of size and methylation pattern.

1) As mentioned before, all fragile X patients are somatic mosaics. However, the individuals with full mutation and premutation are often referred to as *'size mosaics'* in the fragile X literature. This mosaic pattern is observed in 20% to 40% of the male patients.[47,53] As the premutation alleles are normally transcribed and their transcripts normally translated[54,55] (section 1.3), mosaic males are capable of producing FMR1 protein (FMRP) in some cells. Although it has been suggested that such mosaic males might have a higher IQ,[56] their intellectual impairment seems similar to males with a full mutation only.[47,57] This suggests that the proportion of cells in the brain producing FMRP is inadequate (see section 1.5.2 and chapter 3.1).

Males have been described with somatic mosaicism for a full mutation and a (partial) deletion of the *FMR1* gene in a proportion of their leucocytes.[57-60] Those males had a similar degree of mental retardation to males with a full mutation only.

2) Individuals with intercellular variations for the methylation status of a full mutation are called *'methylation mosaics'*. The proportion of leucocytes with an unmethylated full mutation may vary from low (≈ 10%) to 100%. A difference in cognitive functioning might therefore be expected. Intellectually normal males with a high proportion (>60%) of

leucocytes with an unmethylated full mutation have been reported (chapter 3.3).[56,61-67] FMRP production in the brain of those males is apparently sufficient for normal intellectual functioning. Remarkably, the majority of those males have minor physical features which are characteristics of the fragile X syndrome. This may suggest variation in the effect of the unmethylated full mutation on different tissues (chapter 3.3).[67] Immunocytochemistry showed that three males with normal cognition and unmethylated full mutations were found to have normal FMRP production in lymphocytes.[65,67] However, others reported severely reduced FMRP levels in clonal fibroblasts (200 to 285 repeats), from a patient with an unmethylated full mutation.[64] It is remarkable that, in the males referred to above, an expanded CGG repeat at the *FMR1* locus is not associated with hypermethylation of the repeat and its flanking region. The critical length of the repeat for hypermethylation is situated around 200 CGG units. It is therefore conceivable that some mutations with sizes between full mutation and premutation might not undergo methylation. However, several mutations with more than 400 CGG repeats but without hypermethylation have been reported.[56,62,65-67] This suggests that, in addition to the repeat length, other factors are involved in hypermethylation. It may be that an unknown factor or factors required for hypermethylation at the *FMR1* locus is deficient in males with this special subclass of mosaicism. This might also explain the occurrence of methylation mosaicism within some families.[61,65,67]

1.2.2 Mutations outside the CGG repeat

Whereas the mutations within the CGG repeat occur stochastically and are subject to a new genetic mechanism, the mutations in other regions of the *FMR1* gene follow the Mendelian rules of de-novo and inherited X-linked mutations. Interestingly, the frequency of the latter *FMR1* mutations is probably orders of magnitude below that of the CGG amplification mutations. In part, this may be a distortion due to the difficulty of detecting individual mutations because of the need to sequence the *FMR1* gene. On the other hand, the expected clinical and familial pattern would have attracted the attention of either clinicians or molecular geneticists if mutations outside the CGG repeat were frequent.

The mutations outside the CGG repeat which have been observed most commonly are large deletions (partial or complete) of the *FMR1* gene, with or without surrounding regions. At present, 8 (unrelated) fragile X patients with a partial or complete deletion of the *FMR1* gene - in all somatic cells studied - have been reported.[68-77] The first intragenic mutation in the *FMR1* gene, a *de novo* point mutation (an Ile367Asn substitution) in exon 11, was reported in a profoundly retarded male with fragile X features.[78] The missense mutation resided in one of the important domains of the FMR1 protein (see section 1.3.2).[79] Intragenic mutations may result in an absence of FMRP and thereby cause the fragile X syndrome. One single *de novo* base pair deletion in exon 5 and an inherited two base pair substitution in exon 2 resulting in a loss of protein production have been described.[80] Both male patients showed the mental retardation and physical features characteristic of the fragile X syndrome. Those observations also support the hypothesis that the absence of FMRP is in fact the main cause of the Martin-Bell phenotype.
It is remarkable that, at present, only three such mutations in the coding region of the *FMR1* gene have been identified. It is for the future to show whether these mutations are rare or whether they have simply been overlooked as intragenic mutations might give a different clinical phenotype and are technically difficult to detect. If these mutations occur *de novo*, the X-linkage could remain undetected and geneticists will not be encouraged to sequence the *FMR1* gene.

1.3 RNA/protein studies

To understand the effect of a gene mutation, it is essential to study the resulting protein expression, as the absence of a gene product or its altered function may give clues to the clinical phenotype.
The translation of the *FMR1* gene starts distal to the CGG repeat[44,81] and results in a 70-80 kDa protein. Studies in lymphoblastoid cell lines showed that the normal and premutated *FMR1* alleles are transcribed equally, resulting in *FMR1* transcripts with the same half-life

of 12 hours and the same level of FMR1 protein expression.[54,55] Alternative splicing in the *FMR1* gene occurs in various human tissues, leading to 24 distinct transcripts.[81-83] Whether those transcripts lead to functional proteins is unclear. The localisation of FMRP is predominantly cytoplasmic.[44,55]

1.3.1 FMRP expression pattern

FMRP expression patterns have been most intensively studied in the brain and testis because those tissues are most frequently affected in fragile X patients (table 2). Expression studies have demonstrated brain localisations of FMRP without as yet clarifying the clinical phenotype. In normal humans, FMRP expression is predominantly found in nearly all neurons of the different brain regions studied, whereas it is absent or reduced in non-neuronal brain cells[55] (Willemsen, *personal communication*). In normal testes, both the primordial germ cells (prenatally) and spermatogonia (postnatally) showed high FMRP expression, whereas in the Sertoli cells and the Leydig cells, expression was found to be low or non-existent. Remarkably, in a proportion of the primordial germ cells and all spermatogonia of fragile X patients, FMRP expression has been found (Willemsen, *personal communication*). This observation concords with the finding of premutation alleles in the sperm of patients.[41]

High FMRP expression has been reported in several other tissues with no primary involvement in the fragile X phenotype.[44,55,84,85] In tissues of mesodermal origin such as dermis, skeletal and cardiac muscle, FMRP expression could not be detected using antibodies specific for FMRP.[55] However, dividing mesodermal cells showed elevated FMRP expression in specific circumstances, such as wound healing or hyperplasia in cardiac muscle.[55]

1.3.2 FMRP function and related proteins

Further characterisation of FMRP revealed that the protein has RNA binding capacity due to characteristic sequence motifs in the protein: an RGG box and ribonucleoprotein particle (RNP) K homology domains (designated KH domains)[86-89](reviewed by Verheij[90]). De

Table 2 *FMRP expression pattern in brain and gonads of (non) affected males*[1]

Organ	Normal		Patient	
	prenatal	postnatal	prenatal	postnatal
CNS				
- neurons	++	++	–	–
- glial cells	+/–	+/–	–	–
Gonads				
- primordial germ cells	++	NA	+[2]	NA
- spermatogonia	NA	++	NA	++
- Sertoli cells	+/–	+/–	–	–
- Leydig cells	+/–	+/–	–	–

NA = not applicable
[1] for references see section 1.3.1
[2] limited number depending on gestational age[40]

Boulle et al [78] reported a patient with a missense mutation that resided in one of those KH domains. This mutation led to normal FMRP production in lymphoblastoid cells from that patient.[79] Whether the severe clinical phenotype in this patient (as compared to the typical fragile X patient) is related to the abnormal FMRP is still in doubt.[78,79] FMRP binds its own *FMR1* transcript and 4% of fetal brain transcripts. It is not known whether the RNA binding is selective.[87] Recent studies revealed a more specific binding of FMRP via RNA with the 60S ribosomal sub-unit, suggesting a function of FMRP in ribosomal function and in translation of certain proteins.[91-94] A nuclear localization signal encoded by the 3′ part of the gene, as well as a nuclear export signal encoded by exon 14, were revealed recently.[95] Eberhart et al postulated an interaction of FMRP with mRNA(s) in the nucleus, followed by export to the cytoplasm as part of a mRNP particle where it associates with ribosomes.[95] A role of the FMRP in the translation machinery could explain the high expression in (some) actively dividing pre- and post-natal tissues (see section 1.3.1).

Although neurons are also dependent on high levels of translation for their function, this also applies to several other tissues with low FMRP expression.

Two genes, *FXR1* and *FXR2* (for FMR1 **cross**-reacting relative) have been identified which are very similar to the *FMR1* gene.[96,97] The *FXR1* gene was mapped to chromosome region 3q28 and is highly expressed in skeletal muscle, the gonads and in proliferating neurons of the developing mouse embryo (based on mRNA in situ hybridization studies).[98] Whereas *FMR1* is expressed in spermatogonia, *FXR1* expression (both mRNA and protein) is observed predominantly in later stages of sperm development[98] (Willemsen, *personal communication*). Like *FMR1* and *FXR1*, the *FXR2* gene (localized on 17p13.1) has two KH domains and is expressed in the cytoplasm.[97] Males with the fragile X syndrome do have normal levels of FXR1 and FXR2 protein in lymphoblastoid cells,[96,97] which makes compensation of the absence of FMRP by those related proteins less likely.

The consequences of the absence of the FMRP have been studied in knockout mice. Those mice showed relevant characteristics, such as learning deficits, hyperactivity and macro-orchidism equivalent to those observed in fragile X patients.[99,100] As in humans, pathological studies of brain and testes of those knockout mice did not show gross abnormalities, except for enlargement of the testes.[99]

1.4 Other growing genes: the 'trinucleotide repeat disorders'

The *FMR1* gene was one of the first examples of a 'novel' class of disease genes: those with trinucleotide repeat expansion as the mechanism of mutation. Repeat amplification has been found to be involved in 13 genetic disorders. The clinical presentation of these disorders depends on the gene damaged by the mutation. In fact, the term 'trinucleotide repeat disorder' is a misnomer, because the only common factor of these disorders is the trinucleotide repeat expansion and the often-observed unusual consequences of intergenerational growth of the repeat. The latter may cause anticipation, the phenomenon of increasing severity during intergenerational transmission of a disorder. Another type of

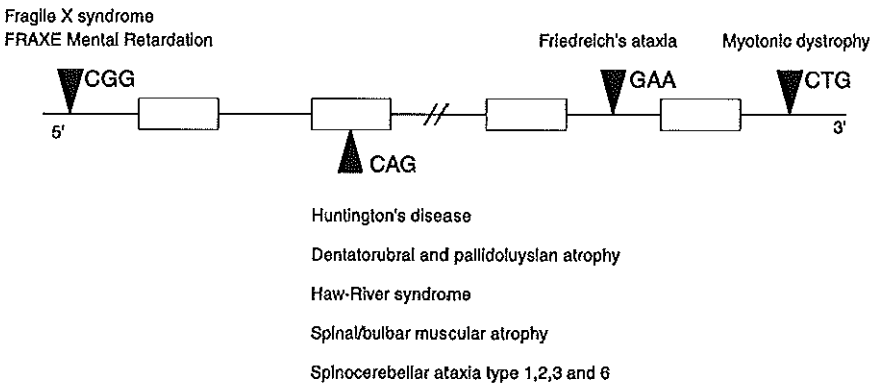

Figure 3 Location of the repeat in relation to the gene for the different trinucleotide repeat disorders.

classification differentiates between the localisation of the mutation in the gene: repeat in the 5′ untranslated region, the 3′ untranslated region, the open reading frame or an intron (fig. 3).

The clinically relevant repeats and their disorders are summarized in table 3:

1) The fragile X syndrome and the FRAXE MR syndrome are both X-linked mental retardation disorders and associated with a fragile site on the X chromosome (FRAXE 600 kb distal to FRAXA). Unlike the fragile X syndrome, the clinical phenotype of the FRAXE MR syndrome appears to be restricted to a mild mental impairment without additional clinical features. The gene involved in the FRAXE MR syndrome, *FMR2*, is also associated with a CGG repeat. Repeat amplification above 200 repeats leads to inactivation of the *FMR2* gene through methylation of the promoter region.[101-104] By contrast with fragile X syndrome, in FRAXE MR syndrome, the amplification of the repeat to a size above 200 repeats can occur through transmission by either sex.[101,105]

2) Myotonic dystrophy is a multisystem disorder, characterized by myotonia, progressive muscle weakness and wasting, cataracts and testicular atrophy. The clinical presentation of this autosomal dominant disease is highly variable both in age at onset and degree of

Table 3 ***Characteristics of disorders with a trinucleotide repeat***

Disorder	major symptoms	prevalence	inher.	gene	local.	repeat	normal repeat size	repeat size in affected	anticipation	M/P	gene product	references[1]
Fragile X syndrome	MR	1/4,000 to 1/6,000 ♂♂	X-linked	*FMR1*	Xq27.3	CGG	6 to 54	>200 +meth	-	+ ♀>♂	FMR1	19,24,42,43
FRAXE mental retardation	MR	rare	X-linked	*FMR2*	Xq28	GCC	6 to 25	>200 +meth	-	-	FMR2	101-104
Myotonic dystrophy	myotonia muscle wasting	1/8,000	AD	*DMPK/ DMAHP*	19q13.3	CTG	5 to 37	50 to >2,0000	+	+ ♀>♂	DMPK/ DMAHP	106-108,112,115,116
Huntington's disease	chorea dementia	1/10,000	AD	*IT15 HD*	4p16.3	CAG	8 to 35	36 to 121	+	+ ♂>♀	huntingtin	119,131,132,245
Dentatorubral and pallidoluysian atrophy/ Haw-River syndrome	ataxia chorea-athetosis dementia	rare	AD	*DRPLA*	12p12	CAG	7 to 23	49 to 75	+*	+ ♂>♀[2]	DRPLA	121,122
Spinal/bulbar muscular atrophy	muscle weakness hypogonadism	rare	X-linked	*AR*	Xq11-12	CAG	13 to 30	40 to 62	+	+ ♂>♀	androgen receptor	118

(Table 3 continued)

Spinocerebellar ataxia type 1	ataxia	rare	AD	*SCA1*	6p22-p23	CAG	6 to 39	41 to 81	+	+ ♂>♀	ataxin-1	123,124
Spinocerebellar ataxia type 2	ataxia	rare	AD	*SCA2*	12q24.1	CAG	15 to 29	35 to 59	+	-	ataxin-2	125-127
Spinocerebellar ataxia type 3 Machado-Joseph disease	ataxia	rare	AD	*SCA3* *MJD1*	14q32.1	CAG	13 to 36	67 to 79	+	+/-	ataxin-3	128,129,133
Spinocerebellar ataxia type 6	ataxia	rare	AD	*SCA6*	19p13	CAG	4 to 16	21 to 27	?	?	α_{1A} Ca^{2+} channel	130
Friedreich's ataxia	ataxia	1/50,000	AR	*STM7*	9q13	GAA	7 to 22	>200	-	?	STM7	134,246

[1] for additional references see section 1.4

[2] not for Haw River syndrome

inher. = inheritance; local. = localisation on the genome;

P/M = sex difference in intergenerational repeat size increment: ♀ > ♂ = repeat size increment greater when transmitted by females

MR = mental retardation; meth = methylation

severity. The molecular defect consists of a CTG repeat in the 3′ untranslated region of the putative gene, *DMPK* (dystrophia myotonia protein kinase-encoding gene).[106-111] However, this CTG repeat also resides in the promoter region of another gene, *DMAHP* (DM associated homeobox protein), which is located downstream of the *DMPK* gene and possibly also involved in the etiology of the muscular dystrophy.[112] In myotonic dystrophy families, an earlier age at onset and increasing severity of the disease in successive generations (anticipation) was observed.[113-116] The identification of the instability of the CTG repeat elucidated the molecular basis of anticipation in subsequent generations of myotonic dystrophy families: most offspring have an earlier age at onset in combination with a larger repeat size than their parents.[117] Moreover, severely affected offspring with the neonatal form of myotonic dystrophy are almost always the progeny of affected mothers.

3) For a large group of neurodegenerative disorders (spinal and bulbar muscular atrophy (SBMA or Kennedy disease),[118] Huntington's disease (HD),[119] dentatorubral and pallido-luysian atrophy (DRPLA),[120,121] Haw River syndrome,[122] spinocerebellar ataxia (SCA) type 1,[123,124] type 2[125-127], type 3 (Machado-Joseph disease)[128,129] and type 6[130]), a CAG repeat in the open reading frame of the gene has been identified, leading to a polyglutamine segment in the gene product. All these disorders are autosomal dominant, except for the X-linked SBMA. Most of these diseases have been associated with genetic anticipation and an influence of the sex of the transmitting parent on repeat size in the offspring.[119,121,123,131-133]

4) Friedreich's ataxia is another neurodegenerative disorder, affecting both the central and peripheral nervous systems. It is the only autosomal recessive disorder associated with a trinucleotide repeat and other mutations in the *STM7* gene.[134] The GAA repeat is located in intron 18, and at present no reduction in mRNA has been detected in affected individuals.[134] The STM7 protein is probably involved in the phosphoinositide pathway, and a dysfunctional protein might cause disturbances of vesicular trafficking or synaptic transmission. Friedreich's disease is another example of how detection of a repeat mutation leads to the detection of a protein, which could either be absent or dysfunctional through a

repeat related mutation or by other (less frequent) mutations in the gene.

The identification of the repeat expansion as a new mutational mechanism has elucidated intriguing aspects, such as anticipation, of several genetic disorders and facilitated the diagnosis of those disorders in patients and detection of carriers within families. Further studies need to be initiated for understanding the mechanism behind the expansion.

1.5 Clinical studies

1.5.1 Phenotype, subphenotypes and associations

In 1943, Martin and Bell were the first to report on 'A pedigree of mental defect showing sex-linkage'.[1] At that time, mental retardation was the only recognised clinical feature of the novel 'syndrome' later to be known as the fragile X syndrome. During the seventies, the clinical phenotype became delineated, e.g. the long face with large protruding ears (fig. 4) and the macro-orchidism.[5-7,135-138] In the same period, the 'old' finding of the marker X

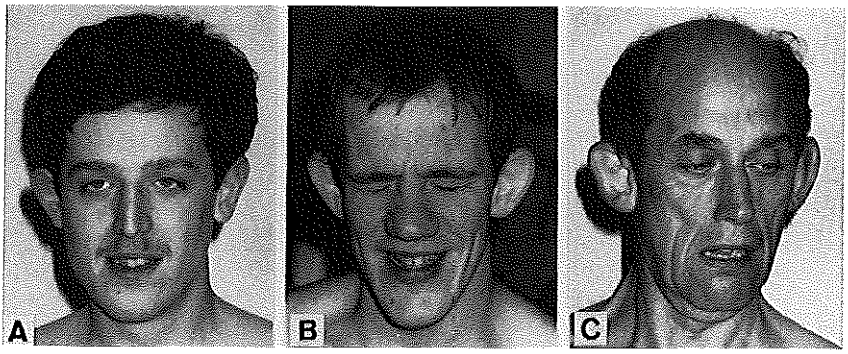

Figure 4 Three affected males with characteristic facial features of the fragile X syndrome. Note the long, narrow face with large, everted ears and the poor eye contact.

chromosome by Lubs[2] in 1969 became associated with this clinical syndrome, leading to the designation fragile X syndrome (see section 1.1).[135-138] Subsequently, the original 'Martin-Bell family' was restudied by Richards et al [12] in 1981 and they found the fragile X site and the typical clinical features. 'Martin-Bell phenotype' became the eponym for the phenotype of affected males. Several additional clinical features were reported in 137 affected boys by Hagerman et al[15]: high arched palate (48%); prominent jaw (28%); strabismus (33%); hyperextensible metacarpophalangeal joints (64%); hand calluses (45%); double-jointed thumbs (41%); single palmar creases (35%); pes planus (65%); scoliosis (20%) and pectus excavatum (43%) (fig. 5). The facial features are often less noticeable, particularly in affected females and children (fig. 6). The macro-orchidism often develops

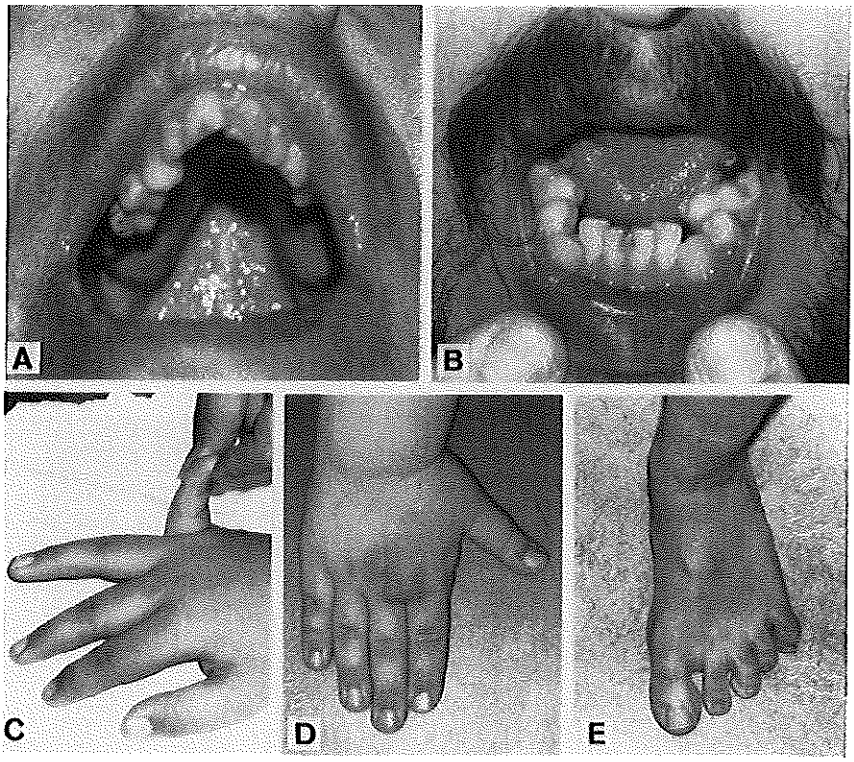

Figure 5 Other clinical features in fragile X individuals: (A) high arched palate; (B) dental crowding; (C) hyperextensible MP joints; (D) hand calluses; and (E) pes planus.

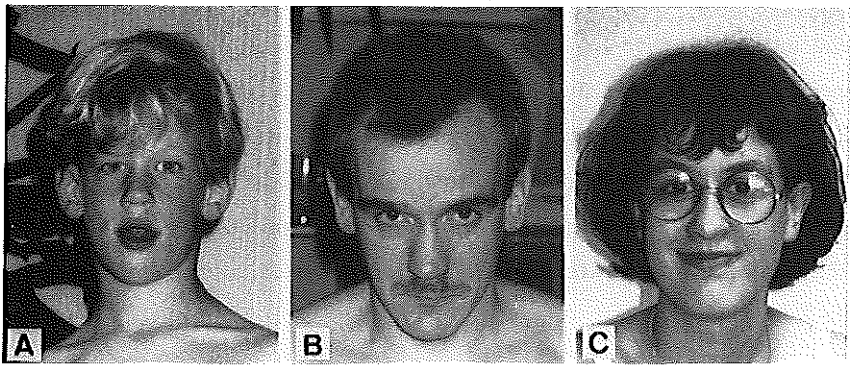

Figure 6 Two affected males and one female with less obvious facial features.

during and after puberty, and is frequently absent in young patients (reviewed by Hagerman[15] and by Fryns[14]). To establish the clinical diagnosis, the use of a score list might be helpful (see appendix).[139-141]

Fragile X children often present with overgrowth, mostly restricted to the head size.[142] The increased head circumference persists into adult life, but the final height of adult males with the fragile X syndrome is below the norm.[142,143] Seizures are observed in approximately 20% of all the fragile X males, with a lower prevalence among adults.[15,144,145]

The separate clinical symptoms as such are not specific for the fragile X syndrome. Of 472 Dutch mentally retarded adult males without a diagnosis but with a normal repeat in their *FMR1* gene, at least 20% had macro-orchidism, 14% large and anteverted ears, 8% a long face with prominent chin or forehead and 20% hyperextensible joints (metacarpo-phalangeal V extension > 90 degrees) (De Vries, *unpublished results*). However, the combination of those features increases the likelihood of the fragile X diagnosis by more than tenfold (chapter 5.1).

Recognition of behavioral features, especially in the younger child, such as hand-flapping, hand-biting, tactile defensiveness, poor eye contact, hyperactivity and perseverative speech are also suggestive for the diagnosis.[15]

The 'autistic-like' behaviour of many fragile X males initiated the debate about whether

autism is associated with the fragile X syndrome.[146,147] Impaired communication and impaired social interactions, two of the DSM-III-R criteria for autism, are significantly more frequent in fragile X males by comparison with mentally retarded controls.[148] A diagnosis of autism was therefore frequent for fragile X males. However, the social and communicative impairments differ in quality in fragile X males from autistic males.[146] The apparent similar frequency of the fragile X syndrome among mentally retarded or autistic populations did suggest a link between autism and mental retardation rather than autism and the fragile X syndrome.[149]

Subphenotypes

The general homogeneity of the fragile X phenotype is contrasted by a few and possibly consistent exceptions. Fragile X males with intellectual disability, truncal obesity, short, broad hands and feet, hyperpigmentation (periorbital, axillary and genital) and hypogenitalism have been reported (chapter 3.1).[150-152] These patients resemble the Prader-Willi syndrome, and this phenotype therefore became known as the Prader-Willi-like subphenotype of the fragile X syndrome[153] (fig. 7). However, the terminology 'Prader-Willi-like' might be confusing and it has been proposed that, instead, obesity should be added to the list of fragile X clinical features.[154]

Some fragile X individuals have general overgrowth that can be confused with the Sotos or cerebral gigantism syndrome (chapter 3.2).[155,156] Both the Prader-Willi- and Sotos-like subphenotypes have been observed separately in fragile X families in which other affected relatives have the classical Martin-Bell phenotype. The 'Prader-Willi-like' subphenotype has also been observed as the only phenotypical expression of the fragile X syndrome within a single family.[151] Although both subphenotypes might result from a neuro-endocrine dysfunction, no major dysfunctions could be detected, except for raised levels of insulin-like growth factor-I and insulin-like growth factor binding protein-3 in only one of the fragile X patients with general overgrowth.[156]

Associations with other clinical syndromes or sequences have been described. Examples are the Robin sequence (micrognathia, glossoptosis and cleft soft palate),[157] the FG

syndrome (congenital hypotonia, macrocephaly, distinctive face and imperforate anus),[158,159] and the DiGeorge anomaly (defects of thymus, parathyroids and great vessels),[160] without definite evidence for a causal relation between those phenotypes and the *FMR1* gene mutation. Several reports concerning the co-occurrence of the fragile X syndrome and sex chromosomal abnormalities like 47,XXY or 45,XO/46,XX[161-166] suggest an increased rate of nondisjunction related to fragile X chromosome.

It has been hypothesized that additional genes adjacent to the *FMR1* gene might be involved in the development of the clinical phenotype in fragile X patients. This hypothesis has been supported by several observations. First, altered replication timing for regions on both sides of the *FMR1* gene (150 kb 5′ and 34 kb 3′ of the expanded CGG

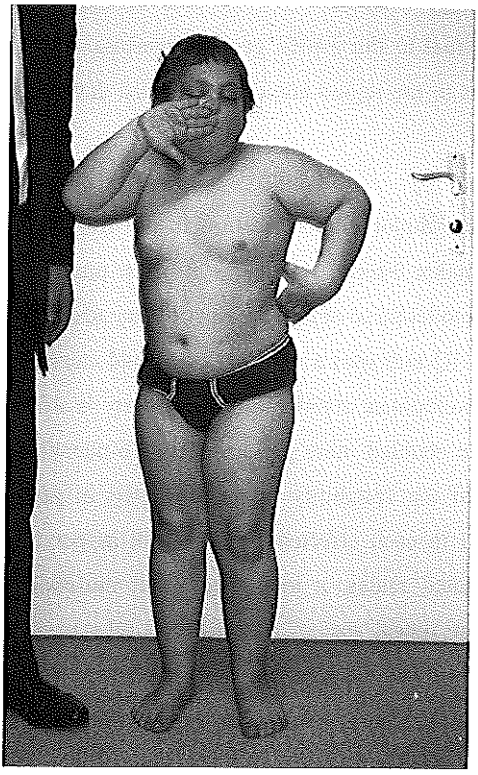

Figure 7 A 9 year-old fragile X patient with the Prader-Willi-like subphenotype. Note the truncal obesity, short stature, short broad hands and feet.

repeat) has been reported.[167] Second, due to 'spreading of methylation', transcription of other adjacent genes might be disturbed resulting in unbalanced protein production and this might influence the phenotype. Reduced iduronate sulphatase (IDS) activity, of which the gene is located ≈ 1.2 Mb distal to the *FMR1* locus,[168] has been reported in fragile X patients.[169] However, direct involvement of nearby genes in the etiology of the classical Martin-Bell phenotype is less likely since mutations have been reported in the coding region of the *FMR1* gene in males with the classical phenotype.[80] At present, no adjacent genes have been identified. Whether such genes might be involved in the etiology of subphenotypes (Prader-Willi-like and Sotos-like), is still unknown. Interestingly, a single atypical case of a boy with the fragile X syndrome with obesity and anal atresia was reported as having a large deletion.[75] The deletion extended from between 160-500 kb distal and 9.0 Mb proximal to the *FMR1* gene, suggesting that, in addition to the deleted *FMR1* gene, other genes in the region could have caused that specific phenotype.

1.5.2 Mental retardation

Intellectual disability, the major clinical feature in the fragile X syndrome, shows some variability in affected males. Mental retardation in most prepubertal boys is moderate (IQ ≈ 35-55) whereas it is moderate to severe in most of the adults (IQ ≈ 20-40).[57,170] Whether this apparent decline in IQ indicates a real loss of mental capacities or merely represents a slowing of cognitive development stabilizing after puberty is still unknown.[170-174]

The cloning of the gene enabled more precise genotype-phenotype correlation studies and insight into the cause of the variability of the IQ levels in patients. The size of the full mutation varies per cell in each affected male as well as per affected individual (section 1.2). However, in affected males, a methylated, full mutation will lead to the mental impairment whatever the repeat size of the mutation (chapter 4.1).[46,57] The fully mutated *FMR1* gene is, generally, hypermethylated and inactivated, leading to a lack of FMR1 protein expression[42-44] (section 1.2). It has been suggested that males with a 'mosaic' DNA pattern (premutation in addition to the full mutation; see section 1.2) - and therefore residual FMR1 protein production - might have better mental functioning compared to

fragile X males with a full mutation only.[56,175-177] However, others found no mean IQ differences between 'mosaic' males and males with a full mutation only (chapter 4.1).[47,57] Apparently, the proportion of brain neurons producing FMRP in 'mosaic' males is insufficient for normal cognitive functioning. This is supported by the observation of a mentally retarded male who had FMRP production in 28% of his lymphocytes due to a partial deletion of the CGG repeat in addition to the full mutation.[60]

The importance of the proportion of cells expressing FMRP for normal mental functioning is shown in males with incompletely methylated full mutations in more than 60% of their leucocytes (chapter 3.3).[46,56,61-63,65,67] These males have normal mental capacities, suggesting that the proportion of brain cells expressing FMRP through unmethylated *FMR1* alleles is decisive for mental capacity. In four of these 'methylation mosaics', a direct relationship between the absence of methylation and the ability to produce FMR1 protein could be observed.[65,67]

Insufficient FMRP production also explains cognitive impairment (IQ < 85) in females who are heterozygous for the full mutation. Before DNA diagnosis became feasible, 35% to 53% of the heterozygous females were found to have mental impairment (IQ < 85).[17,178,179] Using DNA mutation analysis, 52%-82% of the women heterozygous for the full mutation were shown to have mental impairment (IQ < 85) (chapter 4.2).[46,48-50,180] In these heterozygotes, the proportion of abnormal *FMR1* alleles on the active X chromosome (which are unable to produce FMRP) has an influence on cognitive development.[50,180-182] It is still not known whether the FMRP is cell-bound. Apparently, there is no large scale transmission/passage of corrective molecules from FMRP-producing cells to non-FMRP producing neurons. Such metabolic compensation is known to occur in heterozygotes for other X-linked disorders, the Hunter syndrome and the Lesch Nyhan syndrome for example. As the X-inactivation pattern is established early in embryonic life, it is also feasible that certain brain areas are deficient for FMRP. Brain autopsies on female carriers of the full mutation will be needed to test this hypothesis.

1.5.3 Neuroimaging and pathological studies

Although several neuroimaging and a few neuropathological studies have been conducted with fragile X patients, knowledge about structural cellular defects in the fragile X syndrome is still limited.

Reiss et al[183,184] reported on reduced brain size in male fragile X patients shown by cerebral MRI of the posterior cerebellar vermis and increased size of the caudate nucleus, thalamus, and hippocampus. These findings were neither confirmed nor refuted by others. These brain areas might be associated with the attention deficit (cerebellar vermis and hippocampus) and the learning and memory dysfunction (hippocampus) observed in fragile X patients.[184]

Neuropathology studies in three fragile X males' brains revealed immature thin and long dendritic spines in different regions of the brain, with a reduction in mean synaptic contact but with preservation of neuronal density in the neocortex.[145,185,186] Interestingly, in normal mouse brain, high FMRP expression has been observed in the synaptosomes which could be related to the high transcriptional activity necessary for spinal function (Tamanini, *personal communication*). However, abnormally long, thin dendritic spines have also been described in children with chromosomal abnormalities[187] and in retarded children with normal karyotypes.[188] This suggests that abnormal dendritic spines are not a specific neuropathological feature of fragile X patients.

Neuronal heterotopia was observed in two affected males. In addition, one of the males had amyotrophic lateral sclerosis (ALS) and, in the other male, the diagnosis of fragile X syndrome was made in retrospect.[189,190] Heterotopia is a rather non-specific finding in mentally retarded individuals, but it might be related to the epilepsy found in fragile X patients.

In testes, macro-orchidism seems to be associated with increased tubular length and interstitial edema.[6,185,191,192]

Fifty-three years after its original description, the paucity of neuropathological documentation of fragile X patients is a neglected area in the understanding of this type of

mental retardation. It is obvious that larger series of post-mortem studies are required to ascertain the (neuro)pathological defects in the tissues of (primary) interest, namely the brain and testes.

1.5.4 Treatment

Mental retardation and behavioral problems dominate the clinical presentation, and the mental retardation is not amenable to treatment. However, careful medical follow-up and, in some cases intervention is required. The physical and behavioral problems in the fragile X syndrome are generally related to the stage of development of the patient (table 4; reviewed by Hagerman[193]). During infancy, connective tissue abnormalities such as congenital hip dislocations and hernia inguinalis might be present. In later life the connective tissue dysplasia may lead to scoliosis, flat feet and mitral valve prolapse.[194] The mitral valve prolapse requires evaluation by a cardiologist and a recommendation for antibiotic prophylaxis before surgical or dental procedures.

Some children fail to thrive due to gastro-oesophageal reflux, tactile defensiveness or difficulties in sucking.[195] The last of these problems requires attention from a specialized speech therapist or physiotherapist, while the gastroesophageal reflux can be treated by dietary advice and/or medication. Surgery is rarely needed. The frequent otitis media and sinusitis in approximately 50% of the children with fragile X syndrome need adequate intervention (antibiotics and/or polyethylene tubes).[196] Approximately 30-50% of the children with the fragile X syndrome have ophthalmologic problems such as strabismus, myopia or hyperopia. Ophthalmological attention is required here.[112] Seizures observed in approximately 20% of the males and 5% of the females should be diagnosed and treated (see section 1.5.1).

Behavioral problems may be present during different stages of the fragile X patient's life. Attention deficit and hyperactivity are most pronounced at a young age. Although, in general, fragile X patients are very friendly, some show aggressive behaviour in adulthood. Coping with these behavioral problems seems difficult, although behavioral therapy and avoidance of overwhelming stimuli may alleviate the symptoms. In some countries,

Table 4 *Medical problems of males with fragile X syndrome*

Problem	Number of patients evaluated	Percentage of patients with this symptom
Emesis	147	31
Failure to thrive in infancy	138	15
Strabismus	161	36
Myopia or hyperopia	148	22
Hernia	230	15
Joint dislocation	150	3
Orthopaedic problems	171	21
Otitis media	291	85
Sinusitis	43	23
Seizures	288	22
Mitral valve prolapse	79	35
Apnoea	139	10
Autism	211	20
ADHD	224	80
Motor tics	188	19

(adapted from Hagerman, 1996[193])

intervention with medication for the behavioral problems in the fragile X syndrome is common (reviewed by Hagerman[193]). For the Attention Deficit Hyperactivity Disorder (ADHD) symptoms, a variety of drugs have been described, examples being CNS stimulants (such as dextroamphetamine, methylphenidate and pemoline), tricyclics and clonidine. However, adequately controlled studies on the effectiveness of such medications for behavioral intervention in the fragile X syndrome are scarce.

In addition, attention should be paid to the need for special education and training, especially in the younger child. Here, speech therapists or physiotherapists can help with, respectively, language and motor development.

1.6 Molecular diagnosis

Before the cloning of the *FMR1* gene, cytogenetic detection of the fragile site at Xq27.3 (FRAXA), was the only method for (prenatal) diagnosis and carrier detection. However, this method was inadequate for carrier detection: phenotypically normal male carriers and ≈ 50% of female carriers were not detectable with the cytogenetic test. Moreover, other fragile sites located near FRAXA could lead to diagnostic confusion in families expressing those fragile sites: FRAXE (≈ 0.6Mb distal to FRAXA) and FRAXF (1-2 Mb distal to FRAXA).[197-199] With DNA linkage studies, it was possible to ascertain carrier status with a probability up to 99% in the majority of the fragile X families.

1.6.1 DNA analysis

A reliable molecular diagnosis became available after the cloning of the *FMR1* gene and the identification of the CGG repeat amplification as the common cause of the fragile X syndrome. Two major methods (Southern blots and PCR) for DNA analysis of the CGG repeat are used. Different fragment sizes corresponding to different alleles (normal, premutation or full mutation) can be detected with Southern blot analysis (fig. 8 and 9). Since the size of the full mutation varies in the different cells of the individual with the fragile X syndrome, these various enlarged fragments appear as a succession of bands or a 'smear' on a Southern blot (fig. 8, III 1,3, and fig. 9, 2-4,8,9).

The DNA of a premutation in a normal transmitting male shows a slightly enlarged fragment as compared to a normal allele on DNA analysis (fig. 8 I 1, III 4, and fig. 8, 11). Female premutation carriers have the normal allele (normal band) and a premutation (slightly larger band; fig. 8 II 2,3, III 2, and fig. 9 1,6,10). Female full mutation carriers have besides the normal allele, the full mutation ('smear'; fig. 8, III 1, and fig. 9, 8). Different restriction enzymes may be used, such as *Hin*dIII, *Bgl*II and *Pst*I, each with their specific fragment sizes.[200] A double digest, including a methylation sensitive restriction enzyme, enables detection of the methylation of the CpG island in the CGG region. This is a valuable tool in identifying males who have incompletely methylated full mutations

('methylation mosaics', see section 1.2.1). The double digest might also be used in distinguishing between an unmethylated large premutation and a small methylated full mutation.

An alternative method for determining CGG repeat size is the polymerase chain reaction or PCR analysis.[24] However, CGG repeats of more than ≈ 150 units are generally difficult to amplify and will most often not give a detectable product after the PCR. Most laboratories use both methods (restriction enzyme and PCR analysis) for postnatal and prenatal diagnosis.[201-205] When a premutation is found in a chorion villus sample, confirmation in amniotic fluid or a cord blood sample might be considered to ascertain the stability of the premutation during fetal life.[205] In the (near) future when enough information about

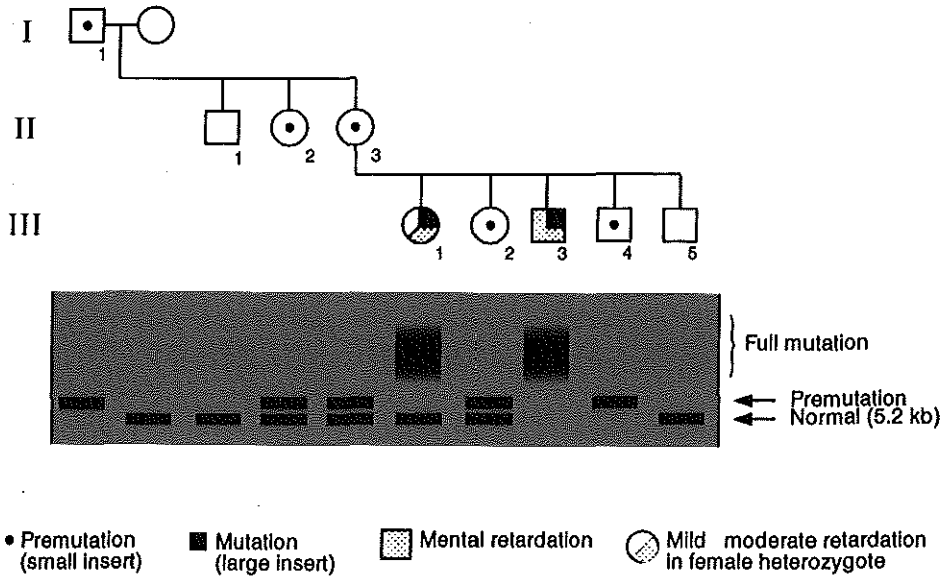

Figure 8 (a) Family pedigree: (square) male; (circle) female; (dot) premutation; (filled upper right box) full mutation; (shaded) intellectual disability. (b) Schematic representation showing DNA analysis of the family above (see text for further details).

stability becomes available, such confirmation might become unnecessary. Absence of methylation of the fully expanded mutation in chorionic villi at 11 weeks of gestational age has been observed in a fetus with hypermethylation of the full mutation in fetal tissue.[43,203] Methylation status is therefore unreliable in an early (< 12 weeks of pregnancy) chorion villus sample because it may differ from the foetal tissue. Nevertheless, methylation status may be useful in amniotic cells.

The methods described above can identify repeat amplifications in the CGG repeat and large deletions in the *FMR1* gene. Subtle mutations outside the CGG repeat will not be detected. At present, only a limited number of mutations in the coding region of the *FMR1*

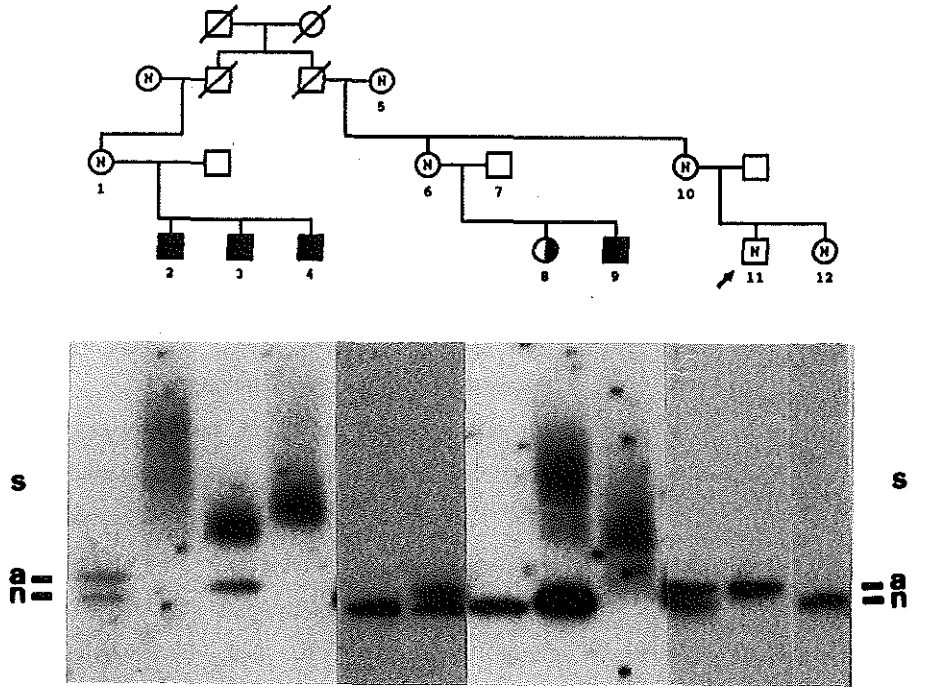

Figure 9 Family pedigree: (square) male; (circle) female; (N) normal female or male carrier of the premutation; (filled square) male with the fragile X syndrome and the full mutation; (half filled circle) female carrier of the full mutation. DNA analysis of the family above: (n) normal fragment; (a) slightly enlarged fragment; (s) various enlarged fragments or 'smear' (see text for further details).

gene were found using sequencing techniques.[78,80] It is conceivable that other rare mutations in the *FMR1* gene will be detected in the future (see section 1.2.2).

1.6.2 FMRP antibody test

Recently, an FMR1 protein antibody test was developed for detecting the presence or absence of FMRP in lymphocytes.[206] This rapid detection method allows the diagnosis of fragile X syndrome in a smear of one drop of blood: cells from fragile X males with a methylated full mutation produce no FMRP[206] (fig. 10). The proportion of cells with FMRP production is a major feature in allowing a distinction to be made between males with the fragile X syndrome and normal individuals. Cells from fragile X males show positive staining in less than 30% of the lymphocytes and can therefore be differentiated from cells of individuals with normal FMRP expression.[207] The test does not detect dysfunctional proteins, neither does it differentiate between normal and premutation alleles.

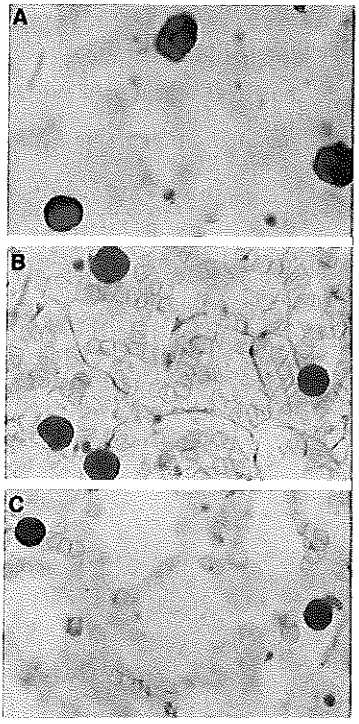

Figure 10 Antibody test for expression of FMRP in a normal male (A), and in a male (B) and a female (C) patient with the fragile X syndrome, using mouse monoclonal antibody 1A1 against FMR1 protein followed by an indirect alkaline phosphatase technique (courtesy of Dr. R. Willemsen, Dept. Clinical Genetics, Rotterdam)

As a result of lyonization and disproportional X-inactivation, there may be staining in females with a full mutation in up to 80% of the lymphocytes studied. There is a considerable overlap with normal females, as 40% of normal females show staining in less than 80% of their lymphocytes.[207] The phenomenon of X-inactivation makes the test less suitable for detecting females with a full mutation. This leads to an undesirable lack of specificity. However, this rapid and cheap antibody test might be

useful in screening for the fragile X syndrome among mentally retarded males or even among newborn males (see section 1.7 and chapter 6.2).
After a diagnosis of a fragile X patient with this antibody test, DNA studies are needed to confirm the diagnosis and to ascertain carrier status in family members.
Recently, the antibody test proved useful in the prenatal diagnosis of at-risk male pregnancies using chorion villi at 12.5 weeks of gestational age[208] and uncultured amniotic fluid cells.[209] As mentioned in the previous section, one should exercise caution when using this technique for prenatal diagnosis because of methylation differences between villi and fetal tissue at week 11. Further studies need to be initiated to validate the antibody test for prenatal diagnosis.

1.7 Prevalence and screening

In the 19th century, a predominance of retarded males over females was noticed.[210] This was confirmed by Penrose in 1938. He explained this phenomenon by implying an ascertainment bias.[211] Retarded males were more frequently institutionalized than retarded females and this may have led to such an overestimation.[212] Lehrke (1974)[213] considered a more appropriate explanation of mutated X-linked genes and associated X-linked mental retardation (XLMR). The identification of the fragile X chromosome initiated a multitude of cytogenetic studies among families with X-linked mental retardation. In approximately one third of those XLMR families, the fragile X syndrome was diagnosed.[138,214-216]
Genetic epidemiological studies of the fragile X syndrome have been widely performed using various selection criteria, e.g. mental retardation, macro-orchidism or autism (reviewed by Webb[217] and by Sherman[218]). The fragile X syndrome has been found in all the ethnic groups studied.[218] Estimates for the prevalence of the fragile X syndrome based upon cytogenetic testing varied from 1/1200 to 1/2600 for males and 1/1700 to 1/4000 for females.[219-222] More precise DNA analysis indicates a lower prevalence for males of 1/4425 to 1/6045 (chapter 5.1).[223-225]

There have been several studies of the mentally retarded using DNA analysis from England, the United States, Australia and the Netherlands (table 5,. chapter 5.1).[224,226-230] The clinical characterisation and the level of IQ was often poorly documented in these patients. Moreover, most studies were inadequately designed to avoid ascertainment biases. The combined results of these studies (irrespective of their shortcomings) showed, in a total of 4,670 mentally retarded individuals, 30 (0.6%) newly diagnosed fragile X patients. The frequency of individuals with an intermediate-size allele is 1.8%. This exceeds the frequency of premutation-size alleles (0.04%).

In the general population, a frequency of approximately 1/250[231,232] has been observed for the premutation-size alleles among females. This relatively high frequency of premutation carriers among females as compared to the estimated prevalence of the fragile X syndrome in males supports the presence of an excess of relatively stable premutation-size alleles. This frequency is mainly based on Rousseau's et al[231] study of anonymous Canadian hospital blood samples (n=10,642). The instability of the premutation-size alleles could not therefore be ascertained using family studies. Another variable might be a founder effect in the French-Canadian population.[233] Additionally, in a Finnish study in Kuopio involving 1,138 pregnant women, 1 in 265 carried a premutation-size allele (> 60 repeats) and 1 in 88 women had an intermediate-size allele (40-60) (Ryynänen, *personal communication*).

As males with the fragile X syndrome usually do not reproduce, the high prevalence of the fragile X syndrome might be sustained by a high mutation rate. For an X-linked gene that is genetically deleterious in males, Haldane (1956) estimated that one third of all cases must be caused by new mutations. Interestingly, 'de novo' mutations of the CGG repeat have not been observed in fragile X families. The polymorphic CGG repeats probably increase in size through many generations. This gives a more or less permanent 'slow-release' delivery of 'new' *FMR1* premutations into the population. The stability of the premutation over 6 or more generations has been shown in at least two fragile X families with a common 18th century ancestor.[34,35] To explain these phenomena, Morton and

Macpherson[234] suggested transitions in steps in a four-allele-model: normal (N, 6-50 repeats), small and relatively stable insert (S), larger but unstable insert (Z) and large insert causing the fragile X phenotype (L). The normal allele mutates at a frequency of 24.7×10^{-5} to an S allele. The S alleles, which have 50 or more CGG repeats, increase slightly over many generations ($\approx$ 90) to a Z allele, possibly through so called 'polymerase slippage' (amplification of the CGG repeat by 1 or 2 extra CGGs during replication due to an error of the polymerase). The Z alleles - which are highly unstable - may change to an L allele within 1 or a few generations, resulting in the fragile X phenotype.

A small diversity of S alleles may account for the founder effects demonstrated in Caucasian and Asian populations by microsatellite studies.[235-243] This hypothetical model predicts a high frequency of relatively stable S alleles among the general population[234,244] which will continuously create new unstable premutation alleles. Precise identification of the various allele classes will have major implications for screening programmes in the general population. For accurate counselling of females identified with a premutation-size allele, knowledge of the allele's stability might be desirable (chapter 6.2).

Table 5 *Overview of DNA screening programs among institutes and schools for the mentally retarded*[*]

	Persons studied			Persons with grey zone allele			Persons with PM	Persons with FM			Characteristics population studied
	♂	♀	♂ + ♀	♂	♀	♂ + ♀ (CGG range)	♂ + ♀	♂	♀	♂ + ♀	
Webb et al (1986), Turner et al (1996) England	219	-	219	-	-	-	-	6	-	6 (2.7%)	mentally retarded children in schools and institutions
Turner et (1986), Turner et al (1996) Australia	472	-	472	-	-	-	-	10	-	10 (2.1%)	mentally retarded children in special schools
Jacobs et al (1992) England	180	74	254	-	-	11 (41-49)	0	4	0	4 (1.6%)	children with special educational needs
Hagerman et al (1994) United States	299	140	439	-	-	-	1 (♂)	1	3	4 (0.9%)	retarded and/or learning disabled children
Slaney et al (1995) England	103	51	154	-	-	-	0	4	0	4 (2.5%)	schoolchildren with learning difficulties
Murray et al (1996) England	1013	-	1013	35	-	35 (41-60)	1 (♂)	5	-	5 (0.5%)	boys with learning difficulties
Meadows et al (1996) United States	888	391	1279	3	7	10 (50-60)	0	1	1	2 (0.2%)	children in special needs education programs
De Vries et al	870	661	1531	10	9	19 (43-60)	0	9	2	11 (0.7%)	mentally retarded children/adults in schools/institutions
Subtotal[**]	**3353**	**1317**	**4670**			**75 (1.8%)**	**2 (0.04%)**	**24 (0.7%)**	**6 (0.5%)**	**30 (0.6%)**	

[*] for references see section 1.7 and chapter 5.1

[**] without (reassessed) studies of Webb et al, 1986 and Turner et al, 1986

PM = premutation; FM = full mutation

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Chapter 2 Aims of the study

Chapter 2 Aims of the Study

Since the recognition of the fragile X syndrome as a separate clinical entity in the late seventies, the phenotype has been delineated as a result of work done in numerous clinical studies (chapters 1.1 and 1.5). The identification of the *FMR1* gene in 1991 made accurate molecular diagnosis possible for the first time (chapter 1.2).

The three major aims of this study are:

1) A clinical and molecular study of fragile X patients with non-classical phenotypes.

2) An assessment of the influence of the genetic defect on cognitive functioning in males and females with a full mutation.

3) A genetic epidemiological study of the fragile X syndrome in mentally retarded individuals.

2.1 The clinical phenotypes of the fragile X syndrome (chapter 3)

The first objective, the further characterisation of the non-classical fragile X patients, involved clinical and molecular studies of:

a) Fragile X patients with clinical phenotypes such as the 'Prader-Willi-like' and the 'Sotos-like' phenotypes (chapters 3.1 and 3.2).

b) Fragile X patients with methylation mosaicism. The full mutation in the *FMR1* gene leads to an absence/reduction of FMR protein production and the question was whether variation in methylation may cause the phenotypic variability. This form of heterogeneity was studied in three males within one family (chapter 3.3).

2.2 The relation between genotype and phenotype with emphasis on mental retardation (chapter 4)

The size of the *FMR1* gene mutation and its methylation status may variously influence mental status in males and females with the full mutation.

Males with 'mosaicism' for the premutation and the full mutation have a proportion of

cells with normal FMR1 protein production and might, therefore, be expected to be less retarded than males with a full mutation in the *FMR1* gene only. The level of mental retardation was studied in relation to the size of the CGG repeat (chapter 4.1).
A wide range of mental development has previously been found in females with a full mutation. We studied the frequency of mental impairment among females with a full mutation as found by molecular analysis. First-degree female relatives (mothers/sisters) without a full mutation served as controls. In addition, the relation between the proportion of normal *FMR1* alleles on the active X chromosome and IQ was analyzed in female carriers of the full mutation (chapter 4.2).

2.3 Screening and diagnosis for the fragile X syndrome (chapter 5)
A genetic epidemiological study in a representative sample of mentally retarded individuals from Dutch institutions and schools for the (severely) learning disabled was performed to establish the prevalence of the fragile X syndrome (chapter 5.1).
The issues addressed in this screening programme were:

1. The prevalence of the fragile X syndrome among ± 3300 mentally retarded individuals, i.e. 3.5% of the total Dutch population of the mentally retarded.
2. The organisation of a programme of this kind in 16 schools and 5 institutes for the mentally retarded with special emphasis on informing all the groups involved.
3. The validation of standardized physical evaluation in order to improve the clinical diagnosis.
4. The evaluation of acceptance and attitudes of relatives towards this type of genetic testing, using pre- and post-test questionnaires and interviews.

The impact on parents and other relatives after the (molecular) diagnosis of the fragile X syndrome was studied using depth interviews after they were informed of the test result, with special emphasis on the transmission of genetic information through participating relatives (chapter 5.2).

Chapter 3 The clinical phenotypes of the fragile X syndrome

3.1 Clinical and molecular studies in fragile X patients with a Prader-Willi-like phenotype
(J Med Genet 1993;30:761-6)

3.2 General overgrowth in the fragile X syndrome: variability in the phenotypic expression of the FMR1 gene mutation
(J Med Genet 1995;32:764-9)

3.3 Variable FMR1 gene methylation of large expansions leads to variable phenotype in three males from one fragile X family
(J Med Genet, 1996;33:1007-10)

Chapter 3.1

Clinical and molecular studies in fragile X patients with a Prader-Willi-like phenotype

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J Med Genet 1993;30:761-6

Abstract

A special subphenotype of the fragile X syndrome is reported which is characterised by extreme obesity with a full round face, small, broad hands/feet, and regional skin hyperpigmentation. It resembles the Prader-Willi syndrome (PWS) and might therefore be named 'Prader-Willi-like'. Unlike the PWS, these PW-like fragile X patients lack the neonatal hypotonia with feeding problems during infancy followed by hyperphagia from toddlerhood. We describe five new fragile X patients and present a clinical update of three previously described patients with the PW-like phenotype. In one family, segregation of either the classical Martin-Bell or the PW-like phenotype was observed and in another family there was repeated transmission of the PW-like phenotype. Previously, one of the patients had been misdiagnosed as having classical PWS, based on clinical findings.

Molecular studies of the FMR-1 gene showed the typical full mutations as seen in fragile X syndrome males. Molecular analysis of the 15q11-13 region, which is deleted in the majority of classical PWS patients, did not show any detectable abnormalities.

In a group of 26 patients with suspected Prader-Willi syndrome but without detectable molecular abnormalities of chromosome 15, one fragile X patient was found.

These clinical and molecular findings illustrate the necessity to perform DNA analysis of the FMR-1 gene in mentally retarded patients presenting with a PW phenotype but without the PWS specific cytogenetic/molecular abnormalities of chromosome 15.

Introduction

The fragile X syndrome is the most frequent form of familial mental retardation. The syndrome occurs in approximately 1/1250 males and 1/2000 females.[1,2] The clinical diagnosis can be confirmed by cytogenetic analysis in folic acid deficient medium, showing a fragile site at Xq27.3.[3,4] The majority of male fragile X patients show the typical Martin-Bell phenotype consisting of mental retardation, long face with large everted ears, and megalotestes.[5-8] Less frequently described phenotypes include short stature, extreme obesity, short, broad hands and feet, and hypogenitalism with hyperpigmentation of the periorbital,

axillary and genital region. Two prepubertal fragile X boys and two adult males were described with this phenotype, which resembled the Prader-Willi syndrome.[9]

Recently, the FMR-1 gene implicated in the fragile X syndrome was isolated and characterised.[10] This gene has a trinucleotide CGG rich repeat in a 5′ exon, which consists of 6 to 54 copies of CGG .[10-11] Premutation alleles are characterised by an increase in the number of triplets to between 52 to 200.[10-14] Male and female premutation carriers are mentally normal and do not show cytogenetic expression of the fragile site Xq27.3.

In male patients the repeat exceeds 200 triplets (designated the full mutation) and the fragile site becomes apparent on cytogenetic testing as well as the clinical features of the fragile X syndrome.

We report five new cases of male fragile X patients with a 'Prader-Willi-like' phenotype and an update of three previously described patients.[9,15]

The recognition of this Prader-Willi-like phenotype among fragile X males prompted us to analyse the FMR-1 gene in 26 patients suspected clinically to have classical Prader-Willi syndrome (PWS) but without any detectable cytogenetic or molecular abnormalities of the 15q11-13 region.

Materials and methods

Seven mentally retarded males with a Prader-Willi phenotype, in whom fragile X syndrome was identified by cytogenetic expression of the fragile site at Xq27.3, were included in the clinical and molecular study.

From another 26 patients cytogenetic and DNA analyses were requested because of clinical suspicion of the classical Prader-Willi syndrome. These patients, who had not shown any detectable molecular abnormalities of the 15q11-13 region, were studied for mutations in the FMR-1 gene.

DNA analysis

The intragenic DNA probe pP2 was used for analysis of the FMR-1 gene.[16] Genomic DNA was isolated from blood leucocytes.[17] DNA (8 μg) was digested to completion with the

restriction enzyme *Eco*RI according to the manufacturer's instructions, separated by gel electrophoresis, and subjected to Southern blot analysis according to standard procedures.[18] The probe was labelled by the random oligonucleotide priming method.[19] After prehybridization and hybridization, the filters were washed in 0.1 × SSC at 65 °C before autoradiography.[18]

DNA analysis of the chromosomal region which is involved in classical PWS, was performed, using standard methods, with markers at GABRB3, D15S9-13 and D15S15-18.[20-22]

Cytogenetic testing

Leukocytes were cultivated under conditions designed to show the fragile site at Xq27.3.[23,24] At least 50 metaphase spreads were examined from each patient.

Results

CASE REPORTS

Case 1

This 15 year old boy (figs 1 and 4) was born at term after a normal gestation and delivery with a birth weight of 3330 g and length of 51 cm. The umbilical cord was wound three times around the neck. His development was slow; at the age of 6 years he was estimated (PEP-test) to be functioning at the level of a 2.5 to 3 years. Up to the age of 10, he was a slender boy (fig 1A), but subsequently he gained weight within a few months without any change in diet. At the age of 12, the fragile X syndrome was diagnosed (fragile site Xq27.3 in 38% of the cells investigated). At this age he had a small phallus with small retractable testes. At 13 years 7 months, his weight was 94.5 kg (+ 6 SD for height), height 173 cm (80th centile), head circumference 57 cm (90th centile), and he had a short arm span (161 cm).[25] He had a full, round face, truncal obesity, short, broad hands and feet with tapering fingers, and narrow, deep nails (fig 1B,C,D). He had normally sized testes for his age (10 ml bilaterally) and sparse pubic hair. His mother and half-sister (who is mildly retarded and slightly obese) both showed expression of the fragile site Xq27.3 in 14% of their blood lymphocytes.

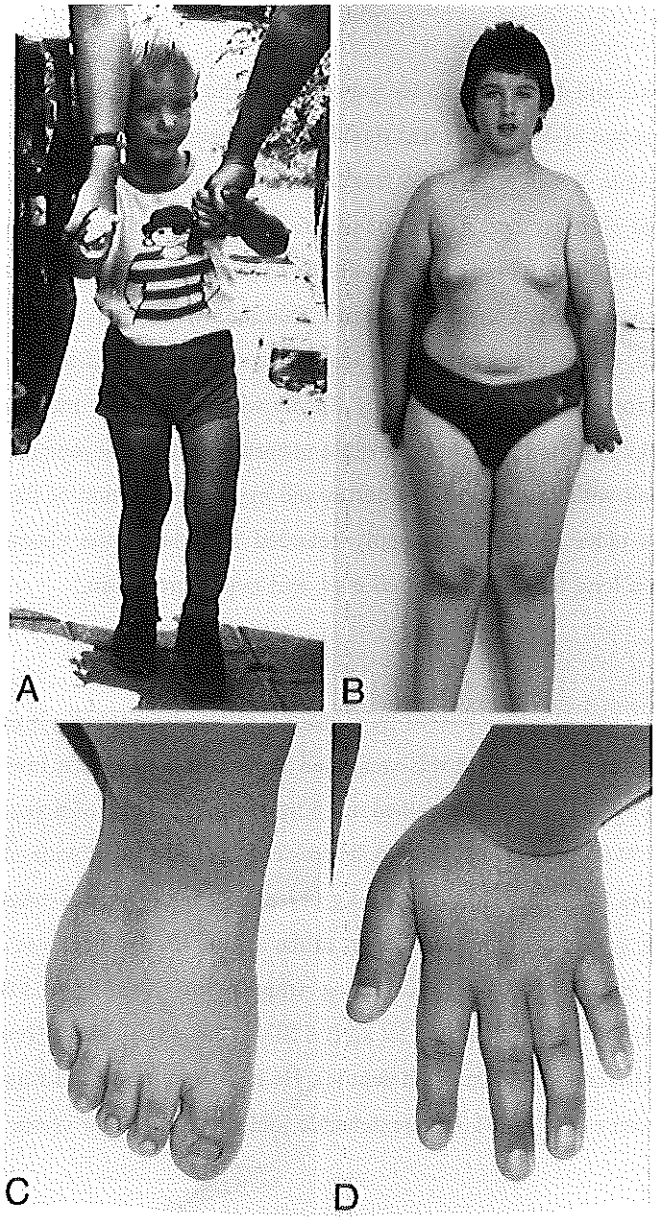

Figure 1 Case 1 (A) at the age of 4 years, showing slender build; (B, C, D) at the age of 12 years showing full body, foot, and hand. Note full round face, truncal obesity, short, broad foot and hand with tapering fingers and narrow, deepset nails.

Case 2

The patient (figs 2 and 4) was born after a normal gestation and delivery with a birth weight of 3425 g. At the age of 2 years, he was found to be mentally retarded and he was diagnosed as having the fragile X syndrome (17% fragile X expression) after a similar diagnosis in his mentally retarded older brother. At the age of 6½ years, he gained weight without change in his diet and became obese within about 5 months. At 9½ years his weight was 44 kg (+ 8 SD for height) and height 129 cm (-2 SD), head circumference 52.5 cm (20th centile). He had a full, round face, ears with a prominent helical root and large lobes, dental crowding, truncal obesity, short, broad hands and feet, stubby fingers with small nails, and short,hyperconvex toe nails (fig 2B,C,D). He had hyperextensible metacarpophalangeal joints and flat feet. His phallus was small with small descended testes and hyperpigmentation of the scrotal skin. His three years older brother is mentally retarded and shows the classical Martin-Bell phenotype with a long face, large everted ears, and macro-orchidism.

Case 3

This boy (figs 3A and 4) was delivered by caesarean section because of a breech presentation with a birthweight of 4000 g. He was weak and floppy during the first few months of life. His development was retarded; he walked at 15 months. At the age of 6 years, he attained a full scale IQ of 50 (WISC-R). At that age, he suddenly gained weight without increased food intake. Two years later his weight was 60 kg (+7 SD for age). He was suspected of having the Prader-Willi syndrome. Nine years later, at the age of 15, the diagnosis of the fragile X syndrome was made by cytogenetic testing (fragile site Xq27.3 in 30% of the cells investigated). By then, his weight was 99 kg (+6 SD for height), height 177cm (40th centile), and head circumference 57.5 cm (80th centile). He had large prominent ears (7.2cm, > 95th centile), large testes (28/16 ml), hyperextensible joints, and a plantar crease.

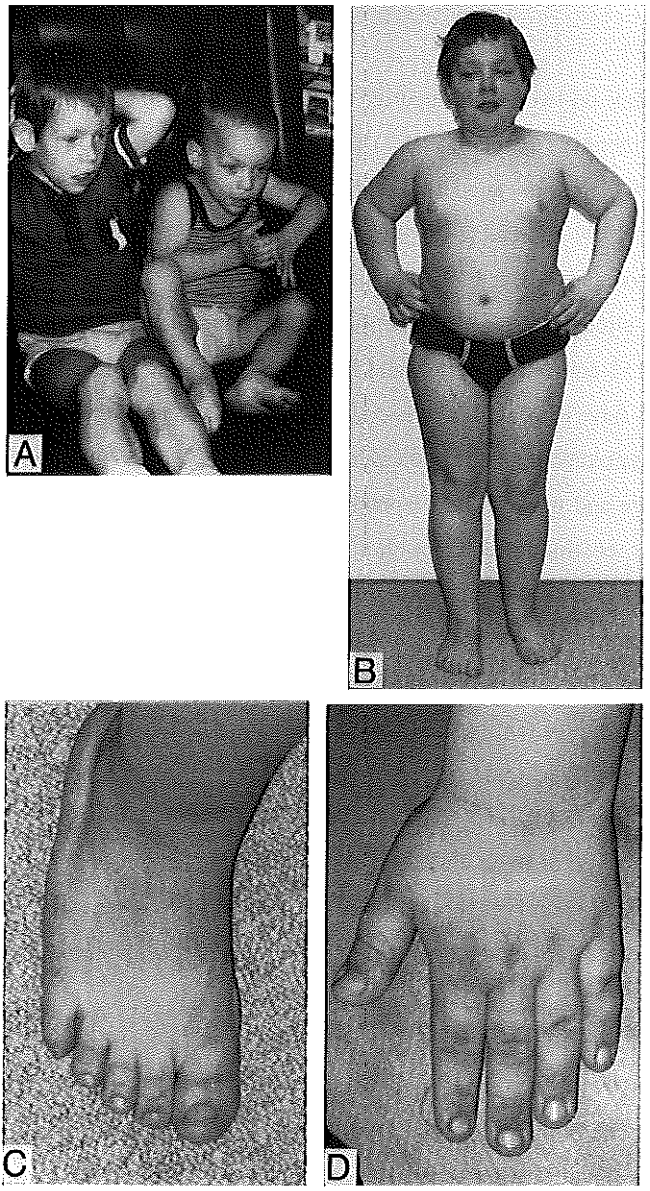

Figure 2 Case 2 (A) on right at the age of 4 years with his brother aged 7 on left. Note the slender build of case 2 and the Martin-Bell phenotype in his brother (long face with large everted ears). (B, C, D) Case 2 at the age of 9 years showing full body, foot, and hand. Note full round face, truncal obesity, short, broad foot with hyperconvex toe nails and hand with stubby fingers.

Cases 4,5,6 and 7

These are an update on two earlier reported patients (cases 4 and 5) and addition of two other affected family members (cases 6 and 7) (figs 3B,C and 4).The two brothers described by Fryns et al[9] in 1987 are now aged 14½ and 12½ years respectively. Their mental function is moderately to severely retarded (IQs 30 and 32, WISC-R). Their phenotype is unaltered from the previous report. The older brother has weight 79 kg (+ 7 SD for height), height 155 (-2SD), and head circumference 54.5 cm; the younger brother has weight 70 kg (+ 6 SD for height), height 153 cm (30th centile), and head circumference 53 cm. Their faces are round and full and they have truncal obesity and short, broad hands and feet with stubby fingers and toes. The skin hyperpigmentation in the periorbital, axillary and genital region became more manifest with age and pubertal development was markedly delayed. In the younger brother the genital status remained identical with a small phallus, hypoplastic scrotum and two small maldescended testes palpable in the inguinal channel. In the older boy the first signs of pubertal development became apparent with the appearance of pubic hair and both testes (8 / 12 ml) were descended in a small scrotum.

Further evaluation of this family showed that two maternal cousins are equally moderately to severely mentally retarded (cases 6 and 7). The diagnosis of the fragile X syndrome has been confirmed in both with fragile X expression in 18% and 12% of the cells. They present with an identical phenotype of truncal obesity, skin hyperpigmentation in the periorbital, axillary, and genital regions, and short, broad hands and feet. They are now 19 and 17 years old, respectively, weights of 98 and 92 kg, heights 172 and 168 cm, and head circumferences 56.5 and 54 cm.

Figs 3B and C shows patients 6 and 7 at the age of 15 and 13 years, respectively. In the older patient, the face became somewhat longer with age. Pubertal development was equally markedly delayed in both and started only after the age of 14 years. Their postpubertal status is normal with normal testes and normal scrotum. The hyperpigmentation of the genital region increased after puberty.

All four patients became obese at the age of 5 to 7 years.

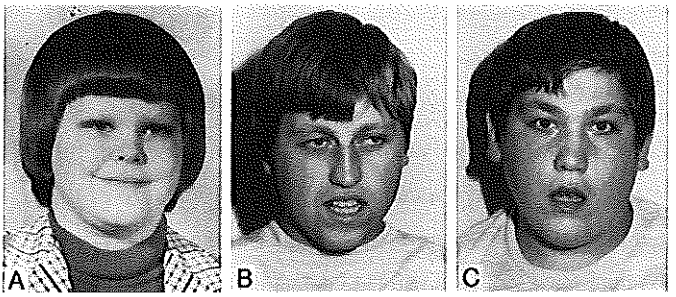

Figure 3 (A) Cases 3, (B) case 6, (C) case 7. Note full round face in all three patients.

Case 8

This 5 year old boy was born at term after a normal pregnancy and delivery with a birth weight of 3100 g. His development was retarded; he walked at 18 months and he spoke only a few words at 5 years. He was obese with cryptorchidism and a small penis. Because he was suspected of having the Prader-Willi syndrome, his DNA sample was referred to our lab for analysis of the 15q11-13 region; however, no specific abnormalities on chromosome 15 were found. He subsequently was studied for the FMR-1 gene mutation, as he belonged to the group of patients with PWS-like clinical features without the specific molecular findings in chromosome 15.

In all patients, thyroid functions, LH, FSH and plasma testosterone levels were normal for their age. The clinical features and molecular findings in cases 1 to 8 are summarised in the table.

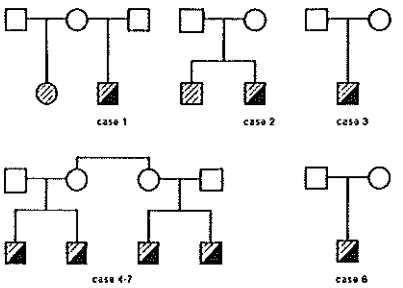

Figure 4 Family pedigrees of cases 1 to 8. Half hatched symbols=patients with fragile X expression and the full mutation in the FMR-1 gene (for cases 6 and 7, fragile X expression only). Half filled symbols= patients with Prader-Willi-like phenotype.

MOLECULAR FINDINGS

In case 1, analysis of the FMR-1 gene showed a full mutation in addition to a premutation allele (insert size 0.4 kb) (fig 5, lane 2). Patient 2 showed a full mutation in addition to a deletion in the FMR-1 gene in a part of the cells (fig 5, lane 3). The size of the deleted part is 250 bp and is located around the CGG repeat.[15] Proximal to the CGG repeat 53 basepairs are deleted and distal to the repeat 178 base pairs are deleted. The allele with the deletion was unmethylated. The patient's brother, with the Martin-Bell phenotype, had a full mutation only and the mother had a premutation (data not shown).

Molecular studies in cases 3, 4 and 5 showed a full mutation of the FMR-1 gene (fig 5, lane 4, 5 and 6). DNA from cases 6 and 7 was not available for study.

In cases 1, 2 and 3 a deletion in chromosome 15q11-13 was not found and maternal disomy of chromosome 15 could be excluded in cases 2 and 3 (data not shown).

From the group of 26 patients with clinical features suggestive of the Prader-Willi syndrome but without the specific cytogenetic/molecular abnormalities of chromosome 15, analysis of the FMR-1 gene showed a full mutation in one patient, case 8.

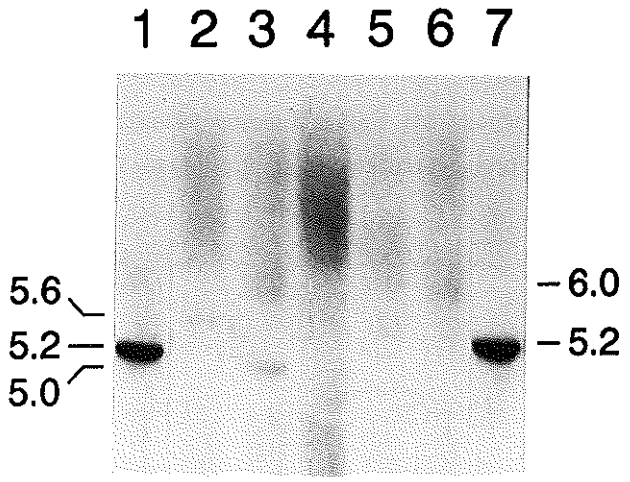

Figure 5 Analysis with probe pP2 of EcoRI digested DNA of two unaffected males (lanes 1 and 7) and five affected males (lanes 2 to 6, cases 1 to 5 in text). In addition to a full mutation, case 1 (lane 2) has a premutation and case 2 (lane 3) a deletion of 250 bp. Cases 3 to 5 (lanes 4 to 6) have a full mutation only.

Discussion

The eight fragile X patients described here show features resembling the Prader-Willi syndrome (PWS), such as truncal obesity, hypogenitalism, and small hands and feet. Consequently, these fragile X patients might be erroneously diagnosed as having Prader-Willi syndrome, as occurred in cases 3 and 8. It is proposed to call this phenotype as 'Prader-Willi (PW) like' to highlight the resemblance between both phenotypes. However, some major differences are observed between the classical Prader-Willi syndrome and the PW-like subphenotype in these fragile X patients. Unlike PWS patients, PW-like fra(X) patients have a normal birthweight and show no hypotonia with feeding problems during infancy (except for case 3). Furthermore, seven patients (cases 1 to 7) developed a sudden gain of weight at the age 5 to 10 years without any change in diet. This is not observed in PWS patients who become obese because of a change in eating pattern which often occurs at a younger age.[26] Another diagnostic difference is the typical fragile X behaviour, including poor eye contact, hyperactivity, short attention span, and perseverative speech,[27] which is expressed by the fragile X patients with the PW-like subphenotype.

It seems that an additional metabolic defect might cause the sudden weight change in these patients. Although routine hormonal investigations in the patients were normal, the typical obesity, short hands/feet and hyperpigmentation suggest an unrevealed metabolic disturbance and further studies have been initiated.

In most patients with the Prader-Willi syndrome, a paternal contribution to 15q11-13 is absent, caused by a deletion in the paternal chromosome or by maternal disomy. Because of the similarity in phenotype, we examined the contribution of maternal and paternal alleles at 15q11-13 in three patients (cases 1 to 3) and no abnormalities were found. Molecular studies of the FMR-1 gene in the PW-like patients showed full mutations. In one patient (case 1) a full mutation with an additional premutation was observed. As this mosaic pattern is seen in around 15% of the fragile X patients,[28] it is not specific for the PW-like phenotype. One patient (case 2) had a full mutation and a deletion of 250 bp in the FMR-1 gene in a part of the cells.[15] Considering the mutation pattern found in other PW-like patients, it is not likely that this deletion is involved in the development of the PW-like phenotype.

Clinical manifestations in the PW-like fragile X patients

	De Vries et al[15]			Fryns et al[19]				
	1	2	3	4	5	6	7	8
Centre of diagnosis	R	R	N	L	L	L	L	P
Present age	15	11	24	14	12	19	17	5
Round full face	+	+	+	+	+	+	+	+
Obesity (>2SD)	+	+	+	+	+	+	+	+
Short stature	–	+	–	+	–	–	–	–
Short,broad hands/feet	+	+	–/+	+	+	+	+	+
Hyperpigmentation								
periorbital	–	+	–	+	+	+	+	–
axillary	–	–	–	+	+	+	+	+
genital	+	+	–	+	+	+	+	–
Age onset weight-change	10	6	7	5-7	5-7	5-7	5-7	ND
Delayed puberty	+	NA	+	+	+	ND	ND	NA
Hormonal investigations								
(TSH,LH,FSH,Test.)	N	N	N	N	N	N	N	N
Fra(X) expression	38%	17%	30%	12%	8%	18%	12%	ND
DNA analysis FMR-1 gene	P+F	D+F	F	F	F	ND	ND	F

+ = present, – = absent, N= normal, ND= no data available, NA= not yet assessable, P= premutation, F= full mutation, D= deletion.
Centre: R= Rotterdam, N= Nashville, L= Leuven, P= Palermo

It is intriguing to observe the occurrence of the classical Martin-Bell phenotype and the PW-like phenotype in two brothers with the fragile X syndrome (case 2, fig 4) and the repeated transmission of the PW-like phenotype in another family (cases 4 to 7, fig 4). At present, there is no molecular explanation for these phenomena. It is conceivable that the mutation at the FMR-1 locus might reduce expression of (nearby) genes. The activity of a gene at the iduronate sulphatase (IDS) locus just distal to the FMR-1 locus is reduced in fragile X patients but a correlation between methylation at the IDS locus and the fra(X) status could not be found.[29]

In the group of 26 patients, referred because of a suspected Prader-Willi phenotype but without detectable molecular abnormalities of chromosome 15, another fragile X patient with

a full mutation in the FMR-1 gene was found (case 8). Of course, such a finding has major implications for the diagnosis and genetic counseling.

These eight (including three previously described) cases of the 'Prader-Willi-like' phenotype confirm this phenotype as a distinct manifestation of the fragile X syndrome, as described by Fryns et al[9] in 1987.

It seems advisable to perform DNA analysis of the FMR-1 gene in all patients presenting with a Prader-Willi phenotype without the specific molecular and cytogenetic findings on chromosome 15q.

Acknowledgments

We thank MN van der Est, L Bakker, WH Deelen and E de Graaff for performing the DNA analysis, and Professor D Lindhout and Dr M Roccella for diagnosing and referring a case. We thank the patients and their families for their kind cooperation.

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Chapter 3.2

General overgrowth in the fragile X syndrome: variability in the phenotypic expression of the FMR1 gene mutation

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J Med Genet 1995;32:764-9

Abstract

The fragile X syndrome, which often presents in childhood with overgrowth, may in some cases show some diagnostic overlap with classical Sotos syndrome.

We describe four fragile X patients with general overgrowth, all of whom are from families with other affected relatives who show the classic Martin-Bell phenotype. Molecular studies of the FMR1 gene in all cases showed the typical full mutation as seen in males affected by the fragile X syndrome. Endocrine studies were unremarkable, except in one case where there were raised levels of insulin-like growth factor-I (IGF-I) and insulin-like growth factor binding protein-3 (IGFBP-3).

These cases illustrate the clinical variability of the fragile X syndrome and the necessity of performing analysis of the FMR1 gene in mentally retarded patients presenting with general overgrowth.

Introduction

The fragile X syndrome is the most common cause of familial mental retardation with an estimated prevalence of 1 in 1250 for males, and 1 in 2000 for females in western countries.[1,2]

Identification of the FMR1 gene in 1991, and the classification of the implication of its full and premutation states, allow definite DNA molecular diagnosis in patients and carriers in nearly all cases.[3] Nearly all fragile X patients show an amplification of the CGG repeat (>200) in the 5′ exon of the FMR1 gene (full mutation), as compared to 6 to 54 repeats in controls and 43 to 200 CGG repeats in unaffected carriers of the premutation.[3-7]

Most affected males present with the Martin-Bell phenotype, a combination of mental retardation, a long, large face with prominent, everted ears, and macro-orchidism.[8-11]

A phenotype less frequently observed in fragile X patients consists of extreme obesity, short stature, stubby hands and feet, and diffuse hyperpigmentation, which has been designated the Prader-Willi-like subphenotype.[12-14]

In addition, a Sotos-like phenotype was reported in 1986 in two fragile X boys featuring large size at birth, unusual length, large head circumference, and minor facial anomalies.[15]

Here we report clinical, endocrine, and DNA studies in four fragile X patients with overgrowth.

Patients and methods

Four mentally retarded males with phenotypic features resembling Sotos syndrome were identified as fragile X positive either by cytogenetic analysis (cases 1, 3, and 4) or by gene mutation analysis (case 2) and are the subjects of this report.

DNA ANALYSIS

Genomic DNA (8 μg) isolated from blood leucocytes[16] was digested with the restriction enzyme *Hin*dIII according to the manufacturer's instructions, separated by gel electrophoresis, and subjected to Southern blot analysis according to standard procedures.[17] The intragenic DNA probe pP2 was used for analysis of the FMR1 gene.[18] The probe was labelled by the random oligonucleotide priming method.[19]

ASSAYS OF IGF-I AND IGFBP-3

IGF-I in serum was determined by specific radioimmunoassay (RIA) after acid Sep-Pak C18 (Wares Associates, Milford, MA, U.S.A.) chromatography.[20,21]

IGFBP-3 levels were determined by RIA. IGFBP-3 was isolated from human plasma using the purification as modified by Martin and Baxter.[22] IGFBP-3 was iodinated using the chloramine-T method (specific activity 50-100 μCi/μg). New Zealand white rabbits were immunised with 110 μg purified IGFBP-3 in complete Freund's adjuvant by multiple subcutaneous injections along the back and proximal limbs. After 80 days an antiserum was obtained from one rabbit, which precipitated 50% of [^{125}I]IGFBP-3 at a 1:10,000 dilution. The antiserum did not crossreact with IGF-I, IGF-II, or IGFBP-1. The assay buffer used in the RIA was composed of 0.05 mol/l sodium phosphate (pH 7.4), 0.01 mol/l EDTA, 0.05% Tween-20, 0.2% BSA, and 0.02% NaN.[22] Standards were prepared from purified IGFBP-3, stored at -70°C. Standard dilutions ranged from 0.06 to 8 ng/tube. Duplicates of serum samples were diluted 1:400 in assay buffer. The incubation mixture

consisted of 100µl assay buffer, 100µl standard or diluted sample, 50 µl antibody (1:16,000), and 50µl tracer (10,000 cpm). After incubation for 18 hours at room temperature in polystyrene tubes, 100 µl Sac-Cel solid phase anti-rabbit coated cellulose suspension (Innogenetics, Nijmegen, The Netherlands) was added. Complex formation was complete after 30 minutes at 20°C. Then 0.6 ml distilled water was added and the samples were subsequently centrifuged at 10,000 *g* for three minutes. Pellets were washed once with 0.6 ml distilled water and counted in a τ-counter (Packard Instrument Co Inc, Downers Grove, IL). The sensitivity of the assay was 0.5ng/ml. Intra-assay variation was 6.9% at 2.52 mg/l and 12.9% at 0.83 mg/l. The interassay variation was 10.8% at 2.88 mg/l and 9.9% at 1.67 mg/l. Serum levels showed parallelism with the purified human as well as recombinant glycosylated human IGFBP-3 (kindly provided by Dr C Maack, Celtrix Pharmaceuticals Inc, Santa Clara, CA, USA).

Results

CASE REPORTS

Physical signs, not mentioned in the case histories, are shown in the table.

Case 1

This boy (fig 1A, B) was born after an uneventful pregnancy and delivery with a birth weight of 3830 g (75th centile) and a height of 52 cm (70th centile). His development was slow, crawling at 12 months, sitting at 15 months, and walking with support at 20 months. At the age of 18 months, he was cytogenetically diagnosed as having the fragile X syndrome (40% fragile X expression) after the same diagnosis in his mentally retarded 4 year old maternal cousin (fig 2). At 2½ years his height was 98 cm (90th centile), weight 23 kg (>+4 SD for age and for height), and head circumference 52 cm (85th centile)(fig 3A, B). He had a full, round face with a high, prominent forehead, broad dental ridges, and normal sized ears. He had large hands (12.2 cm, >98th centile) and large feet (17cm, >98th centile) with short, broad toenails. His phallus and testes were of normal size, but he had a shawl scrotum. There were hyperextensible metacarpophalangeal joints, valgus

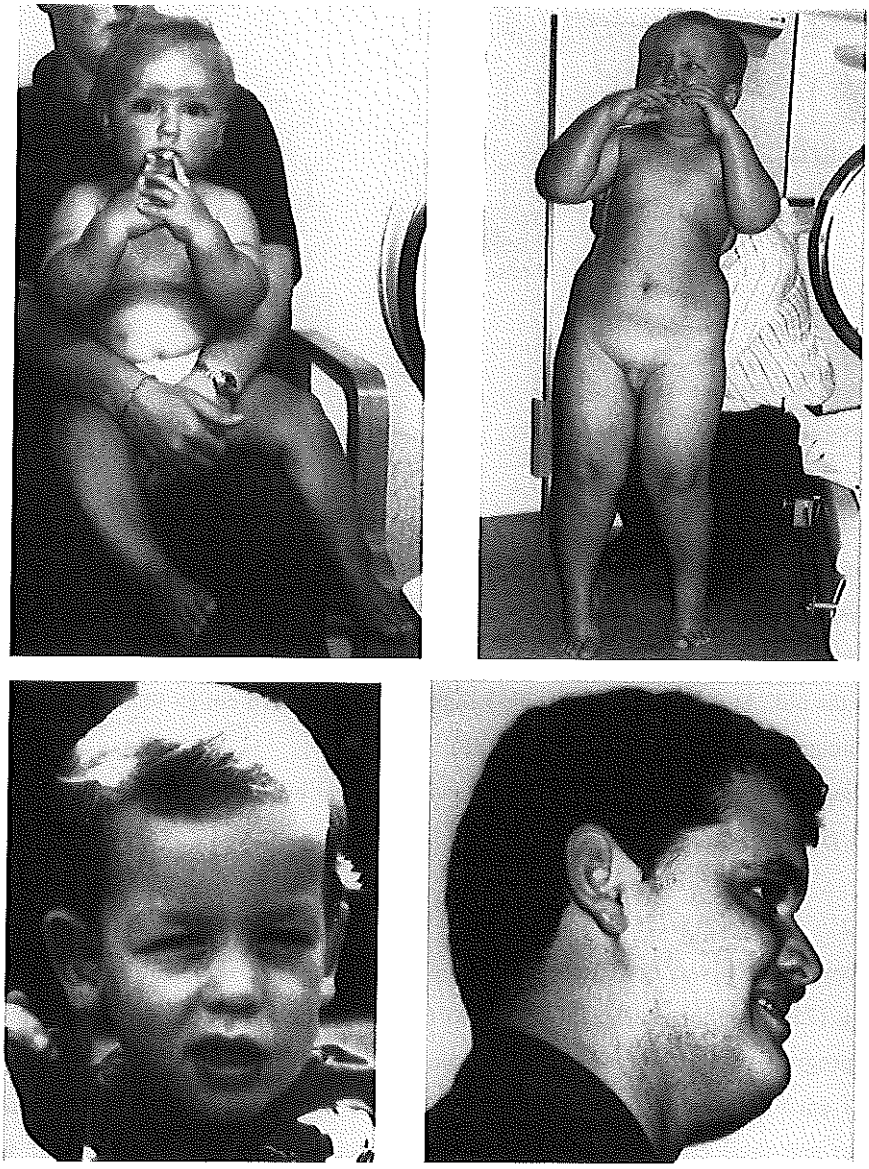

Figure 1 (A) Case 1 at the age of 1 5/12 years developing overgrowth; (B) case 1 at the age of 3 7/12 years showing progression of the general overgrowth.(C) Case 2 at the age of 3 years. Note bitemporal narrowing, frontal bossing, a high hairline, large everted ears and narrow, down-slanted palpebral fissures.(D) Case 3 at adult age. Note long face with high forehead and prominent mandible.

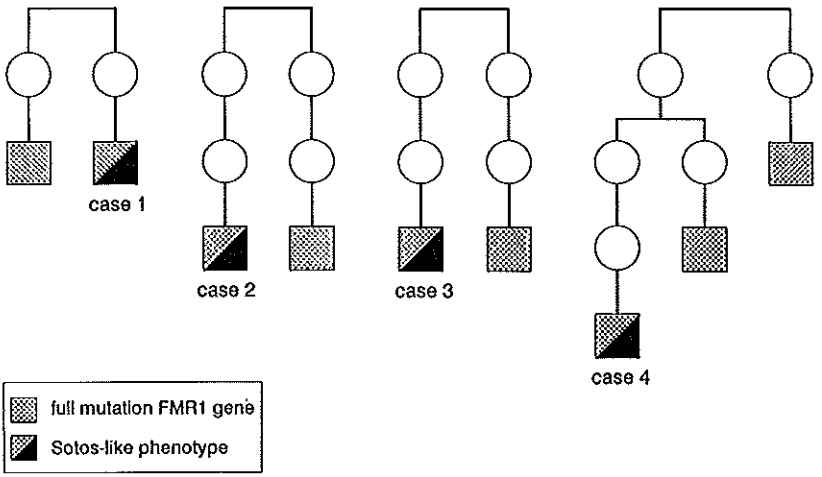

Figure 2. Family pedigrees of case 1 to 4.

position of the knees, and flat feet. The metacarpophalangeal pattern profiles at age 3 and 4 showed relatively long proximal phalanges and metacarpal 1, with normal metacarpals 2-5, middle, and distal phalanges. His bone age was 3 years 3 months when he was 2 years 5 months old. At 4 years 2 months his height increased to 116 cm (> 98th centile) and his headcircumference to 55 cm (>98th centile).

The diagnosis of the fragile X syndrome was confirmed by showing a full mutation of the FMR1 gene.

Case 2

This boy (fig 1C) was born after a normal pregnancy and delivery with a birth weight of 3800 g (75th centile) and height of 52 cm (70th centile). During his first year he developed progressive macrocephaly (head circumference of 48 cm = 50th centile at 3 months and 51 cm > 98th centile at 13 months). At 1 year 8 months his height was 89.5 cm (90th centile) and head circumference 52.5 cm (> 98th centile)(fig 3B, C). The skull showed bitemporal narrowing, frontal bossing, and a high hairline. The palpebral fissures were downward slanted and narrow. He had large, everted ears and a high palate. His phallus was a normal size and there was cryptorchidism. The metacarpophalangeal pattern profile

at 3 years showed relatively short metacarpals and normal proximal phalanges.

The Sotos syndrome and the fragile X syndrome were considered in the differential diagnosis. Analysis of the FMR1 gene showed the full mutation, confirming the fragile X syndrome.

At 3 years, bone age (Greulich and Pyle standards) was in accordance with the chronological age.

The height and head circumference of both parents were normal: mother's head circumference 53 cm, and height 169 cm; father's head circumference 56.5 cm and height 170 cm.

Case 3

This male (fig 1D) was born at term after a normal pregnancy; birthweight was 3950 g (80th centile) and height 56 cm (>98th centile). Neonatal weight gain was above average. He sat alone at 10 months and walked alone at 14 months. At 2 years 10 months he had mastered about 20 single words, his height was between the 90th and 97th centile, and head circumference 52.4 cm (98th centile). He had a broad, large nose and valgus deformity of the feet. A diagnosis of cerebral gigantism was considered at that time. At 13 years of age, the diagnosis of the fragile X syndrome was made by cytogenetic testing (28% fragile X expression).

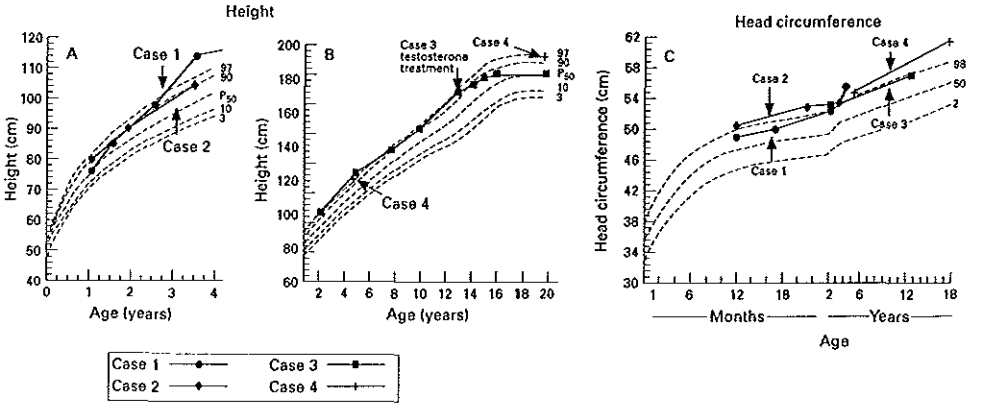

Figure 3 (A, C) Growth and head circumference charts for cases 1 and 2, (B, C) growth and head circumference charts for cases 3 and 4.

At 12 years of age, testosterone enanthate treatment was started in an attempt to limit his final height. At that age, his height was 172.2 cm (98th centile) with a weight of 60 kg (90th centile)(fig 3B, C). His final height after three years of testosterone treatment was 183.5 cm (50th centile). His weight was 97 kg (> 98th centile).

The height and head circumference of both his parents were normal: mother's head circumference 55 cm and height 172 cm; father's head circumference 57.5 cm and height 176 cm.

Case 4

This male (fig 4A, B) was born at term with a birth weight of 3125 g (25th centile). The neonatal period was uneventful. He was found to be obese at the age of 3 months with a weight of 7000 g (98th centile). His developmental milestones were moderately delayed and he was thought to have "mild nonspecific mental retardation" at the age of 18 months. At the age of 4 years 11 months his height was 121 cm (> 98th centile), weight 35.8 kg (> 98th centile), and head circumference 54.2 cm (> 98th centile)(fig 3B, C). He was an obese and retarded child without major abnormalities, except for a large penis with slightly

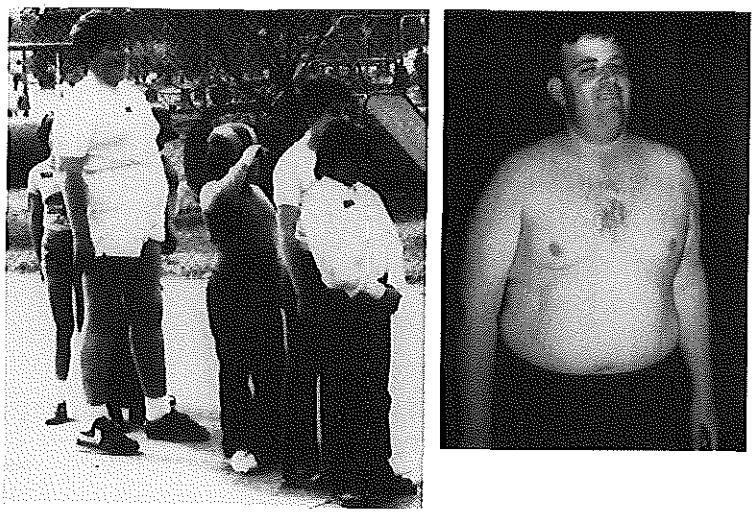

Figure 4 (A) Case 4 at the age of 9 years among classmates, (B) case 4 at adult age. Note the general overgrowth, long face with high forehead, broad chin, large, everted ears and downward slanted palpebral fissures.

enlarged testes for his age. His bone age was 5½ years (7 months advanced according to the Greulich and Pyle standards). Skull *x* rays were normal for age and random growth hormone levels were not elevated (< 2 ng/ml). The diagnosis of cerebral gigantism was considered.

At the age of 18, the diagnosis of fragile X syndrome was made by cytogenetic testing (18 % fragile X expression). Two male relatives (the son of the maternal grandmother's sister and the son of the sister of the maternal great grandmother) (fig 2) were also affected by the fragile X syndrome (including FMR1 gene full mutations) but showed the Martin-Bell phenotype without any symptoms of overgrowth.

At 24 years of age, height was 193 cm (>P98) and head circumference 61 cm (+4SD). The height and head circumference of both his parents were normal, except for father's head circumference: father's height 180 cm and head circumference 60 cm (> 98th centile); mother's height 168 cm and head circumference 57 cm.

MOLECULAR AND ENDOCRINE FINDINGS

Molecular studies showed a full mutation of the FMR1 gene in all cases, with an additional premutation in cases 1 and 3 (fig 5).

In case 1, both the insulin-like growth factor-I (IGF-I) and insulin-like growth factor binding protein 3 (IGFBP-3) were raised. At the age of 2 years 9 months the IGF-I was 138 ng/ml (>+2 SD) and IGFBP-3 was 2.14 mg/l (>+1 SD), at the age of 3 years 7 months the IGF-1 was 85 ng/ml (>+1 SD) and IGFBP-3 was 2.52 mg/l (>+2 SD). His

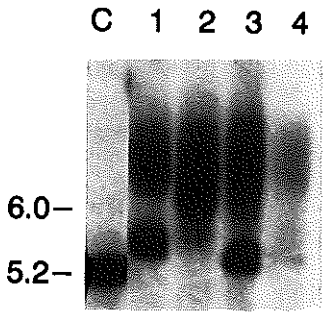

Figure 5 Analysis with probe pP2 of HindIII digested DNA of one unaffected male (lane 1) and cases 1 to 4 (lanes 2 to 5). In addition to a full mutation, cases 1 and 3 have a premutation.

thyroid function, GH, LH, FSH, and plasma testosterone levels were normal.
In case 2, the IGF-I and IGFBP 3 were both normal at the age of 3 years 4 months, 82 ng/ml and 1.16 mg/l respectively.
Additional endocrine studies could not be performed in cases 3 and 4.
The clinical, molecular, and endocrine findings of cases 1 to 4 are summarised in the table.

Discussion

Overgrowth as a feature of the fragile X syndrome has been reported in childhood, but mostly relating to head size.[23-26] Head circumference, in comparison with the normal population[24-27] and with non fragile X retarded males,[28] was found to be increased in children and adults. One study reported an increased head circumference in fragile X males as infants and children but not as adults.[23] Another study showed an increase in height (> 95th centile) of nine out of 29 fragile X boys in childhood, with normal height in adult fragile X males.[25] Several groups have described normal height in young fragile X patients, with adults being below mean normal height.[24,27-29]
The four present cases are special in that the enlarged head circumference, together with extreme overall body overgrowth, was samilar to that described earlier in two fragile X boys.[15] The latter observation led to the proposition that a "Sotos-like" phenotype of the fragile X syndrome might exist. This study extends these observations in respect to the overgrowth. In all four cases reported here, a full mutation in the FMR1 gene was found with an additional premutation in cases 1 and 3, providing a clear diagnosis of the fragile X syndrome. The two adult cases (3 and 4) had previously been diagnosed as a Sotos syndrome at a time when the fragile X syndrome was unknown. Originally, the Sotos syndrome was characterised by large body size and early accelerated growth in combination with acromegaloid features, advanced bone age, developmental delay, and a non-progressive neurological disorder.[30] Developmental delay was observed in all four present cases (mental retardation is the major feature of the fragile X syndrome) and other features

Clinical manifestations in the fragile X patients with symptoms of overgrowth

	1	2	3	4	all
Current Age (y)	4	3	29	26	
Birth weight (g)	3830	3800	3950	3125	
Adult height (cm)	X	X	184*	193	
Obesity (> 2 SD for age and height)	++	–	+	++	3/4
General features of the Sotos syndrome[31], in cases 1-4					
Large body size and early accelerated growth	+	+	+	+	4/4
Acromegaloid features	+	–	+	+	3/4
Advanced bone age	NS	–	X	NS	0/3
Developmental delay	+	+	+	+	4/4
Facial features of the Sotos syndrome[31], in cases 1-4					
Frontal bossing	+	++	–	–	2/4
High hairline	+	++	+	+	4/4
Prominent jaw	–	+	+	++	3/4
Downward slanted palpebral fissures	+	+	–	+	3/4
Facial flushing	+	+	–	–	2/4
Dolichocephaly	–	+	+	++	3/4
High palate	+	+	+	++	4/4
Laboratory findings					
Growth hormone	N	N	X	N	3/3 normal
Insulin-like growth factor-I	↑	N	X	X	1/2 elevated
Insulin-like growth factor binding protein-3	↑	N	X	X	1/2 elevated
FMR1 gene	P+F	F	P+F	F	4/4 FM

+ = present, – = absent, N= normal, P= premutation, F= full mutation, X= not assessable, NS = not significant (advancement of 10 months and 7 months, respectively)
* *After testosterone therapy*

of the Sotos syndrome are also apparent in the present cases (table), including large body size (all cases) and acromegaloid features (cases 1, 3, and 4). The advanced bone age in cases 1 (10 months advanced) and 4 (7 months advanced) could be considered within the normal variation of the population.

Sotos syndrome is further characterised by additional features (for review see 31) which are helpful for clinical differential diagnosis (table 1).

The facial appearance in case 2 shows some similarities to Sotos syndrome; however he lacks acromegaloid features and advanced bone age. In cases 1, 3, and 4 the facial

features, which are difficult to observe owing to extreme obesity, seem atypical of the Sotos syndrome. Obesity is rarely observed in patients with the Sotos syndrome[31] but is occasionally seen in fragile X patients.[12-14] Other diagnostic differences are normal or high normal range birth weights (all cases) and normal birth heights (cases 1 and 2; case 4 unknown), which are clearly less extensive than in Sotos syndrome patients. Only case 3 had a birth height of 56 cm (> 2 SD) as has been observed in most patients with Sotos syndrome.[31]

The metacarpophalangeal pattern profile (MCPP) in case 1 corresponds with the profiles observed in patients with Sotos syndrome,[32-34] while the MCPP in case 2 resembles the profiles seen in fragile X patients.[34]

The phenomenon of general overgrowth prompted endocrine studies in the two younger patients. Raised IGF-I and IGFBP-3 was found in case 1, perhaps related to the extreme overgrowth. Raised IGF-I by bioassay has been found before in Sotos patients.[35,36]

The family of IGFs and their binding proteins are involved in growth processes, including cartilage development, tissue regeneration and ovarian function.[37,38] Early menopause has been reported in fragile X carriers[39] and has been linked recently to the observation of increased dizygotic twinning among offspring of these carriers.[40] The latter phenomenon might also be related to local IGF-I function.

The general overgrowth in the present cases might be considered as a distinct manifestation of the fragile X syndrome. Although "Sotos-like" might seem a descriptive term for this variant of the fragile X syndrome in the present cases, this term might be both confusing and lacking precision in the absence of the full characteristics of Sotos syndrome in all cases.

The fragile X syndrome is phenotypically heterogeneous with some patients showing a tendency to general overgrowth (presented here) and another minority of fragile X patients presenting with phenotypes suggesting the Prader-Willi syndrome.[12-14] This has important implications for clinical and differential diagnosis.

In conclusion, the present four cases illustrate the clinical variability of the fragile X syndrome and the necessity of performing analysis of the FMR1 gene in mentally retarded

patients presenting with general overgrowth, and in cases suspected of having Sotos syndrome.

Acknowledgments

We thank S Mohkamsing for performing the DNA analysis and Drs A Schuller and G J Bruining for helpful advice. We thank the patients and their families for their kind cooperation.

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Chapter 3.3

Variable FMR1 gene methylation of large expansions leads to variable phenotype in 3 males from one fragile X family

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J Med Genet 1996;33:1007-10

Abstract

The fragile X syndrome is caused by an expanded CGG repeat (> 200 units, full mutation) at the 5′ end of the FMR1 gene, which is associated with methylation of a CpG island upstream of the FMR1 gene and down regulation of the transcription.

We describe three related males with full mutations in the FMR1 gene, as defined by size, but with different percentages of unmethylated alleles (±90%, 35% and 15%, respectively) as studied in leucocytes. Normal mental status was observed in the male who showed 90% lack of methylation, whereas his two cousins were retarded. The mentally normal male did show some minor facial features of the fragile X syndrome; the FMR protein was detectable in 75% of his leucocytes. In all three cases, the proportion of unmethylated FMR1 genes corresponded to the percentage of leucocytes showing FMR1 protein production. Our results indicated a direct relationship between methylation and the ability to produce FMR protein.

These cases will be discussed in relation to phenotypic effects of incompletely methylated full mutations in the FMR1 gene as observed by others.

Introduction

In the FMR1 gene,[1-4] which is involved in the fragile X syndrome, a polymorphic CGG repeat is present in the first exon. The number of the CGG repeats varies between 6 and 54 repeats in the normal population. Phenotypically normal male and female premutation carriers of this X-linked disorder have repeats in the range 54 to 200. A full mutation is defined by a repeat length > 200. The full mutation is associated with methylation of the CpG island upstream of the FMR1 gene and with the subsequent shut down of gene transcription and, consequently, the absence of the FMR1 protein.[3,5] The latter is regarded as the major cause of mental retardation in male and female fragile X patients.[5] Recently, Feng *et al*[6] suggested a reduced translation of unmethylated FMR1 alleles with > 200 repeats, leading to diminished FMR1 protein production as a direct result of the repeat size.

We report a mentally normal male with an FMR1 gene trinucleotide repeat expansion of

> 200 repeats and an almost complete (±90%) lack of methylation. His two retarded cousins also had full mutations, as defined by size, but showed higher degrees of methylation. The FMR protein was assayed in leucocytes of these three related males. Our data indicated that methylation and not the length of the repeat is the primary determinant of diminished FMR1 protein production.

Patients and methods

The three males who are the subjects of this report were cousins from one fragile X family. The family was ascertained through the brother of case 3, who was referred to our Department of Clinical Genetics for genetic counselling.

DNA ANALYSIS

Genomic DNA was isolated[7] from blood leucocytes and fibroblasts, digested with *Hin*dIII or *Hin*dIII + the methylation sensitive enzyme *Eag*I, and hybridised with probe pP2 according to standard protocols.[8] The autoradiograms were made in duplicate and with different exposure times, and analysed by densitometry using a scanner (HP, scanjet IICX).

PROTEIN ANALYSIS

Blood smears were made from one drop of blood within two hours after collection. Slides were air dried, frozen, and stored at -80°C. The FMR1 protein was visualised by using mouse monoclonal antibodies 1A1 against FMR1 protein followed by a second incubation step with goat antimouse immunoglobulin conjugated with biotin (DAKO) according to the procedures described previously.[9]

DETERMINATION OF IQ LEVELS

The Wechsler Adult Intelligence Scale (WAIS) was used to test the intellectual abilities by one examiner (AD) who was not informed about the genetic status of the persons tested. The verbal, performance, and full scale IQ scores were calculated.

Results

CASE REPORTS

Case 1

This 29 year old man (fig 1A) was examined because of a family history of the fragile X syndrome. He showed a normally proportioned male phenotype, height 193 cm (98th centile) and head circumference 58 cm (90th centile). He had a long face with a prominent chin, high forehead, normally sized but everted ears, and dental crowding in the lower jaw. Testicular size was normal (25ml/25ml). Apart from the facial characteristics, no other fragile X features, for example, behavioral symptoms, were seen.

His full scale IQ level (WAIS) was 101 points with a verbal scale IQ score of 108 and performance scale IQ score of 92. His sister with normal FMR1 genes had a full scale IQ of 115 and the sister with a premutation had a score of 96.

Chromosome studies in folic acid deficient medium[10] showed a normal 46,XY karyotype in cells of case 1, without expression of the fragile site at Xq27.

Case 2

This 27 year old mentally retarded cousin of case 1 (fig 1A) had several physical features of the fragile X syndrome, including high, broad forehead, periorbital fullness, macro-orchidism (>35 ml), hyperlaxity of small and large joints with pes planus and genu valgum, a simian crease on the left palm, and soft velvety skin. Typical fragile X behavioural characteristics avoidance of eye contact, handflapping, and handbiting. Because of his refusal to speak with strangers, IQ testing was not possible; he attended a special school for the mentally retarded.

Case 3

The other mentally retarded cousin (fig 1A) of the same age (27 years) had a long, narrow face, prominent chin, flat feet, and macroorchidism (50 ml/50 ml). Typical fragile X behaviour included avoidance of eye contact, obsession with neatness, handrubbing, and

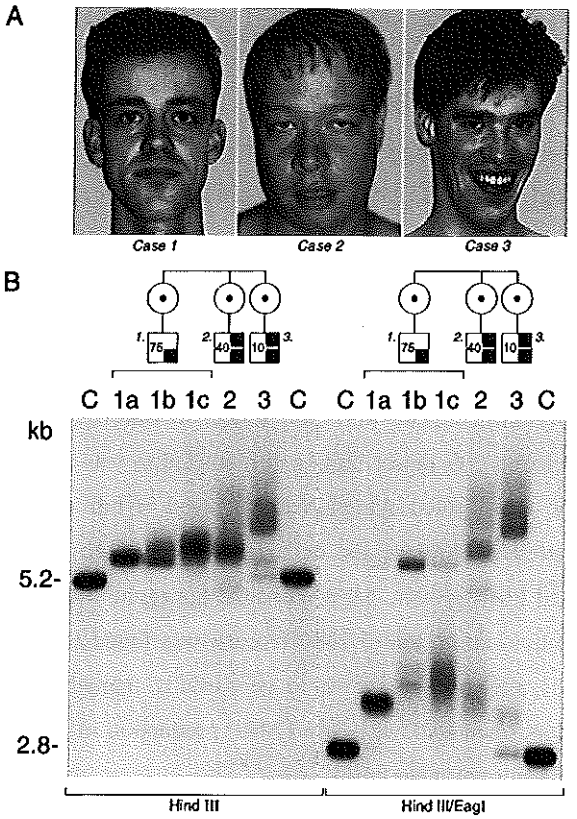

Figure 1 (A) Cases 1, 2 and 3. Note a long face with a prominent chin and high, broad forehead in case 1, high, broad forehead and periorbital fullness in case 2, and a long, narrow face with a prominent chin in case 3.

(B) Pedigree of cases 1-3. Filled lower right box = minor or major fragile X physical features present, filled upper right box = mental retardation present, number in left half = percentage of the leucocytes expressing the FMR protein, and analysis with probe pP2 of HindIII (left) and HindIII/EagI (right) digested DNA of case 1 (lane 1a, fibroblasts; lane 1b, EBV transformed lymphoblasts; lane 1c, leucocytes), case 2 (lane 2, leucocytes), case 3 (lane 3, leucocytes) and several normal male controls (lanes C).

murmuring to himself. Twenty percent expression of the fragile site at Xq27 was observed in lymphocytes cultured in folic acid deficient medium.[10]

MOLECULAR FINDINGS

In case 1, leucocytes with an expansion of 200 to 800 CGG repeats of the FMR1 gene were found by DNA analysis. Double digestion with *Hin*dIII and the methylation sensitive restriction enzyme *Eag*I showed that 90% of the alleles were unmethylated (as estimated by densitometry; fig 1B, lane 1c, right). To assess whether cells with such alleles were able to express FMR protein (FMRP), further studies were initiated. The FMRP was detectable in 75% of the leucocytes by antibody testing in a bloodsmear (normal male control 90%). Apart from cells derived from fresh blood, cultured fibroblasts and an EBV transformed lymphoblastoid cell line were also tested. In fibroblasts the size of the CGG repeat was estimated to be 200 repeat units and methylation was absent (fig 1B, lane 1a, left and right). EBV transformed lymphoblasts showed a full mutation (size 200-300 repeats) and methylation in 70% of the cells (fig 1B, lane 1b). Less than 5% of the lymphoblasts expressed FMR protein. Most probably, the results reflect clonal effects.

In case 2, a full mutation, sized 200 to 1300 repeats, was found in his leucocytes. By the methylation assay, the alleles were shown to be partially unmethylated (35%) (fig 1B). Protein analysis in bloodsmears showed 40% FMRP production in his leucocytes (controls: fragile X male 2% and normal male 90%).

In case 3, the leucocytes showed a full mutation in the FMR1 gene (size 400-1600 repeats, fig 1B). Absence of methylation was found in 15% of the alleles. The protein antibody test showed 10% of the leucocytes expressing the FMR protein.

All three mothers of these males were carriers of a premutation.

Discussion

The cases described here belong to a subgroup of subjects from fragile X families with 'methylation mosaicism'.[11] In a large multicenter study, 'methylation mosaicism' was observed in 3% (15/500) of the males with a full mutation; two males had large unmethylated mutations (>230 repeats), but additional molecular and clinical data were not reported.[12] Table 1 is an overview of clinical and molecular data of 14 currently known

Table 1 Overview of 'incompletely methylated full mutations'

	References													
	13			14			11, 15		6	16		Current family		
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	case 1	case 2	case 3
Age	36	45	43	12	13	22	29	8	1.6	74	72	29	27	27
No in original report	II.6	II.2	II.3	2	3	25	III.5	14	III.2	7298	7241			
DNA (leucocytes)														
Expansion size full mutation[a]														
smallest	170/	130/	100/	160/	115/	150/	130/	230/	266[b]	170/	100/	200/	200/	400/
largest	530	200	270	1270	850	1250	470	300		340	1500	800	1300	1600
Unmethyl. full mutation (%)	55	97	97	100	100	100	60	90	100	100	100	90	35	15
FMR protein														
Western Blot (%)						35[c]			12[b]	red.[c,d]	red.[c,d]			
Cells expressing FMRP (%)										100	100	75	40	10
Phenotype														
MR[e]	+	–	–	–	–	+/–	–[f]	+/–[f]	NK	–[f]	–[f]	–	+	+
Physical features	yes	minor	minor	minor	minor	minor	minor	yes	minor	no	no	minor	yes	yes
Fragile X (%)	11	0	0	4	5	4	1-4	3		6	13	0		20

[a] *Number of repeats, if necessary, estimated from the sizes (kb) in the original reports.*
[b] *Fibroblast cell line.*
[c] *Lymphoblast cell line.*
[d] *Reported reduced but not quantitated.*
[e] *Mental retardation: + = IQ<70 ; +/– = 70<IQ<85 ; – = IQ>85.*
[f] *Mental status clinically estimated without specific IQ testing.*
NK=not known.

cases.[6,11,13-16] The presence of facial characteristics of the fragile X syndrome with normal mental capacities is interesting (case 1 of this report and other reports, table 1). Rousseau *et al*[15] reported a normally functioning male with 130 to 470 CGG repeats and lack of methylation in 60% of his leucocytes, who also displayed some minor fragile X features. These observations suggest different thresholds for phenotypical expression of the full mutation in different tissues or during different critical times in development. Remarkably, the combination of normal intellectual development and facial fragile X features has also been observed in female obligate carriers with cytogenetic expression,[17] suggesting a more common phenomenon.

The methylation of a CpG island upstream of the FMR1 gene has been associated with the lack of transcription in males with a full mutation. Studying 'methylation mosaics' might shed light on mechanisms concerning translation of unmethylated expanded FMR1 alleles. The existence of this special genotype indicates that amplification occurs before methylation.[18] So far, few FMRP studies have been performed in tissues of 'methylation mosaics'. Recently, Feng *et al*[6] reported markedly diminished FMRP production in fibroblast clones from transcripts with more than 200 repeats, suggesting a hindrance for 40S ribosomal subunit migration along the >200 repeats. However, in the three cousins reported here a direct relation is seen between the percentage of unmethylated full mutation in leucocytes, protein production, and cognitive function, suggesting normal translation of expanded unmethylated FMR1 alleles. This is also supported by the observation of Smeets *et al*[16] of two mentally normal males with unmethylated full mutations and FMR1 protein expression, not only in all leucocytes, but also in a lymphoblast cell line with a single repeat length of 400. This observation and our own also show that the clonal effects observed in cell lines make them less informative for routine diagnostic studies.

The mental retardation of case 2, who has FMRP production in 40% of his leucocytes suggests that protein expression at and below this level is insufficient for normal cognitive functioning. This is in line with another report of a retarded male with the fragile X phenotype who had 28% FMR1 protein expression owing to a deletion in a proportion of

his cells[19]. The observed situation is reminiscent of females heterozygous for the full mutation in the FMR1 gene who are affected in 60-75% of the cases[12,20] with a positive correlation between the proportion of normal FMR1 alleles on the active (unmethylated) X chromosome and IQ.[20] Although FMR protein expression studies have not been performed in brain tissue of female full mutation carriers or methylation mosaic males, it seems that in neither case does compensation of deficient brain cells by cells expressing the normal protein occur to a sufficient level.

The present data suggest that methylation is directly involved in down regulation of transcription and indicate that transcripts with more than 200 repeats can normally be translated into FMR protein in vivo. Further studies might focus on developing a technique of selective demethylation at the FMR1 locus in order to restore transcription and translation. However, as an FMR protein level at least over 40% of normal is likely to be required for normal cognitive development, as far as can be judged from leucocytes, this will be difficult to achieve.

Acknowledgements

We are grateful to Prof. Dr. H. Galjaard and the Foundation for Clinical Genetics, Rotterdam for their continuous support.

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Chapter 4 The relation between genotype and phenotype with emphasis on mental retardation

4.1 Mental status and fragile X expression in relation to FMR-1 gene mutation
(Eur J Hum Genet 1993;1:72-9)

4.2 Mental status of females with an FMR1 gene full mutation
(Am J Hum Genet 1996;58:1025-32)

Chapter 4.1

Mental status and fragile X expression in relation to FMR-1 gene mutation

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Eur J Hum Genet 1993;1:72-9

Abstract

The fragile X mental retardation syndrome is caused by unstable expansion of a CGG repeat in the FMR-1 gene. Clinical expression is associated with a large expansion of the CGG repeat. The mutation in the FMR-1 gene and the cytogenetic expression of the fragile site at Xq27.3 have been studied in 52 fragile X male patients. The percentage of the cytogenetic expression of the fragile site at Xq27 positively correlates with the mean size of the full mutation in the FMR-1 gene ($p < 0.0001$) irrespective of the presence of additional premutation alleles. We noted a less frequent occurrence of additional premutation alleles in adult patients compared with juveniles, suggesting a continued mitotic instability in life.

Additionally, the level of mental retardation has been ascertained in 35 patients using the Stanford-Binet or Terman-Merrill test of general intelligence. The presence of a full mutation in the FMR-1 gene seemed decisive for the occurrence of mental impairment in the patient. No correlation is observed between the degree of mental retardation and the size of the full mutation. The degree of mental retardation seemed not to be influenced by the presence of premutation alleles in part of the cells in addition to a full mutation.

One patient is descibed with the "Prader-Willi like" subphenotype of the fragile X syndrome, showing a deletion in the FMR-1 gene in a part of his cells in addition to a full mutation.

Introduction

The fragile X syndrome is the most common cause of familial mental retardation. Its prevalence in Western societies is estimated to be 1/1,250 for affected males and 1/2,000 for females [1,2]. Most affected males have as main clinical features: mental retardation, macro-orchidism and a long narrow face with everted ears which is designated as the Martin-Bell phenotype [3-5]. The clinical diagnosis in male patients can be confirmed by cytogenetic testing, showing the fragile site at Xq27.3 in 2-60% of the cells [6,7].

Recently, a gene (FMR-1) related to the mental retardation was isolated [8]. It was shown that the fragile X syndrome is caused by elongation of a small DNA fragment, containing a repeat of the trinucleotide CGG located in the 5′ exon of the FMR-1 gene [8-12]. Most likely, the increase in size is due to amplification of this repeat sequence. Normal X chromosomes have between 6 and 46 copies of CGG, which are stable during meiosis, and a nearby CpG island is unmethylated on active X chromosomes [12]. Premutations alleles are characterized by an increase of the number of triplets to 56-200. Premutations do not cause mental retardation, the carrier males or females do not show cytogenetic expression of the fragile site and the CpG island remains unmethylated. This premutation sequence is unstable during meiosis. In the full mutation, the repeat sequences exceed the size of 200 triplets and often show somatic heterogeneity; the fragile site becomes apparent in cytogenetic testing in males as do the clinical features of the fragile X syndrome. The CpG island has become methylated. About 15% of patients have a full mutation in the majority of their cells but carry a premutation in a minority of the cells [9].

Several studies have shown that the majority (2/3) of the male fragile X patients are moderately or severely retarded [13]. However, in fragile X patients, profound or borderline mental retardation have also been reported. Cross-sectional and longitudinal data indicate that IQ levels diminish with age in most patients. The majority of the prepubertal boys function at a moderate level (IQ ± 35-55) and most of the adults are severely retarded (IQ ± 20-35) [13]. This decline in IQ does not indicate a loss of mental capacities, but merely represents a slowing of cognitive development, resulting in a stabilisation of intellectual abilities after puberty [14-16].

We investigated whether there is a correlation between the percentage of cytogenetic expression of the fragile site at Xq27.3 and the insert size in the FMR-1 gene. Secondly, we questioned whether there is a relation between the mental capacities of the fragile X patient versus the type of mutation in the FMR-1 gene.

Materials and methods

Fifty-two mentally retarded boys and adults in whom fragile X syndrome was confirmed by cytogenetic expression of the fragile site at Xq27.3 were included in the study.

DNA analysis

The intragenic DNA probe pP2 was used for DNA analysis of the FMR-1 gene [17]. Genomic DNA was isolated from blood leucocytes [18]. DNA (8 μg) was digested to completion with the restriction enzymes EcoRI or EcoRI and EagI according to the manufacturers instructions, separated by gel electrophoresis and subjected to Southern blot analysis according to standard procedures [19]. The probe was labelled by the random oligonucleotide priming method [20]. After prehybridization and hybridization, the filters were washed to 0.1 × SSC at 65 ° C prior to autoradiography [19].

Cytogenetic testing

Blood samples were cultivated under conditions designed to demonstrate the fragile site at Xq27.3 [7,21]. At least 50 metaphase spreads were examined from each patient.

Determination of IQ levels

IQ testing was performed on 35 patients, aged 2-26 years (mean of 13.2 ± 6.7 years). Each individual had been tested at least once during the last year with the Stanford-Binet, or Terman-Merrill test of general intelligence [22] and a Dutch scale for adaptive functioning [23]. These two measures yielded the level of mental retardation according to the guidelines of the AAMD [24].

Almost all patients had been repeatedly tested with the Stanford-Binet test over an extended period which allowed identification of a significant decline in IQ scores. A significant decline is defined as a regression of at least 16 IQ points (= 1 SD).

Results and discussion

FMR-1 gene mutation in patients

DNA of fragile X patients was studied to determine the size of the insert in the FMR-1 gene. DNA analysis showed multiple fragments with different insertions larger than 500 bp that appear as a fuzzy band or smear on a Southern blot (fig. 1, lanes 2, 4, 5 and 7). The patients with the full mutation were all somatic mosaics for the insertion in the FMR-1 gene. Some of the fragile X patients had, in addition to the full mutation, a single abnormal band which corresponds to an insertion smaller than 500 bp and has the size of a premutation allele (fig. 1, lanes 3 and 6).

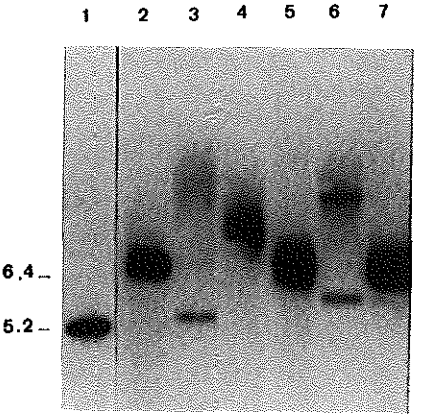

Figure 1 Analysis with probe pP2 of EcoRI digested DNA of an unaffected male (lane 1) and 6 affected males (lanes 2-7). Patients 2, 4, 5 and 7 have a full mutation only, and patients 3 and 6 have a premutation in addition to a full mutation.

The patients can be classified into two different groups according to their banding pattern: (1) full mutation (often appearing as a smear); (2) full mutation together with a premutation allele.

The second group consists of 28% of the patients. This number is higher than the 15% described by Rousseau et al. [25]. This may be explained by the relatively low mean age (19.1 ± 13.7) of our patients. In patients with an age < 20 years (n = 35) we observe 12 patients with a premutation (34%), while in the remaining patients (n = 17) we found only

2 patients with a premutation (11%) (Fischer's exact test, p = 0.06). This suggests an age-dependent process whereby in adult males the premutation tends to expand completely to a full mutation due to continued mitotic instability in life.

There is a CpG island immediately proximal to the exon of the FMR-1 gene containing the insert and this is methylated in individuals with full mutation who have the clinical symptoms of the fragile X syndrome [9].

The methylation level of this CpG island was studied by using the methylation-sensitive restriction enzyme EagI. The CpG island was completely methylated in the males with a full mutation. No fragments resulting from EagI activity were seen in DNA analysis of these

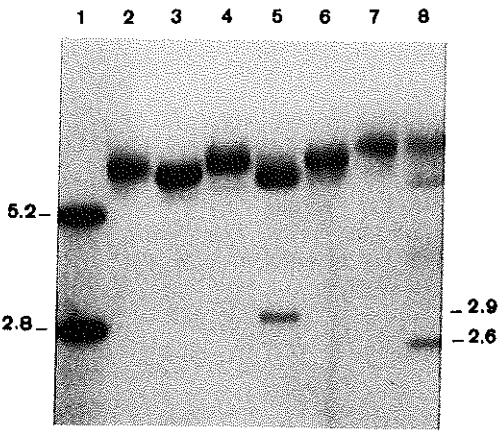

Figure 2 Analysis with probe pP2 of EcoRI plus EagI digested DNA of a normal female and 7 affected males (lanes 2-8). The female (lane 1) shows a 2.8-kb band and a 5.2-kb band of the active and inactive X chromosome, respectively. Patient 5 has an unmethylated fragment with an insert of 100 bp and patient 8 has an unmethylated fragment with a deletion of 250 bp.

males (fig. 2, lanes 2, 3, 4, 6 and 7). In all the males with a premutation in addition to the full mutation, lack of methylation was seen in the premutation allele. DNA analysis of these males showed distinct fragments due to EagI activity (fig. 2, lanes 5 and 8).

FMR-1 gene mutation and fragile X expression

We studied in 36 male patients whether there is a relation between the percentage of cytogenetic expression of the fragile site at Xq27.3 and the mutation in the FMR-1 gene.

Patients with a full mutation only (n = 28) had a cytogenetic expression between 2 and 60% (mean 23.1 ± 16.1). The patients with full mutation and premutation (n=9) showed cytogenetic expression of the fragile site between 1 and 40% (mean 17.6 ± 11.5).

If the fragile X expression is influenced by the size of the inserts, one might expect a higher percentage of fragile X expression among the patients with full mutation in the FMR-1 gene in comparison with the patients who have an additional premutation. This may be conceivable because normal transmitting males have a premutation only in the FMR-1 gene in absence of cytogenetic expression. Our results show that the mean percentage of fragile X expression is lower in the group with a premutation but the difference between both groups is not significant. The cells with a premutation account for a small minority of the total cells. The cells with large inserts appear to determine the fragile X expression, therefore a possible relation between the size of the full mutation and the percentage of fragile X expression was

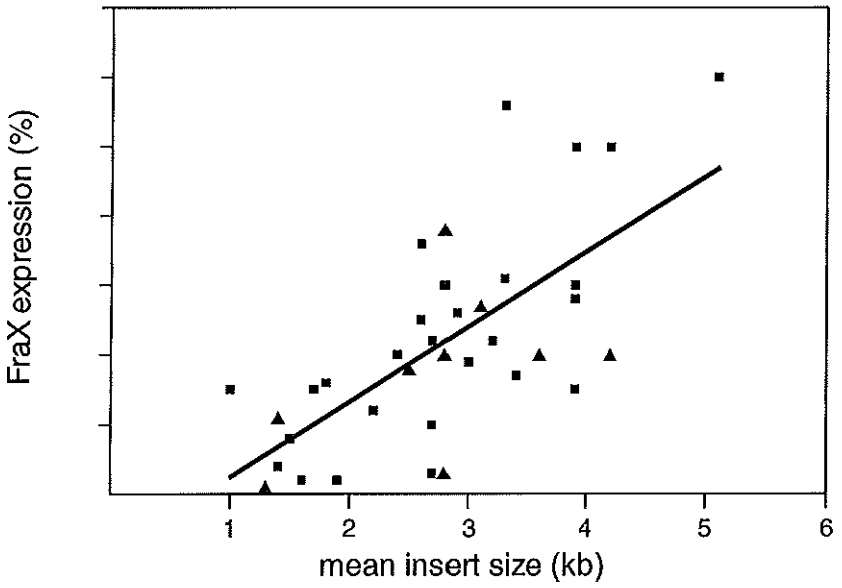

Figure 3 Mean insert size of the full mutation in the FMR-1 gene related to the percentage of the fragile X expression in 36 patients. ■ = patients with a full mutation only; ▲ = patients with a premutation and a full mutation.

investigated. We examined the relation between the mean size of the inserts of the full mutation and the percentage of the fragile X expression in the same 36 patients. The mean insert size of the full mutation has been ascertained by averaging the smallest and the largest detectable inserts. A positive correlation between mean insert size and the cytogenetic expression was clearly demonstrated (fig. 3) ($r = 0.68$, $p < 0.0001$). In the CGG repeat a number of breakpoints are located that are detected in somatic cell hybrids with induced breakpoints in the fragile site [11, S.T. Warren, pers. commun.]. From fluorescence in situ hybridization experiments with probes crossing the fragile site it is suggested that fragile X expression is the result of a break in one of the chromatids [26]. Therefore, we conclude that the size of the insert is a causal factor in the generation of the fragile site and that the percentage of cytogenetic expression is determined by the size of the full mutation.

FMR-1 gene mutation and mental status

The mental status of the fragile X patients was studied in relation to the type of mutation in the FMR-1 gene in 35 boys and adult males with fragile X syndrome. This group is partly overlapping with the group of patients described in the earlier section. We compared the mental status of the two groups (patients with full mutation only and patients with an additional premutation).

We estimated the size of the smallest detectable insert by determining the beginning of the smear on Southern blot in patients with a full mutation only. In patients with a premutation we defined the size of the premutation band as the size of the smallest insert. In fig. 4, the smallest detectable insert in the FMR-1 gene is shown in relation to the IQ level of the patient. There is no obvious clustering. Patients with an additional premutation were found with a relatively high IQ as well as with a lower IQ ($n = 12$, mean age = 11.6 ± 5.5 years, mean IQ = 45.3 ± 8.8). The same was seen for patients with a full mutation only ($n = 23$, mean age = 14.3 ± 6.8, mean IQ = 44.1 ± 10.0). The young, prepubertal, patients (< 14 years, $n = 21$) in both groups have a higher mean IQ than the older patients (≥ 14 years, $n = 14$): mean IQ = 48.4 ± 9.1 versus 38.7 ± 6.9. This age efect on IQ has been reported before [13].

Looking at the two different groups separately, a correlation between the smallest insert in the FMR-1 gene and IQ could still not be observed. Therefore the IQ level seems not to be related to the smallest insert in the FMR-1 gene in patients. A similar pattern was observed when the mean insert size of the full mutation in the FMR-1 gene is used instead of the smallest insert (data not shown).

In males with full mutation only, the CpG island proximal to the FMR-1 gene is fully methylated (Fig. 2, [9]) and the FMR-1 gene is inactivated [27]. The CpG island proximal to the FMR-1 gene in premutation alleles is unmethylated; incomplete methylation at the CpG island may, therefore, have an effect on the level of mental retardation in these patients. The FMR-1 gene with the unmethylated CpG island still produces mRNA [27] and through this a normal protein may be produced; therefore a difference in mental functioning between both groups might be expected. This difference is not observed.

Several explanations can account for the fact that patients with partial transcription of the FMR-1 gene are not less retarded than the patients with no transcription at all. Firstly, the level of transcription may not reach the necessary threshold to provide normal levels of

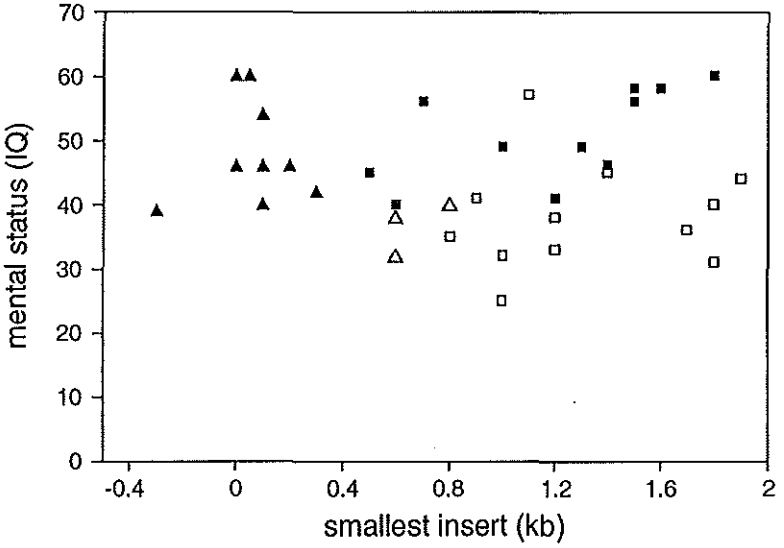

Figure 4. Smallest detectable insert size in the FMR-1 gene related to the IQ level of 35 patients. ■ = *patients with full mutation only;* ▲ = *patients with a premutation and a full mutation. Filled symbols: age <14 years; open symbols: age ≥ 14 years.*

protein. The protein might be cell-bound so that low levels within deficient cells cannot be compensated by normal levels in protein-producing cells. In addition, the expression is only found in a minority of cells; so the absence of expression in a large number of cells may have a dominant effect, resulting in the fragile X phenotype. Secondly, mRNA expression does not necessarily ensure a normal FMR-1 protein. Thirdly, studies of lymphocytes may not be representative for other tissue. The mRNA expression could still be absent in the appropriate tissue (e.g. brain) or at the stage critical for the development of the fragile X phenotype.
Although the numbers are small our data suggest no relation between partial transcription of the FMR-1 gene and the mental status of the patient. It seems therefore that the presence of the full mutation in the FMR-1 gene is decisive for the mental impairment. This feature distinguish the fragile X syndrome from myotonic dystrophy. In both disorders enlargement of DNA fragments, in the disease locus, are found in affected individuals [28-30]. However, in myotonic dystrophy the increase in size seems to correspond with increasing severity of the disease within families.
During several years, a follow-up IQ testing was performed, so the IQ development could be observed. In the study group 7 patients (mean age 15.7 ± 2.7 years) had a significant IQ decline (> 16 points) and their mutation pattern was studied. Five patients with a significant IQ decline had only full mutation. The remaining two patients had a premutation in addition to a full mutation. So a significant IQ decline is not restricted to patients with a full mutation only, but can occur in patients with an additional premutation as well.
One patient was observed with a deletion in the FMR-1 gene in a part of the cells in addition to a full mutation. Deletions in the FMR-1 gene have not been reported before. The DNA analysis of the patient with a deletion of 250 bp is shown in figure 2 (lane 8); the fragment with the deletion was not methylated. The deletion is located around the CGG repeat (fig. 5). Proximal to the CGG repeat 53 bp are deleted and distal to the repeat 178 bp are deleted. The patient showed expression of the fragile site Xq27.3 in 17 % of the blood lymphocytes and had an IQ level of 39 points. On physical examination, he showed the following features: short stature, obesitas, short broad hands and feet and hypogenitalism with hyperpigmentation of the genitals. This pattern of features has been described before in two other fragile X boys

Figure 5 DNA sequence of the PstI fragment containing the CGG repeat [12]. The CGG repeat is underlined. The box indicates the deleted basepairs in the FMR-1 gene in the patient.

[31] and has been designated as the 'Prader-Willi-like' subphenotype of the fragile X syndrome. Besides a normal allele, the mother had an allele with an premutation, no deletion was detected.

Several conclusions can be drawn from this study. Firstly, in patients with an additional premutation the percentage of fragile X expression is not significantly lower compared to patients with a full mutation only. Our data suggest an age-dependent process whereby in adult male patients the number of cells carrying a premutation tends to diminish due to continued mitotic instability in life.

Secondly, the mean insert size of the full mutation in the FMR-1 gene positively correlates with the percentage of fragile X expression. This suggests that the size of CGG repeat in the FMR-1 gene is a causal factor in the generation of the fragile site at Xq27.3 and that expression of the fragile site increases with the number of CGG repeats.

Thirdly, the presence of the full mutation seems decisive for the mental impairment. So males who have a premutation in addition to a full mutation seem as severely mentally retarded as males with the full mutation only.

Acknowledgments

We express our gratitude to M.N. van der Est, L. Bakker and W.H. Deelen for their excellent technical assistance, Drs. E. Bakker, B.A. van Oost, H. Meyers and T. Hulsebos for providing some of the DNA samples and Ir. W.C.J. Hop for the statistical support.

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Chapter 4.2

Mental status of females with an FMR1 gene full mutation

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Am J Hum Genet 1996;58:1025-32

Summary

The cloning of the FMR1 gene enables molecular diagnosis in patients and in carriers (male and female) of this X-linked mental retardation disorder. Unlike most X-linked disorders, a considerable proportion of the female carriers of a full mutation in the FMR1 gene is affected. In this study, the intelligence quotients (IQs) were ascertained by the Wechsler Adult Intelligence Scale in 33 adult females with a full mutation, with 28 first-degree adult female relatives (mainly sisters) without a full mutation as controls. Seventy-one percent of the females with a full mutation had IQ scores below 85. In paired analysis, no significant correlation could be detected between the IQs of the females with a full mutation and those of their first-degree female relatives, reflecting a dominant effect of the FMR1 gene full mutation in the mental development of females. Considering females with a full mutation only, we observed a significant relation between the proportion of normal FMR1 alleles on the active X chromosome and IQ. We present a model to explain this relationship.

Introduction

The fragile X syndrome is the most common single cause of inherited mental retardation, affecting (according to earlier estimates) ± 1/1,250 males and ± 1/2,000 females (Webb et al. 1986). Recently, a lower incidence was reported of ± 1/4,000 for males (Turner et al., in press). A fraction of the X-chromosomes in affected males show, on cytogenetic testing, a fragile site at Xq27 when cells are cultured in a folic acid-deficient medium (Lubs 1969; Sutherland 1977).

The cloning of the FMR1 gene enabled direct DNA diagnosis in both male and female fragile X patients (Verkerk et al. 1991; Oberlé et al. 1991; Yu et al. 1991; Fu et al. 1991). The FMR1 gene contains a variably sized CGG trinucleotide repeat at the 5′ end, which has a length of between 6 and 53 repeats in normal individuals. Patients have >200 CGG repeats, the so-called full mutation, which is associated with methylation of a CpG island upstream of the FMR1 gene and with absence of gene transcription (Oberlé et al. 1991;

Pieretti et al. 1991). Normal carrier males and females, with an exception for normal females with a full mutation, have an intermediate repeat number of between 43 and 200, the so-called premutation, with an unmethylated CpG island in front of the FMR1 gene and normal transcription (Pieretti et al. 1991).

The fragile X syndrome is transmitted as an X-linked semidominant disorder, since males and females may be affected. In earlier studies, the cognitive profile in females with the fragile X syndrome has been related to fragile X chromosome expression. Some groups found a significantly negative relationship between the percentage of fragile X expression and intelligence quotient (IQ) (Chudley et al. 1983; Wilhelm et al. 1988). Others found no such relation between IQ and fragility (taking ≥ 2 % of fragile X cells as evidence for the carrier status) (Cronister et al. 1991).

Before the cloning of the gene, the reported incidence of intellectual deficits (IQ < 85) among heterozygous females ranged from 35% to 53% (Sherman et al. 1985; Hagerman et al. 1992). Besides the limitations of cytogenetic methods for the diagnosis of heterozygosity in females, most of those earlier studies also lacked an adequate control group. Recent studies based on DNA mutation analysis, show mental impairment in 52% to 82% of the females with a full mutation (Rousseau et al. 1991a; Rousseau et al. 1991b; Smits et al. 1994; Taylor et al. 1994), whereas Reiss et al. (1993) showed that the premutation in females did not affect their intellectual development.

Molecular analysis of the FMR1 gene in carrier females enabled a reliable determination of X-inactivation. A relation between the ratio of active normal X chromosomes to normal inactive X chromosomes and full-scale IQ was reported (Rousseau et al. 1991b; Abrams et al. 1994). Abrams et al. (1994) reported a relation between the size of the FMR1 gene mutation and IQ, which had also been observed in a multicenter study by Rousseau et al. (1994). However, another study could not confirm this observation (Taylor et al. 1994).

We report the IQs and profiles in females with the full FMR1 mutation in comparison with their first-degree female relatives without a full mutation. Secondly, the size of the full mutation and the proportion of normal active X chromosomes in leucocytes were studied in relation to IQ and age of the females with a full mutation.

Subjects, Material and Methods

Subjects and controls

Thirty-three females with a full mutation in the FMR1 gene, aged 20-70 years (mean 39.7 ± 14.4 years), from 23 fragile X families were included. All families were ascertained through a male index patient (usually a first-degree relative) and were studied after informed consent was obtained. The study was approved by the Medical Ethical Committee of the University Hospital, Rotterdam. One female did not want to participate (not counted). Twenty-one females were paired to a sister with a normal FMR1 genotype (aged 22-72 years, mean 41.3 ± 14.5 years). In case more female control sibs were available, the female with the best age match was included in the study. In the absence of a control sister, the mother with a premutation was included as a control (n = 7; aged 48-68 years, mean 54.7 ± 7.1 years). For five females with a full mutation from large sibships, a unique control was not available, because every control had only been used once. Those 5 females were excluded from paired analysis (leaving 28 pairs) but were included in the molecular association studies.

Determination of IQ levels

In all 61 females, the IQ and profile were ascertained by using the Dutch version of the Wechsler Adult Intelligence Scale (WAIS) (Stinissen et al. 1970). IQ testing was performed at the home of females by one examiner (AW) who was not informed about the genetic status of the women.

DNA Analysis

The intragenic DNA probe pP2 was used for DNA analysis of the FMR1 gene (Oostra et al. 1993). Genomic DNA was isolated from blood leucocytes (Miller et al. 1988). DNA (8μg) was digested to completion with the restriction enzymes *Hin*dIII and *Eag*I according to the manufacturer's instructions, separated by gel electrophoresis, and subjected to Southern blot analysis according to standard procedures (Sambrook et al. 1989). The probe was labeled by the random oligonucleotide priming method (Feinberg and Vogelstein

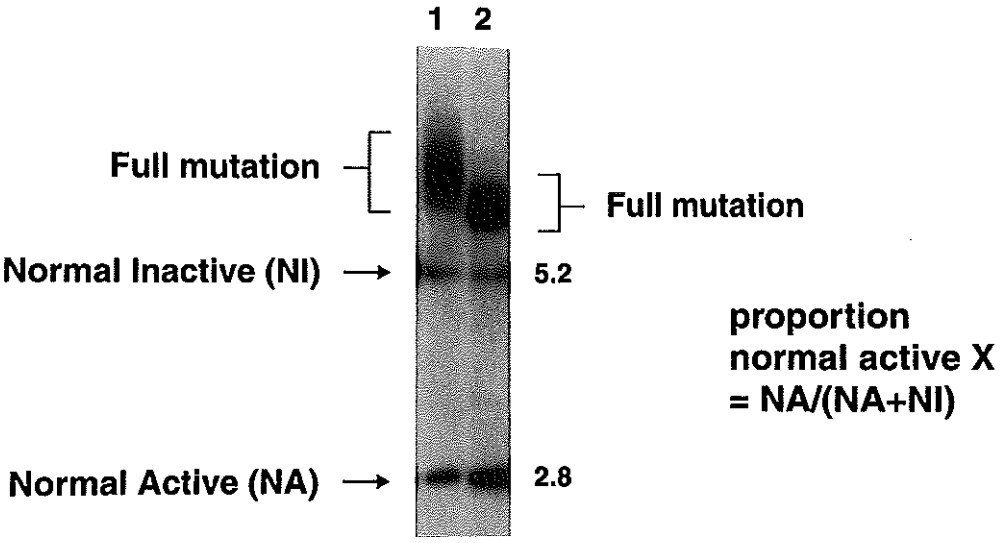

Figure 1 Analysis with probe pP2 of HindIII and EagI digested DNA of two females with a full mutation reflecting the proportion of normal FMR1 alleles on the active X chromosomes (NA) and normal FMR1 alleles on the inactive X chromosomes (NI).

1983). After prehybridization and hybridization, the filters were washed to 0.1 × SSC at 65 ° C prior to autoradiography (Sambrook et al. 1989). Several autoradiograms (2-3) with different exposure times were made.

The size of the full mutation was estimated by averaging the smallest and largest detectable expansion. Sizes were estimated by determining the beginning and the end of the smear on Southern blot in carriers of the full mutation (fig. 1).

The percentages of normal FMR1 alleles on the active X chromosomes (NA) and normal FMR1 alleles on the inactive X chromosomes (NI) were ascertained by densitometry using a scanner (HP, scanjet IICX). The proportion of normal FMR1 alleles on the active X chromosome (NA) versus normal FMR1 alleles on the inactive X chromosome (NI) was calculated using the equation: NA/(NA+NI) (fig. 1) (Rousseau et al. 1991b).

In females with a mosaic pattern of the FMR1 gene mutation (full mutation with an additional premutation), estimation of the size of the expansion and the proportion of normal active X will be hampered by the additional premutation. Therefore, females with a

premutation in addition to the full mutation (n = 6) were excluded from the comparison of the size of the full mutation and the X-inactivation ratio with IQ scores.

Data Analysis

A paired t-test for continuous data was applied to test for significant differences between the female carriers of the full mutation and their controls. The bivariate relationship between variables was estimated by means of Pearson's product-moment correlation coefficient (r). This correlation coefficient was considered to be an indicator of relative importance, while the corresponding P value represents the level of significance statistically. Adjusting for age, the partial correlation coefficient was the indicator of relative importance.

Results

IQ and profile in females with the full mutation versus controls

The mean full-scale IQ (FSIQ), performance IQ (PIQ) and verbal IQ (VIQ) of the different groups studied are shown in table 1. The 28 female carriers of a full mutation had significantly lower mean FSIQ, PIQ, and VIQ scores than their paired controls with a normal FMR1 gene or with a premutation (for all means $P < 0.001$, paired-sample t-test). There was no significant difference of FSIQ between the females with a full mutation only (n = 27) and the females with a full mutation and an additional premutation, the so-called mosaics (n = 6) (r = 0.22, P = 0.21; table 1). Also, no significant difference of FSIQ, PIQ, and VIQ was observed between the females with a normal FMR1 genotype and the females with a premutation (r = −0.1, P = 0.61; r = −0.17, P = 0.39, respectively, r = −0.02, P = 0.9).

Paired analysis: females with a full mutation compared with the controls.

In figure 2, each pair (female with a full mutation and control) and each females' IQ are shown. In the group of 28 pairs, 20 (71%) of 28 carriers of the full mutation had an IQ

Table 1 Summary of the IQ data of the subjects and controls

	Subjects		Paired control 1st degree relatives					
	FM[a] only (n=27)		PM[b] + FM[a] (n=6)		N[c] (n=21)		PM[b] (n=7)	
	X	SD	X	SD	X	SD	X	SD
Age (years)	39.1	13.7	42.3	18.1	41.3	14.5	52.3	7.1
Full scale IQ	74.1	18.4	84.2	26.8	116.0	18.4	111.6	22.5
Performance IQ	81.9	19.7	90.0	28.9	119.1	18.8	111.4	23.7
Verbal IQ	72.3	16.3	82.2	22.1	111.2	18.0	110.3	19.2
Proportion normal active X	0.71	0.17						

[a] *full mutation FMR1 gene;* [b] *premutation FMR1 gene;* [c] *normal FMR1 gene*

lower than 85 points. When the more strict criteria for mental retardation (IQ < 70 points) was used, 14 (50%) of 28 carriers of the full mutation (50%) were mentally retarded, whereas none of the control females had an IQ < 70 points.

Sixteen (57%) of 28 carriers of the full mutation scored > 30 IQ points (2 SD) lower than their control. This ratio remained nearly unchanged when only the 21 sister-sister pairs are considered (62%). Remarkably, two females with the full mutation had a higher IQ than their controls (fig. 2). No significant correlation could be detected between the FSIQ of the females with the full mutation and the FSIQ of the controls (r = 0.22, P = 0.27). Likewise, there was no significant correlation for the VIQ of the females with a full mutation and the VIQ of the controls (r = 0.07, P = 0.74), but the PIQ correlation was significant (r = 0.39, P = 0.04).

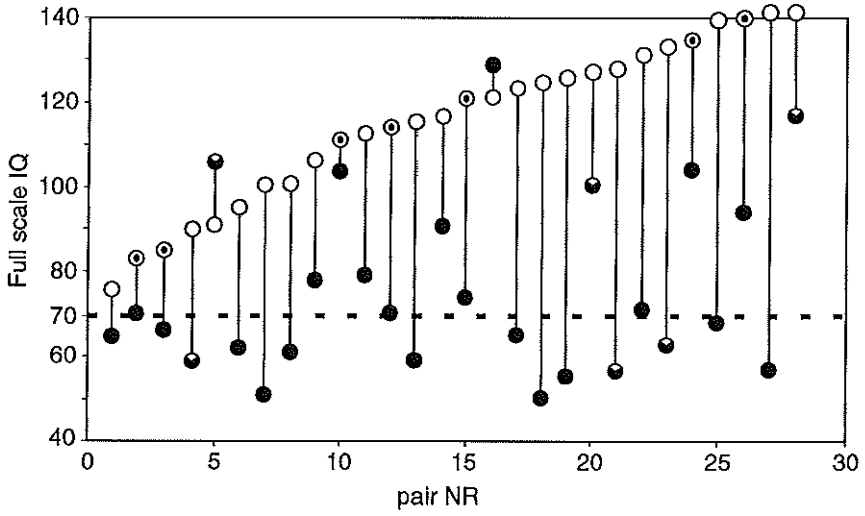

Figure 2 IQ level of the females with a full mutation (filled or 2/3-filled symbols) compared with their first degree relatives (open symbols). Females with full mutation only are represented by filled circles, females with a premutation and a full mutation by 2/3-filled circles, sisters with normal FMR1 genes by open circles, and mothers with a premutation by open circles with a dot.

The size of the FMR1 gene mutation and mental status in females with the full mutation only.

For females with a full mutation only, the FSIQ, PIQ and VIQ scores were negatively related with age ($r = -0.43$, $P = 0.03$; $r = -0.39$, $P = 0.05$; and $r = -0.43$, $P = 0.03$). Therefore, the further data were, in addition, analyzed with adjustment for this age-dependent effect. Since the results of those analyses did not differ from the analyses without adjustment for age, we choose only to present unadjusted data.

The mean of the average sizes of the full mutation was 1.6 ± 0.6 kb ($n = 27$; females with full mutation only). No significant relation was found ($r = -0.16$, $P = 0.42$) between average size of the full mutation and FSIQ (figure 3), or with one of the subtests, PIQ and VIQ ($r = -0.19$, $P = 0.34$; and $r = -0.13$, $P = 0.52$, respectively). A similar result was observed with the smallest or largest size of the full mutation (data not shown).

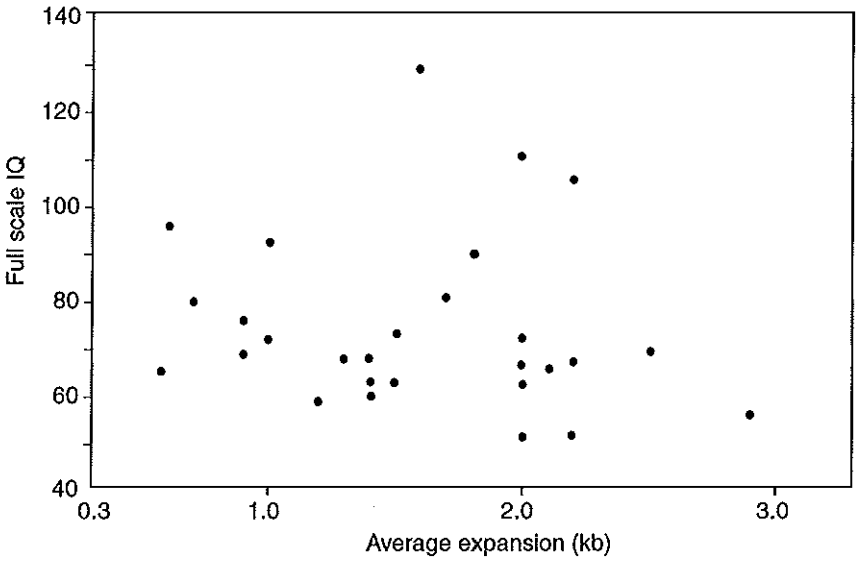

Figure 3 Average expansion size of the full mutation in the FMR1 gene related to the full scale IQ in females with a full mutation only.

The proportion of normal FMR1 alleles on the active X chromosome and mental status in females with the full mutation only.

The average of the proportions of the normal FMR1 alleles on the active X chromosome (0.71 ± 0.17) in females with a full mutation only, was significantly > 0.5 (n = 27, P << 0.001). As an internal control, we measured the control females with normal FMR1 genes (n = 21) who showed 0.50 ± 0.09 X-inactivation.

In figure 4, the proportion of normal FMR1 alleles on the active X chromosome in relation to FSIQ is shown for females with a full mutation only. The (moderate) relation between the proportion of normal FMR1 alleles on the active X chromosome and FSIQ is significant (r = 0.45; P = 0.02). The relation with the proportion normal active X was significant for the PIQ (r = 0.56, P = 0.003). However, a significant relation between the proportion of normal active X and VIQ was not observed (r = 0.31, P = 0.11).

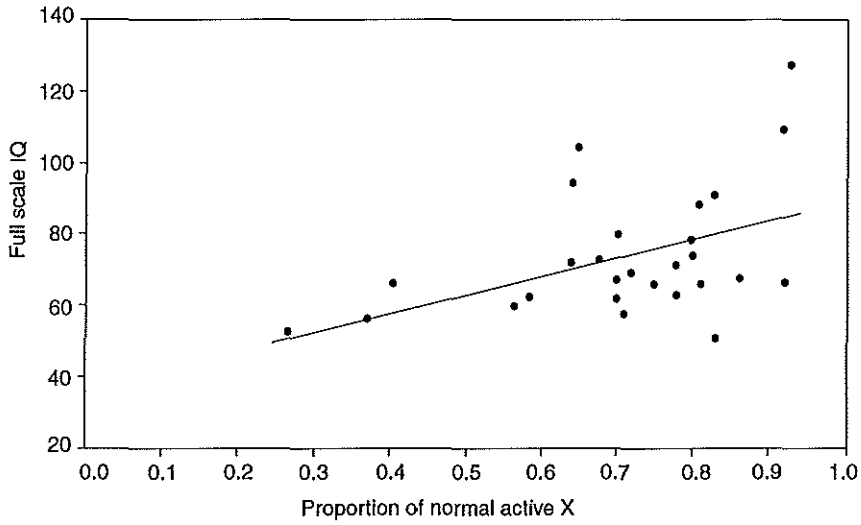

Figure 4 The proportion of normal FMR1 alleles on the active X chromosome related to full scale IQ in females with a full mutation only.

No significant relation could be detected between the proportion normal active X and age in the 27 females with a full mutation only ($r = -0.14$, $P = 0.50$; data not shown).

Discussion

The full mutation in FMR1 gene in females has a dominant effect over other factors involved in intellectual development.

In the current study, 71% (20/28) of the females with a full mutation in the FMR1 gene had IQ scores < 85, representing borderline or mild/moderate retardation. Other groups also using DNA analysis of the FMR1 gene reported lower percentages, varying from 52% to 65%, of mental impairment (IQ < 85) among the females with a full mutation (Rousseau et al. 1991b; Rousseau et al. 1994; Taylor et al. 1994) (table 2), except for

Smits et al. (1994) who reported 82%. However, in three of these studies the mental development was clinically estimated without accurate IQ testing (Rousseau et al. 1991b; Smits et al. 1994; Rousseau et al. 1994).

Using strict criteria for mental retardation (i.e. IQ below 70 points), 50% (14/28) of the females with a full mutation in this study were mentally retarded. This is higher than the observation of Taylor et al. (1994), who reported 22% (5/23) of the females to have an IQ < 70. Their results might be biased by the use of four different IQ tests and the low average age of the subjects they studied. Further, they could not exclude an ascertainment bias because of 'unavoidable selection for motivation to come to the clinic' (p. 513), which is a major problem in several comparable studies. The present study avoided such a bias, both by selecting the families through a male index patient and by visiting the families at their homes. Although the possibility that females with a full mutation, ascertained through a male index patient, might be functioning on a different cognitive level as females with a full mutation without an affected, first-degree, male relative might be conceivable, at present no difference of this type is known.

The first-degree female relatives without a full mutation in the FMR1 gene (mainly sisters without an FMR1 gene mutation) served as references in this study, allowing an intrafamilial assessment of the effect of the full mutation. The effect of the full FMR1 mutation is reflected in the IQ difference between females with the full mutation and their controls. Fifty-seven percent (16/28) of the women with a full mutation scored significantly lower (> 30 points) than their control, which indicates a dominant effect of the full mutation in the FMR1 gene over shared environmental and genetic factors involved in mental development. This is supported by the observation that both FSIQ and VIQ of the females with a full mutation were uncorrelated to the controls' FSIQ and VIQ. It is remarkable that a significant relation between PIQ of females with a full mutation and their controls was found.

Molecular characteristics of the full mutation and the mental status

The full mutation in the FMR1 gene in females can be characterized by different features, such as size of the mutation and localization on the active or inactive X chromosome. No relation between the size of the full FMR1 mutation, whether characterized by smallest, average, or largest size, and the IQ level of the females was observed in this study. These findings are inconsistent with earlier reports by Abrams et al. (1994) and Rousseau et al. (1994), but they are in line with the reports from other groups (Rousseau et al. 1991b; Taylor et al. 1994; Reiss et al. 1995) (table 2). Because a relation between size and IQ level could not be detected in males (De Vries et al. 1993, Loesch et al. 1993), it would be unlikely to observe such relationship in females. This corresponds with the characteristics of the full mutation in the FMR1 gene. Independent of the size of a full mutation, the full mutation is associated with methylation of a CpG island in front of the FMR1 gene, leading to an absence of FMR1 protein.

As in other X-linked disorders, lyonization may influence the phenotypic expression of the normal allele in females with a full mutation in the FMR1 gene. Earlier studies using cytogenetic techniques in lymphocytes explored whether the normal X chromosome is more frequently the active (or early replicating) X chromosome in carrier females with a normal intelligence compared to carriers with an intellectual impairment. Although some studies confirmed the relation between a high percentage of normal active X chromosomes and normal intelligence (Knoll et al. 1984; Paul et al. 1984; Wilhelm et al. 1988), others did not (Fryns et al. 1984; Nielsen et al. 1983). With molecular techniques, the proportion of normal FMR1 alleles on the active X chromosome can be estimated more accurately using methylation assays. The cells with normal active FMR1 alleles are actually capable of producing FMR1 protein. In the 27 females with a full mutation only, the normal FMR1 alleles were significantly more frequent on the active X chromosome in intellectually higher-functioning females than in females with a mental impairment. This confirms earlier findings (Abrams et al. 1994; Rousseau et al. 1991b; Reiss et al. 1995) but contradicts two other studies (Rousseau et al. 1994; Taylor et al. 1994) (table 2). In the study of Rousseau et al. (1994), the intelligence level was clinically estimated without

Table 2 Summary of molecular and IQ data in female carriers of the full mutation: overview of the literature and the present study

	N	Age (years)	Controls	IQ Test	IQ < 85 (%)	Relationship		
						size of FM[a] to IQ	X-inactivation[b] to IQ	X-inactivation[b] to age
Rousseau et al. (1991) (France)	27	all	Women PM[c]	Clinical estimate	52	?	Yes (+)	Yes (+)
Taylor et al. (1994) (Denver, USA)	23	3-58	Mothers	Test (4)[d]	65	No	No	Yes (+)
Abrams et al. (1994)[e]	31	4-27	Mothers (+ fathers)	Test (2)[d]	?	Yes (−)	Yes (+)	No
Reiss et al. (1995)[e] (Baltimore, USA)	29	6-17	Mothers + fathers	WISC[f]	?	No	Yes (+)	?
Rousseau et al. (1994) (collaborative)	170	all	Women N[g] and PM[c]	Clinical estimate	59	Yes (−)	No	Yes (+)
Present study	33	20-70	Sisters (+ mothers)	WAIS[h]	71	No	Yes (+)	No[i]

[a] full mutation FMR1 gene; [b] proportion of normal active X, as described by Rousseau et al. (1991b); [c] premutation FMR1 gene; [d] number of different IQ-tests used; [e] study-groups partly overlap; [f] WISC: Wechsler Intelligence Scale for Children; [g] normal FMR1 gene; [h] WAIS: Wechsler Adult Intelligence Scale; [i] adults only; (−) or (+): negative or positive correlation

specific IQ testing, and therefore the relation might have been missed. Taylor et al. (1994) included females with a mosaic pattern (premutation and full mutation). Although we did not observe a difference of the FSIQ between females with a full mutation versus mosaics, the effects of an additional premutation on the phenotype have not been fully assessed in larger controlled studies. Therefore, including 'mosaic' females might influence the results. Another reason for excluding 'mosaic' females is the inability to establish the percentage of normal active X chromosomes accurately in these cases.

We observed an age-independent, skewed X-inactivation in leucocytes favoring the X-chromosomes with normal FMR1 alleles over those with a full-mutation FMR1 allele both in females with normal and subnormal intelligence level. This suggests a positive selection for leukocytes able to produce FMR1 protein earlier in life. Some groups (Rousseau et al. 1991b; Rousseau et al. 1994; Taylor et al. 1994) observed an age-dependent selection in leukocytes: a higher proportion of active X chromosomes carrying the normal FMR1 gene in elderly women with a full mutation compared to younger females. Because only adults were included in the present study, we could not observe such an age-dependent selection.

In each cell, the X inactivation occurs at random in the late blastocyst stage (Nesbitt 1971; McMahon et al. 1983; Lyon 1994). The precursor cells for both brain and blood formation are the embryonic ectodermal cells, which undergo X inactivation around the second week of embryonic development. The X-inactivation distribution is rather consistently equal when different tissues within one individual are compared, both in mice and men (Nesbitt 1971; Fialkow 1973, McMahon et al. 1983; Kolehmainen and Karant 1994). Because we observed females with a high proportion of normal FMR1 alleles (>0.80) with either a normal or a subnormal IQ level, the currently observed relation is likely influenced by the age-dependent selection for normal FMR1 expression in leukocytes. We hypothesize that the proportion of inactivation of the normal X chromosome at the late blastocyst stage in some individuals is < 0.5 and in others > 0.5. Individuals with a lower proportion of normal active X chromosome in the brain will have subnormal intelligence, while individuals with a higher proportion will be normal. There will be a relation between the proportion of normal FMR1 alleles on the active X chromosome in the brain and IQ. In

leucocytes, however, there is selection for normal X chromosomes, leading to a change in the proportion of normal X chromosomes. This change is more likely to be higher in individuals with a low proportion of normal X chromosomes, disturbing the relationship between proportion of normal alleles and IQ. The presently observed relation between a higher proportion of normal FMR1 alleles on the active X chromosomes in leucocytes and a higher intelligence in females with a full mutation might still be a reflection of the distribution of X-inactivation in brain cells. To prove this hypothesis, we propose to study the X-inactivation pattern in other tissues that might lack this selection phenomenon. Studies on protein expression in neuronal cells of either females with a full mutation or heterozygous knock-out mice might give the relevant clues.

The currently observed relation between molecular parameters and the FSIQ were also significant for the subcomponent PIQ but not for VIQ. This is in line with a recent report about girls with a full mutation, in which a stronger relation between the proportion of normal active X was suggested for PIQ than for VIQ (Reiss et al. 1995). Since the PIQ is less influenced by cultural factors than the VIQ (Cattel 1963; Horn 1968), the PIQ seems preferable over the VIQ and FSIQ as an indicator of mental developmental capabilities in molecular-cognitive association studies.

The mental status in females with the full mutation is influenced by several factors. Besides the full mutation in the FMR1 gene, other genetic and environmental factors may play, to an unknown extent, a role in intellectual development. In this first study of comparing full mutation FMR1 female carriers with their noncarrier sisters, those shared factors were less significant, reflecting a dominant effect of the FMR1 gene full mutation on mental development in a majority of females with a full mutation. This finding will be relevant in the genetic counselling of carriers of (pre-)mutations of the fragile X syndrome. X-inactivation studies during prenatal diagnosis in female pregnancies with a full FMR1 mutation will not allow a reliable prognosis of the intellectual development of such child. Henceforth, parents will have to decide upon the general risk estimates for intellectual impairment in female pregnancies with a full mutation as observed in this study.

Acknowledgements

We are thankful to Prof. Dr. H. Galjaard and the Foundation for Clinical Genetics, Rotterdam for their continuous support, and Prof. Dr. B.A. van Oost for providing some of the DNA samples and his comments on the manuscript.

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Chapter 5 Screening and diagnosis for the fragile X syndrome

5.1 Screening and diagnosis for the fragile X syndrome among the mentally retarded: an epidemiological and psychological survey

5.2 DNA testing for the fragile X syndrome: implications for parents and family

Chapter 5.1

Screening and diagnosis for the fragile X syndrome among the mentally retarded: an epidemiological and psychological survey

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Summary

Background. The fragile X syndrome is an X-linked mental retardation disorder caused by an expanded CGG repeat in the first exon of the Fragile X Mental Retardation (FMR1) gene. Its frequency, X-linked inheritance and consequences for relatives all prompt for diagnosing this disorder on a large scale in all affected individuals.

Methods. A screening for the fragile X syndrome has been conducted in a representative sample of 3352 individuals in schools and institutes for the mentally retarded in the South-West Netherlands, using a brief physical examination and the DNA-test. The attitudes and reactions of (non)consenting parents / guardians were studied by (pre- and post-test) questionnaires.

Findings. 2189 individuals (65%) were eligible for testing, since they had no valid diagnosis, nor cerebral palsy or a previous test for the FMR1 gene mutation. 70% of the parents / guardians consented for testing (1531/2189). Besides 32 previously diagnosed fragile X patients, 11 new patients (9 males and 2 females) were diagnosed. Scoring physical features was effective in pre-selection (sensitivity 0.91 and specificity 0.92). Major motives to participate in the screening were: the wish to obtain a diagnosis (82%), the hereditary implications (80%) and the support of research into mental retardation (81%). 34% of the parents / guardians will seek additional diagnostic work-up after exclusion of the fragile X syndrome. The prevalence of the fragile X syndrome was estimated at 1/6,045 for males (95% CI 1/9,981 to 1/3,851). Based on the actual number of diagnosed cases in The Netherlands, it is estimated that over 50% of the fragile X cases are undiagnosed at present.

Interpretation. This first comprehensive genetic epidemiological study of a representative sample of mentally retarded individuals using DNA techniques demonstrated the feasibility and utility of clinical pre-selection, and a lower prevalence of the fragile X syndrome than in previous studies based on cytogenetic analysis. This type of genetic screening requires careful pretest information and shows

high acceptance and realistic appraisal by parents / guardians. The low rate of introduction of DNA diagnosis for this disorder underlines the need for improvement of diagnosis for this inherited type of mental retardation.

Introduction

The identification of genes and their mutations has facilitated direct molecular diagnosis for numerous genetic disorders.[1] The criteria for introduction of new diagnostic procedures, such as target-groups and an active or passive approach, are still under debate for several genetic disorders, including the fragile X syndrome.[2-8] The fragile X syndrome screening program presented here gives a model for actively introducing a new DNA diagnostic procedure and, moreover, as a method to obtain accurate prevalence data.

The fragile X syndrome is characterized by X-linked mental retardation with additional features like a long face with large protruding ears, macro-orchidism and eye gaze avoidance.[9,10] Affected males and most of the affected females show a fragile site at Xq27.3 in a percentage of the cells tested under special culture conditions,[11] that method was used until the cloning of the gene. The first estimates of the prevalence of the fragile X syndrome, based on cytogenetic testing, varied from 1/1000 to 1/2600 for males and 1/2000 to 1/4000 for females.[12,13]

The cloning of the Fragile X Mental Retardation (FMR1) gene in 1991[14-16] enabled an accurate molecular diagnosis. Affected individuals have expanded CGG repeats (>200) in the first exon of the FMR1 gene (the so called full mutation). This expansion is accompanied by hypermethylation of the repeat and its upstream region resulting in a shutdown of transcription and absence of the FMR protein.[17-19] In the normal population, the (CGG)n repeat varies from 6 to 54 units.[20] Phenotypically normal carriers of a repeat size from 43-200 are called premutation carriers.[20] Female carriers of a premutation or full mutation have an increased risk of affected offspring. Male carriers of a premutation will transmit this usually unaltered to their daughters.

Screening for the fragile X syndrome by DNA analysis was offered to mentally

handicapped individuals in schools and institutes for the mentally retarded in the South-West of the Netherlands. We analyzed the acceptance by parents / guardians of mentally retarded individuals, feasibility of such a screening program and the prevalence of the fragile X syndrome in the Dutch population. The pre- and post test attitudes and expectations of consenting and non-consenting relatives were studied.

Patients and methods

Since 1992, a screening program for the fragile X syndrome has been conducted in five institutions giving residential care (1869 individuals) and sixteen special schools (1483 individuals) for the mentally retarded individuals in South-West Netherlands. Persons without a known cause of their mental handicap, without cerebral palsy (with quadriplegia) and without previous DNA mutation analysis of the FMR1 gene (based on medical records and previous medical investigations) were eligible for a brief physical examination and venapunction for DNA analysis of the FMR1 gene. Parents / guardians were informed by letter and through information meetings. After the parents / guardians' written consent, the subjects were included in the study. Organizations for parents / relatives were informed prior to the onset of the program. Also, the medical, nursing and teaching staff of the various institutes and schools were informed in separate meetings. Parents / guardians of newly diagnosed patients were offered genetic counselling and asked for participation in a follow-up study. The study was approved by the Medical Ethical Committee of the Erasmus University and University Hospital Dijkzigt, Rotterdam and the respective institutional Ethical Review Committees.

Physical examination

Each individual was scored for 8 fragile X features (including large / prominent ears, elongated face, body habitus, family history of mental retardation, hyperextensible finger joints, soft / smooth skin, behavioral features and macro-orchidism) by the first author (BdV) using a score adapted from Laing et al.[21] Additionally, the height and head circumference and dysmorphic features, not related to the fragile X syndrome, were

recorded. The individuals were divided into low, moderate and high risk groups for having the fragile X syndrome: "low" when dysmorphic features suggested another diagnosis than fragile X syndrome, "moderate" in the absence of specific dysmorphic features, "high" in the presence of fragile X syndrome characteristics. Intellectual functioning, e.g. profound / severe (IQ below 30), moderate (IQ 30-50) or mild mental retardation (IQ 50-70) was established by each individual's psychologist, by IQ testing in schoolchildren or clinical estimation in the institutionalized individuals.

DNA analysis

A 10 ml blood sample was obtained from each individual and genomic DNA was isolated from blood leucocytes.[22] PCR analysis of the CGG repeat was performed according to Fu et al.,[20] using modifications.[23] In all males without a fragment in the normal range (6 to 54 CGG repeats) and females without two distinguishable normal fragments, additional Southern blot analysis on *Hin*dIII digested DNA, using the intragenic probe pP2, was performed.[24]

Questionnaires to consenting and non-consenting parents / guardians

The acceptability of the screening program and the (anticipated) implications of test results was assessed in a pre- and post-test questionnaire study. A sample of consenting parents / guardians (n=1090) received a pre-test questionnaire, after taking the blood sample in their relative, and a post-test questionnaire, three weeks after obtaining the test result. A reminder was sent after three weeks. Non-consenters (n=435) received a questionnaire to ask them about their motives. A translation of the questionnaires is available on request.

Statistical analysis

The data were analyzed with version 6.0 of SPSS for Windows and the software Confidence Interval Analysis (CIA) compiled by Gardner & Altman. The data are presented as proportions or percentages with 95 percent confidence intervals. Differences between groups were assessed with the Chi-square test and considered significant for $P < 0.001$.

Results

Study population, physical examination and DNA-testing

65.5% (2170/3313) of the mentally retarded individuals were eligible for testing. Reasons for exclusion of the other 1143 individuals included: an earlier diagnosis of the fragile X syndrome (32/1143; 2.8%) or its exclusion (36/1143; 3.1%) or a causative diagnosis, such as Down syndrome (474/1143; 41.5%) or other valid diagnosis, including cerebral palsy, confirmed by medical records (601/1143; 52.6%) (percentages of total in Table 1). Seventy percent (1520/2170) of the parents / guardians of eligible patients consented for participation. The uptake of the test was higher in the five institutions than in the sixteen special schools (74.4%; 95% CI 71.9 to 76.9% respectively 64.6%; 95% CI 61.6-67.6%).

For 39 of the 3352 individuals the level of intellectual development could not be ascertained and those individuals were excluded in those analyses for which this level was required.

1501 of the 1531 tested individuals (including 11 with an unknown level of mental retardation who were not included in Table 1) had an CGG repeat in the normal range (<43 repeats). For 12% of the males and 59% of the females the PCR-test result was inconclusive and an additional Southern blot analysis was done. Although no individuals with a premutation were detected, 19 individuals (1.2%) had an allele with a size in the 'intermediate range' (43-60 CGG). Eleven fragile X patients (0.7%) were newly diagnosed (9 males and 2 females). Seven of those resided in an institution and 4 attended a special school. Ten of 11 detected cases were in the group of 134 cases with a high risk for having the fragile X syndrome (based on physical examination, sensitivity 0.91 and specificity 0.92, odds of being affected having a "high risk" phenotype 1:12). Moreover, all newly diagnosed male patients showed the "high risk" phenotype (Table 2).

Estimated prevalence of the fragile X syndrome

The prevalence of the fragile X syndrome was estimated for the various levels of mental retardation in male individuals studied i.e. mild (IQ, 50 to 70) and moderate / severe

Table 1 *Overview of study sample: gender and level of mental retardation*

	Moderate/severe		Mild		total*	
	♂	♀	♂	♀	No	(%)
Not eligible for testing						
Fragile X syndrome	24	0	6	2	32	(1.0)
FMR1 mutation excluded	15	6	14	1	36	(1.1)
Down syndrome	230	204	20	20	474	(14.3)
Other chromosomal abnormality	18	29	2	6	55	(1.7)
Metabolic disorder	12	13	3	7	35	(1.1)
Cerebral palsy	133	100	8	6	247	(7.5)
Other	91	76	55	42	264	(8.0)
subtotal	**523**	**428**	**108**	**84**	**1143**	**(34.5)**
Eligible for testing						
Non participants	213	194	161	82	650	(19.6)
Participants	533	461	333	193	1520	(45.9)
subtotal	**746**	**655**	**494**	**275**	**2170**	**(65.5)**
general total	**1269**	**1083**	**602**	**359**	**3313***	

**without 39 patients with an unknown level of mental retardation*

retardation (IQ less than 50) (Table 3).

The estimation of the population prevalence is restricted to the data from males in this study because females with a full mutation in the FMR1 gene have an intellectual development varying from severely retarded to normal. The latter group was not included in this study among the mentally retarded.

In the group of mildly retarded males (n_{tot}=602), 4 fragile X patients (f_p) were newly diagnosed among the participants (n_p=333) and 6 fragile X patients (f_{ne}) had been previously diagnosed among the individuals who were not eligible for testing (n_{ne}=108) (see Table 3). Assuming the same relative prevalence in the nonparticipating group

Table 2 *Phenotype of newly diagnosed fragile X patients*

Phenotype suggestive for fragile X syndrome	Frequency fragile X syndrome		Row total
	♂	♀	
Low	0/223	0/251	0/474
Moderate	0/555	1/368	1/923
High	9/92	1/42	10/134
Column total	9/870	2/661	11/1531

(n_{np}=161) as in the participating group (namely 0.01201), the total prevalence in the sample of mildly retarded males was ((333+161)*0.01201+(108*0.05555))/602=0.0198. According to an estimate of 30,000 mildly retarded males in The Netherlands,[25] one may expect a number of 595 mildly retarded fragile X males in this population (95% CI 309 to 1038).

In the sample of moderately / severely retarded males (n_{tot}=1269) 5 fragile X patients (f_p) were newly diagnosed among the participants (n_p=533) and 24 fragile X patients (f_{ne}) had previously been diagnosed among the patients who were not eligible for testing (n_{ne}=523). Assuming the same relative prevalence in the nonparticipating group (n_{np}=213) as in the participating group (namely 0.00938), the total prevalence in the sample of moderately / severely retarded males was ((533+213)*0.00938+(523*0.04589))/1269=0.0244. According to an estimate of 27,000 moderately / severely retarded males in the Netherlands,[25] we estimated a number of 659 fragile X male patients (95% CI 451 to 932) with a moderately / severely mental handicap.

For The Netherlands with 7,586,000 male residents,[26] a total number of 1255 males with the fragile X syndrome will lead to a prevalence of 1/6,045 for males (95% CI 1/9,981 to 1/3,851).

Table 3 Prevalence of the fragile X syndrome, estimated for males in the Dutch population

	Study-sample								Population	
	participants			non-participants	not eligible			total		
Level of retardation	n_p	f_p	p_p	n_{np}	n_{ne}	f_{ne}	p_{ne}	n_{tot}	p_{tot}*	N	F**
Mild	333	4	0.01201	161	108	6	0.05555	602	0.0198	30,000	595
Moderate/severe	533	5	0.00938	213	523	24	0.04589	1269	0.0244	27,000	660
Total	866	9		374	631	30		1871		57,000	1255

Numbers (n) , frequencies (f) and prevalences (p) for particants ($_p$), non-particants ($_{np}$), not eligible ($_{ne}$) or total ($_{tot}$) cases.

* $p_{tot} = ((n_p+n_{np})*p_p +(n_{ne}*p_{ne}))/n_{tot}$.

** $F= p_{tot}*N$.

Using a similar analysis for Down syndrome in our male study sample (20 mildly and 230 moderately / severely retarded males with Down syndrome), a prevalence of 1/1,288 for Down syndrome males was found (95% CI 1/1,538 to 1/1,087). This is similar to data from the United Kingdom.[27]

The prevalence of the fragile X syndrome did not differ significantly between the mildly retarded males and the moderately / severely retarded males (0.0198 respectively 0.0244).

Motives for participation or non-participation

Pre-test attitude responses from consenting parents / guardians

The response rate was 79% (860/1090), most of them parents (71%). Eighty-four percent had discussed the DNA-test with relatives and would inform them about the result. Major motives to participate were: the wish to have a diagnosis, the hereditary implications, and the support of research into mental retardation (Table 4). Eighteen percent of the respondents (95% CI 15 to 21%) expected that the fragile X syndrome would be diagnosed in their retarded relative, 30% were uncertain (95% CI 27 to 34%) and 52% did not expect the diagnosis (95% CI 48 to 55%). Six percent had intrusive thoughts and / or feelings about the test and its outcome (95% CI 5 to 8%). Parents / guardians of schoolchildren expected significantly more often that a diagnosis would improve the care for their retarded family member than parents / guardians of institutionalized individuals (Table 4).

Post-test attitude responses from consenting parents / guardians.

The response rate was 66% (681/1030; to 51 parents / guardians a follow-up questionnaire could not be sent and the parents / guardians of the newly diagnosed were offered genetic counselling). One-third of the respondents (35%) were relieved by the exclusion of the fragile X syndrome (95% CI 31 to 38%). One-third were not relieved (95% CI 29 to 37%) and 5% (95% CI 3 to 6%) were even disappointed. Eighteen per cent still worried about possible genetic implications for their family (95% CI 15 to 21%). The majority (87%) had informed their relatives about the test result.

After the exclusion of the fragile X syndrome in their relative, the parents / guardians of

*Table 4 Motives for (non) participation, in parents / guardians of mentally retarded individuals in schools and institutes**

	Schools % (95% CI)**	Institutes % (95% CI)	Total % (95% CI)
Consenting parents / guardians			
Pre-test	N=325***	N=535	N=860
Wish to have a diagnosis	88 (85 to 92)	78 (75 to 82)	82 (79 to 85)
Hereditary implications	79 (74 to 83)	81 (78 to 85)	80 (78 to 83)
Support research into mental retardation	72 (67 to 77)	87 (85 to 90)	81 (79 to 84)
Expecting better care after fra(X) diagnosis	68 (63 to 73)	47 (43 to 52)	55 (52 to 59)
Post-test	N=248	N=433	N=681
Will seek further investigations ('active')	43 (37 to 50)	28 (24 to 32)	34 (30 to 37)
Will use new diagnostics when offered ('passive')	78 (72 to 83)	57 (53 to 62)	65 (61 to 69)
Non-consenting parents / guardians	N=55	N=98	N=153
Blood test is too stressful for family member	42 (27 to 58)	73 (61 to 83)	61 (52 to 70)
'Definite' cause mental handicap is known	27 (15 to 41)	54 (42 to 65)	44 (35 to 52)
Any possible cause of retardation is different from fragile-X syndrome	56 (38 to 74)	69 (56 to 80)	64 (54 to 74)

* Percentages reflect agreement to a statement; ** 95 percent confidence interval
*** number of questionnaires obtained

school children were significantly more willing to pursue further investigations both actively as well as passively than the parents / guardians of institutionalized individuals (Table 4). Respondents (80%; 95% CI 77 to 83%) appreciated the test and would recommend others to participate in such program.

Attitude responses from non-consenting parents / guardians

The response rate was 35% (153/435). Non-consenters differed only by having significantly higher education levels than consenters (high school level and above: 64% (95% CI 56 to

72%) and 47% (95% CI 43 to 50%), respectively). The majority (78%) had discussed the DNA-test with others. Main reasons for non-consenting were the opinion that a definite cause of the mental handicap in their relative was already known, or the conviction that any possible cause must be different from the fragile X syndrome (Table 4). The test was considered as too stressful for their relative, significantly more often among non-consenting parents of institutionalized persons (Table 4). Non-participation was neither influenced by fear for possible consequences of the test (9%; 95% CI 4 to 16%) nor by religion (6%; 95% CI 2 to 12%). Generally, non-consenters were not opposed to genetic testing (72%; 95% CI 64 to 80%). One third even considered the future use of other diagnostic options if these would become available (95% CI 24 to 42%).

Discussion

This first comprehensive genetic epidemiological study of a representative sample of male and female mentally retarded individuals from a population (the Netherlands) of $15x10^6$ inhabitants, using DNA techniques for the fragile X syndrome, indicates a prevalence of the fragile X syndrome in 1/6,045 males in the general Dutch population. This is considerably lower than the previously considered 1/1,000 - 1/2,600[12,13] but similar to recent reports of 1/4000 - 1/5000 (Australia and England).[28,29] However, sample-sizes of these recent studies did not allow very accurate estimates, neither was a representative sampling of mentally retarded males achieved. The earlier high estimates were obtained using cytogenetic studies, with possible confounding by other fragile sites in this region of the X-chromosome or false positives.[28]

In the Netherlands, among 7.6 million males, 1255 males with the fragile X syndrome may be expected, probably without a difference in the distribution over the mildly and moderately / severely retarded males. However, the seven clinical genetic centres, covering the whole country, identified ± 450 male cases so far (Oostra, unpublished data). This suggests an underdiagnosis of > 50 percent. In our study, 1/4 of the fragile X patients were newly diagnosed cases. This study included both institutionalized (all ages) and non-

institutionalized (< 21 years) , but no non-institutionalized adult retarded individuals. Most people in the latter group work in sheltered workshops and live with their relatives or in sheltered homes. The fragile X syndrome is likely to be most underdiagnosed in this group, due to lack of diagnostically oriented medical care for these individuals. Improvement of genetic diagnosis in these settings is important, also for counselling of the families. Selection of male patients for FMR1 gene analysis might be facilitated by evaluation of dysmorphic features, since the presence of fragile X features was found to increase the yield of positive molecular diagnosis a tenfold in males (table 2). Such clinical pre-selection is less efficient for females because of the variability of expression of the full FMR1 mutation in females.[9,10]

Genetic carrier screening may be done at young adult age, especially to identify and inform female carriers of the pre- and full mutation prior to parenthood. One study in the French Canadian population suggested a frequency of premutation carrier females of 1/259.[30] Alternatively, screening for the fragile X syndrome might start -as presented here- among (young) mentally retarded individuals, which will allow families of newly diagnosed cases the option of avoiding the birth of a subsequent affected child. However, genetic screening programs are under debate for reasons of privacy, the risk of medicalization, risk of losing insurance and lack of treatment options. In introducing a screening program among the mentally retarded, a careful assessment of the acceptability by the families directly involved is of primary importance. The present study showed informed consent by parents or guardians in 71% of the eligible patients which is in accordance with other reports.[29,31-33] However, a high uptake is only one of the parameters of acceptance. Motives for consenting were the wish to have a diagnosis, the possibility of hereditary implications and the support of research into mental retardation. In general, there was openness in the family about having the relative tested: the majority of consenting parents / guardians discussed both the DNA-test and its result with others. The pre-test expectations of the consenting parents / guardians seems realistically reserved, as only a minority actually expected a diagnosis of the fragile X syndrome in their relative. However, none of the newly diagnosed cases had been anticipated by the parents / guardians. Most parents of the newly

diagnosed patients felt relieved by the resolution of uncertainty and the lack of direct responsibility for the retardation. They acknowledged the genetic nature of the condition and informed their relatives, as was found elsewhere.[34]

The test procedure did not cause undue anxiety among the participants, and most were interested in future diagnostic studies when these become available. One third of consenting parents / guardians arranged for additional diagnostic investigations and genetic counselling, as was reflected by a sharp increase in referrals for clinical genetic and dysmorphological work-up.

The observations in the non-consenting group should be interpreted with caution, given the low response. Reluctance was felt because of "stressful" blood-sampling. That might be alleviated, in the future, by the FMR1 protein test which requires only a few blood drops[35] or by a test using DNA isolated from a mouth-wash or cheek-brush.[29,31] The majority of the non-consenters believed that a "definite" cause for the handicap had already been established, however vague that diagnosis might have been. However, non-consenters agreed with the general principle of performing DNA- and other diagnostic investigations among the mentally retarded.

Several goals of a diagnostic program, i.e. establishing a cause for mental retardation, fuller information and choice for parents and relatives, are obviously achieved in this study. Even in a North West European country with well-developed diagnostic facilities more than 50% of fragile X cases seem undiagnosed at present. This reflects the slow rate of introduction of new diagnostic facilities in the care of the mentally handicapped. In a period of DNA technology and fears of genetic discrimination, this study shows that parents / guardians of individuals with mental handicaps have a realistic idea about potentials and limitations of new technologies, if adequately informed. The fear of health care authorities and others regarding adverse effects of studying larger groups of mentally handicapped individuals may be alleviated by the realistic appraisal of those directly involved.

Acknowledgements

We are grateful to Prof.Dr. G. Turner and Ms. H. Robinson for sharing their experience with the fragile X study in New South Wales, to Prof.Dr. H. Galjaard for his continuous support and his comments on the manuscript, and the Foundation of Clinical Genetics for their financial support.

Appendix

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Chapter 5.2

DNA testing for the fragile X syndrome: implications for parents and family

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Abstract

The fragile X syndrome is an X-linked semi-dominant mental retardation disorder caused by the amplification of an CGG repeat in the 5′ UTR of the FMR1 gene.

Nineteen fragile X families in which the mutated FMR1 gene segregated were evaluated. The implications of the diagnosis for parents and family were studied by pedigree research, interviews and questionnaires.

Information about the heredity of fragile X syndrome was only disseminated by family members to a third (124/366) of the relatives with an a priori risk of being carrier of the fragile X syndrome. Twenty-six percent (94/366) of the relatives were tested. Transmission of information among first degree relatives seemed satisfactory but sharply dropped off with increasing distance of the genetic relationship, leaving 66% uninformed. The latter is especially disadvantageous in X-linked diseases. Of the tested individuals 42% (39/94) had a premutation and 18% (17/94) had a full mutation. On the average, in one family one new fragile X patient and two new carriers were found.

Feeling responsible and capable to inform is a general problem in transmission of genetic information through relatives, and reduced acquaintance and contact with more distant relatives immediately reduces the effectivity of information transfer.

Introduction

The fragile X syndrome is a common cause of familial mental retardation with an estimated prevalence of 1/4000-1/6000 for males in western countries.[1-3] Main clinical features in males are mental retardation, macro-orchidism and a long narrow face with large everted ears.[4-6]

The mutation involved in this X-linked disorder is characterised by the amplification of a trinucleotide (CGG) repeat in the 5′ UTR of the FMR1 gene.[7,8] Normal persons have between 6 and 54 CGG repeats, phenotypically normal carriers of the premutation have between 52 and 200, and affected individuals have more than 200 CGG repeats in their

FMR1 gene, the so-called full mutation.[9] The cloning of the FMR1 gene in 1991 made determination of carrier status by DNA mutation analysis in both males and females possible.[10] The fragile X syndrome is an X-linked recessive disorder with some special features as 52%-82% of the carrier females with a full mutation show mental impairment[11-13] and as also males can be carrier of the premutation. Those so called 'normal transmitting males'[14] do transmit the premutation through phenotypically normal daughters to their grandchildren who are at risk of being affected.

A fragile X diagnosis in a mentally retarded individual will allow better support of behavioral and psychological problems related to the fragile X syndrome.[15,16] However, the diagnosis has also far-reaching implications for parents of the affected individual such as considerations regarding future offspring. Furthermore, it may be relevant to inform relatives about the hereditary aspects of the fragile X syndrome. So far, few reports studied the impact of the fragile X diagnosis on parents and family and the effectiveness of disclosure of information to relatives. Other studies on informing the family through relatives such as in cystic fibrosis[17,18] or balanced chromosomal translocations,[19-21] showed the general ineffectiveness and problems of such an approach.

We studied the implications for parents and family after a diagnosis of the fragile X syndrome. Parental adjustment, the dissemination of information in the family, the uptake of genetic counselling and/or DNA testing by family members at risk for being carrier of the fragile X syndrome are reported.

Methods and subjects

Between 1991-1995, nineteen fragile X families became newly identified by DNA mutation analysis of the FMR1 gene and were counselled at our department by one counsellor (BdV). In all families but one, the indexpatient was male. The ages of the index patients at the moment of diagnosis varied from 3 to 57 years. All families remained in contact with our department for at least one year. The relatives with an a priori risk of being a carrier of the fragile X syndrome had to be informed by the consultants about the

possibility for counselling and DNA-test.

The study was approved by the Medical Ethical Committee of the Erasmus University and University Hospital Dijkzigt.

Uptake DNA test and test results

Relatives were divided into four groups based on their relationship to the fragile X patient: first, second, third or fourth degree relatives. In each group the number of relatives informed about the risk by one of the primary consultants was determined based on information given by the family during the counselling process. The uptake of DNA-mutation analysis and its result were evaluated.

DNA analysis

Genomic DNA (8μg) isolated from blood leucocytes[22] was digested with the restriction enzyme *Hin*dIII according to the manufacturer's instructions, separated by gel electrophoresis and subjected to Southern Blot analysis according to standard procedures.[23] The intragenic DNA probe pP2 was used for analysis of the FMR1 gene.[24] The probe was labelled by the random oligonucleotide priming method.[25] PCR analysis of the CGG repeat was performed according to Fu et al.,[26] using modifications.[27]

Interviews

The impact of test results and genetic counselling was evaluated in interviews with the parents of newly diagnosed patients by the psychologist of the team (AT). The interview addressed issues collected from a review of the literature, and our own clinical experience. The interviews were semi-structured and a checklist helped to complete coverage of the following areas were: personal development, coping with stressing events, experience and coping with fragile X syndrome and personal risk, intimate relationships, and anticipating the test-outcome. The interviews took one to two hours and the first 45 minutes were audio-taped with parents consent.

Twenty-three parents, representing 14 families (nine couples and five single parents), were

interviewed. Results of the interviews of the 14 families are described as "couples". The remaining five families were not accessible (by moving to another area (n=4) or declining an interview (n=1)). The participating parents consisted of 12 mothers, all carrying the premutation, and 11 fathers.

Psychological questionnaires

In addition to the interviews, parents completed a questionnaire that assessed: experiences with the fragile X syndrome, attitude towards the fragile X syndrome, Impact of Event Scale (IES), the Beck Hopelessness Scale (BHS) and the Hospital Anxiety and Depression Scale (HAD). One female carrier did not complete the questionnaire because it provoked intrusive, unwanted feelings and one father because of language problems.

The Impact of Event Scale (IES) is a reliable, self report scale used to measure the current degree of subjective impact, experienced as a result of a specific life event (in this case, fragile X syndrome).[28] The IES estimates the influence of a stressor on two dimensions: (1) intrusion of unwanted ideas and thoughts into consciousness and (2) conscious denial-avoidance. The IES consists of seven items that form the intrusion subscale (score range 0-35) and eight items that form the denial-avoidance sub-scale (score range 0-40).

The Beck Hopelessness Scale consists of twenty true/false statements used to measure hopelessness or the pessimistic expectations one has for his/her future. A score of 9 or higher (range 0-20) is indicative of depression and possible suicidal behaviour.[29,30]

The Hospital Anxiety and Depression Scale is a self-report instrument for screening for clinically significant anxiety and depression, and provides a valid measure of the severity of these mood disorders. A score above 10 on either scale (score range 0-21) is indicative for severe anxiety or depression.[31]

Due to small sample size, data of these questionnaires were not further analyzed at group level. However, scores on BHS and HAD were considered as indicative for psychological well being.

Results

Uptake DNA test and test results

Up to the fourth degree the 19 families studied, consisted of 504 relatives: 251 females, 248 males and five relatives of whom the sex had not been recorded. The 19 indexpatients, 78 relatives without any risk of inheriting the mutation, 36 mentally normal children (age under 18), and the five relatives with unrecorded sex were excluded from further analysis. The remaining group of 366 relatives consisted of 41 first degree, 68 second degree, 97 third degree and 160 fourth degree relatives (table 1). All first degree relatives (41/41) were informed about their risk of being carrier of the fragile X syndrome. In second, third and fourth degree relatives these percentages were respectively 59% (40/68), 39% (38/97) and 3% (5/160). Overall 34% (124/366) of the relatives were informed about their carrier risk. Almost half of the uninformed relatives (103/242) lived abroad.

Overall, 26% (94/366) of the relatives were tested for carriership. The participation was highest among first degree relatives: 90% (37/41). In second degree relatives 37% (25/68) were tested and in third degree relatives 30% (29/97) applied for DNA testing. In the group of fourth degree relatives three out of 160 relatives at risk were tested for carrier

Table 1 Overview of informed and tested persons in a group of 366 relatives derived from 19 families with a risk on being carrier of fragile X syndrome

	Relation to patient with fragile X syndrome				
	1st degree n=41	2nd degree n=68	3rd degree n=97	4th degree n=160	total n=366
relatives informed	41 (100%)	40 (59%)	38 (39%)	5 (3%)	124 (34%)
relatives tested	37 (90%)	25 (37%)	29 (30%)	3 (2%)	94 (26%)

Table 2 DNA testresults in relatives of fragile X patients

	Relation to patient with fragile X syndrome								
	1st degree		2nd degree		3rd degree		4th degree		total
test result	♀	♂	♀	♂	♀	♂	♀	♂	
Normal	6	8	8	0	11	4	1	0	38 (40%)
Premutation	14	0	13	3	4	3	2	0	39 (42%)
Full mutation	7	2	1	0	3	4	0	0	17 (18%)
Total	27	10	22	3	18	11	3	0	94

status.

In the group of tested relatives 40% (38/94) had normal FMR1 genes, 42% (39/94) had the premutation and 18% (17/94) the full mutation (11 males and 6 females)(table 2). In the latter group, 14 persons were mentally retarded (11 males and 3 females).

Interviews

Experience before test result

Retrospectively, most parents (11/14) reported underestimation of the problems of their child by health care professionals and/or school teachers, and this reawakened resentments towards health care in general. The prominent feeling was, especially for mothers, that they had to convince others that something was wrong with their child. Four participants felt abandoned by family and friends.

Before a definite diagnosis, different explanations for the problems in their child were believed; lack of oxygen (3) or brain injury after traumatic delivery (6), age over 40 years

of the father (1), a disease in the mother (1), etc. The desire to learn more was counteracted by the fear for new stressful and nonproductive medical procedures. Also, most medical specialists usually did not have suggestions how to cope with the child's anxiety, panic and behavioral problems.

The influence of the affected child on family life was pervasively strong. The most difficult decisions were about schooling or admission to an institution, and throughout there was a lack of emotional and social support for these experiences (11/14 parents). Learning to love their retarded child and its associated strong affections made them more acutely aware how their child will be lifelong dependent on them. The fear about reduced awareness of the future society of the needs for the handicapped is frequently (12/14 parents) expressed. One couple (both with extreme pessimistic future expectancies as measured by the BHS) hoped to survive their child. Another couple anticipated the future by developing a training program to make their child more independent from the parents.

Experience after the diagnosis of the fragile X syndrome

Long years of uncertainty, medical shopping, guilt feelings were ended by the diagnosis. Moreover, the limited possibilities of the child, his restless behaviour and anxious moods were now to become confirmed as the reality.

As some couples (10/14) did only communicate the test results and the clinical genetics department's offer for genetic counselling to their close relatives, others (4/14) have communicated the test result to relatives and were also able to inform and support their relatives.

A few couples (2/14) were afraid to burden relatives with the information and to provoke adverse reactions. Both coping with the test result and informing relatives about the diagnosis and its hereditary aspects at the same time was regarded as a problem. Some reported resentments and disapproval by relatives (5/14), while most experienced positive reactions. Parents emphasized the need for additional support in regard to disclosure of the hereditary aspects to the family.

Table 3 Post-diagnosis attitude of parents towards fragile X syndrome (n=21)

	Agree	
	n	(%)
I was relieved by the DNA test result in my child	15	(71)
The testresult has improved the relationship with my child	11	(52)
I am worried about the implications of the testresults for the family	10	(48)
The family has a right to know about the inheritance of the fragile X syndrome	20	(95)
It is difficult for me to inform relatives about the prevalence of the fragile X syndrome in the family	5	(24)
I (we) feel responsible to inform other relatives about the inheritance of the fragile X syndrome	21	(100)
I (we) encourage relatives at risk to have themselves tested for carriership of the fragile X syndrome	20	(95)

Psychological questionnaires

The most important result was the expression of relief (a cause was found) in 15/21 parents (table 3). About half of the parents (11/21) stated that the diagnosis had improved their relationship with their affected child.

The impact of this diagnosis for relatives was generally acknowledged. The majority of the parents (18/21) informed close relatives within a few days after the diagnosis. The family's right to know about the genetics was generally acknowledged (20/21), including the responsibility to inform their relatives at risk (21/21) and to encourage them to be tested (20/21)(table 3). Half of the parents worried about the consequences of the result for their relatives and a quarter found it difficult to inform their relatives.

The majority of parents (13/21) agreed with abortion when the fetus shows a severe

disease, but a minority (8/21) found termination acceptable for Down syndrome or fragile X syndrome.

On the BHS, IES and HAD questionnaires two couples and one single female carrier expressed great fears and pessimistic expectations. Both couples had BHS scores higher than 9 which indicates an increased risk on depression or possible suicidal behaviour. Three carriers had extreme high Intrusion scores on the IES which reflects suffering from untoward feelings and thoughts about fragile X syndrome. Their HAD scores were higher than 10 indicating a severe depressive and anxious mood. Although the group is small, three out of twelve interviewed parents (25%) have reported extreme psychological problems.

Discussion

The diagnosis of the fragile X syndrome usually meant the end to a long period of uncertainty, anxiety among parents and disbelief by professionals. Parents were relieved and could adjust their expectations towards the affected child. However, little support was experienced, neither from family or friends, nor from professionals, especially not for the problems associated with raising a mentally retarded child. The family life was strongly affected in different ways and nearly all parents expressed their worries about the future. Moreover, some participants reported severe psychological problems. Therefore, it is essential to pay attention to psychological support and follow-up evaluation of the impact of a fragile X diagnosis.

The cascade screening within families in combination with counselling is a way to detect carriers and patients. In the present study, in more than half of the tested relatives mutations in the FMR1 gene (pre- or full mutation) were detected, however, on the average, only one new fragile X patient and two additional carriers were diagnosed per index patient. The latter low yield is probably caused by the low number of individuals informed and tested per family. Over all, only one third of the relatives at risk became informed. However, first degree relatives were completely informed within a short period of time but second and further degree relatives were not approached in the initial period

after the diagnosis. More relatives might have become informed later on, but our center did not receive later "spontaneous" requests for carrier testing in more distant relatives because of information on one of the index cases. The pattern of loose-tied relations between relatives, migration and influences of communication styles and family conflicts contribute to the poor transmission through the family grape-vine. Such family dynamics can play an important role when dealing with an inheritable disease.[32-35] Feelings like denial, blame and guilt can interfere with a good diffusion of information. Nearly all parents are initially well aware of the impact of the fragile X syndrome on the family and their responsibility to transmit the information. However, the result is influenced by inability to inform and/or reach relatives, their perception and understanding etc. Counselling on a genetic diagnosis should also address these family dynamic aspects of the parent's responsibility to inform relatives. Standard information procedures should be developed to assist parents in informing their relatives. Genetic associate nurses might play an important role to extend the family contacts. Besides family dynamics, the high migration ratio certainly influenced the ability of the consultants to inform their relatives.

McConkie-Rosell et al. (1995) gave some useful guidelines to facilitate the disclosure of information, such as informing different branches of the family by different relatives.[36] If the genetic counsellor takes the initiative to inform relatives at risk, more individuals would be able to consider genetic counselling and DNA testing. However, this approach bypasses the principle of medical confidentiality which might be solved through obtaining permission to contact the relatives by the genetic counsellor.

A different way for identifying individuals at risk, is active screening for the fragile X syndrome among the mentally retarded, for example in special schools and institutions.[37,38] Subsequently, the fragile X diagnosis in a patient would allow genetic counselling and DNA testing in relatives.

It is most important that first degree relatives (parents and sibs) are properly informed and that they are supported in disseminating the information to other relatives. The genetic counsellor should play an important role in optimizing and supervising the process of transmission of genetic information.

Acknowledgments

We thank the parents for their kind cooperation, W. Deelen, C. Jansen and S. Ramlakhan for their technical assistance, and Professor H. Galjaard and the Foundation for Clinical Genetics, Rotterdam for their continuous support.

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Chapter 6 General discussion and prospects

6.1 Clinical studies and FMRP function

6.2 Diagnosis and screening for the fragile X syndrome

6 General discussion and prospects

Since the first report of the fragile X syndrome as a familiar sex-linked mental retardation in 1943, clinical and molecular diagnosis of the disorder have greatly improved. The recognition of a fragile site on Xq27.3 opened the way to a confirmation of the clinical diagnosis in specific X-linked mental retardation families with macro-orchidism and other specific dysmorphism. Clinical, cytogenetical and a large number of extended family studies identified the phenotype and special features of the inheritance. It became possible to explain the unique phenomenon of, for example, normal transmitting males and the 'Sherman paradox'[1,2] after the cloning of the *FMR1* gene in 1991. The premutation concept became confirmed by the nature of the CGG repeat at the 5' end of the *FMR1* gene and its intergenerational amplification. Although the precise mechanisms for the repeat amplification are unknown,[3,4] the characterisation of the FMR1 protein led to detection of its widespread expression in nearly all tissues studied. At the cellular level, both nuclear and cytoplasmic localisations were found. The protein seems to shuttle between nucleus and cytoplasm and it can bind to ribosomes via mRNA. The question of why the major clinical symptoms are mostly restricted to brain and testis remains unanswered.

6.1 Clinical studies and FMRP function

(Over) growth

Clinical studies may also give clues to the protein function. The fragile X syndrome is seen as an early overgrowth syndrome which is mainly restricted to head size, suggesting a dysfunction starting prenatally and continuing into early life. A minority of fragile X patients show a tendency to general overgrowth - partly resembling the Sotos syndrome - while others present with a phenotype suggesting the Prader-Willi syndrome (chapters 3.1 and 3.2). It is still not known whether the overgrowth is the result of a prenatal and/or postnatal effect of the lack of FMRP absence in some tissues (e.g. certain parts of the brain). Macro-orchidism is most prominent during and after puberty when it becomes

manifest in more than 50% of the male fragile X patients.[5] No consistent hormonal abnormalities have been observed in fragile X patients.[6] The common enlargement of testes may be the result of an increased local response to normal pubertal hormone secretion. It is not known whether the low cyclic AMP levels in platelets and lymphoblastoid cell lines from fragile X patients[7-9] relate to such abnormal intrinsic responses. Cyclic AMP is an important intracellular mediator through which numerous hormones stimulate their target tissues. Whether other cell types have also low cyclic AMP levels is as yet unknown.

FMRP and its potential role in transcription[10-13] may explain growth abnormalities in the fragile X syndrome. The increase in FMRP expression in dividing mesodermal cells supports this: high transcription activity is required during cell growth and/or division.[14,15] If there is a mutation, a growth delay or an overgrowth as in fragile X patients may be observed. However, overgrowth might also be a result of inadequate or delayed responses of certain parts of the brain (e.g. the hypothalamic-pituitary system) to physiological feedback mechanisms. Earlier onset of puberty in males and females with the fragile X syndrome compared to normal supports the hypothesis of hypothalamic-pituitary dysfunction,[16] although others could not confirm this.[17,18] The family of IGFs (insulin-like growth factors) and their binding proteins are of interest for the phenotype of the fragile X syndrome: IGFs are involved in growth processes, including cartilage development, tissue regeneration and ovarian function.[19,20] It has been suggested[21] that local IGF-I function plays a role in the - as yet poorly understood - premature ovarian failure in carriers of the premutation[17,22-24] and increased dizygotic twinning among their offspring.[25] In addition, elevated IGF1 (somatomedin) and IGFBP3 have been reported in the blood of one fragile X boy with extreme overgrowth (chapter 3.2).[21] The link with the *FMR1* gene mutation remains conjectural.

Intellectual impairment

Intellectual disability is the most consistent clinical symptom in males with the methylated full mutation. Whether the mutation and the absence of the FMRP already affects mental

development from the prenatal stage onwards is not known but it seems likely. No gradual decline in mental capacities in fragile X patients has been observed.[26] Analysis of the effects of a full mutation on brain function has been limited to IQ measurements in patients and repeat sizes in their lymphocytes. The CGG repeats are notorious for occurring in length- and methylation-variation somatic mosaics. Occasional males have been observed with milder retardation or no retardation when they have a significant number of cells with unmethylated full mutations, showing that eventual methylation status is inversely related to FMRP production and, subsequently, mental impairment (chapter 3.3).

FMR1 protein expression in less than 30% of lymphocytes is associated with mental retardation and other clinical symptoms.[27,28] Size mosaicism (combined presence of premutation and full mutation) does not mitigate mental retardation (chapter 4.1). In females, the mental retardation ranges from absent to severe in full mutation carriers: there is a positive relation of the proportion of lymphocytes with a normal *FMR1* allele on the active X chromosome and mental development (chapter 4.2).

Neuroimaging and histological studies of brain tissue have isolated areas of the brain which are possibly involved in the etiology of the mental retardation. There is an increase in the size of the caudatus, thalamus and hippocampus[29,30] (chapter 1.5.3). The hippocampus has a major role in memory function (and learning) by translating short-term into long-term memory. The latter results from changes in the synapses and/or synaptic connections. Such a process requires considerable turnover of proteins and therefore translation. As the FMRP is thought to be involved in translation, its absence might well lead to inadequate hippocampal function and impaired long-term memory. The hippocampus is also involved in processing sensory information through connections with the sensory cortex and the limbic system. The behavioral features in fragile X patients - such as tactile defensiveness, eye gaze avoidance and hyperactivity - might well be a consequence of hippocampal dysfunction.

6.2 Diagnosis and screening for the fragile X syndrome

Molecular diagnosis in the fragile X patient and ascertainment of carriers became highly precise after the identification of the *FMR1* gene with its CGG repeat. Genetic counselling as well as prenatal diagnosis in families with the fragile X syndrome became more accurate.

It is still an open question if large scale testing of, for example, the mentally retarded or young adult females should be organised.[31-38]

Testing mentally retarded individuals leads to diagnosis and improved support for the newly diagnosed fragile X patients (chapter 1.5.4; table). Such programmes have been in place since the cytogenetic test method was introduced. DNA testing in institutes and the special school population is much more precise, especially for further testing for (pre)mutation carriers in the family, as in this study (chapter 5).[39-44] These programmes are highly welcomed by parents/guardians and well accepted by the staff of schools and institutes. The relatively low 'yield' of such programmes (≈ 1% of the tested patients have the fragile X syndrome) may be significantly increased by pre-selection based on physical features (chapter 5.1). A simple scoring system may be used during the medical intake of an individual in a school or institution for the mentally retarded. If molecular diagnosis for the fragile X syndrome is routinely performed in those selected individuals, large screening programmes will become less necessary. A limitation in screening of the mentally retarded is that many of these live at home with their parents or in small sheltered living facilities, receiving medical care only from their GPs. Some of them visit daytime occupational facilities, but it is generally difficult to approach them through a central agency. Another limitation in such screening is the insufficient dissemination of information to relatives about the genetic implications of a diagnosis. There is consensus that all members in a fragile X family should be offered testing ('cascade screening'). The effectiveness of that approach relies on the efforts of the parents/guardians to inform other relatives about the risk of being a carrier and the option of carrier testing. After detection of a new case of the fragile X syndrome, this information is not disseminated to all the family members at

*Table Screening options for FMR1 gene mutations**

			test method		considerations		
	target group	expected yield	DNA	protein antibody	advantages	disadvantages	pitfalls
Prior to pregnancy	fertile women	1/ 250 PM	+	–	identification females at risk several options	difficult to contact unfamiliar with disorder	(in)stability intermediate or PM sized alleles
Prenatal	pregnant women	1/ 250 PM	+	–	identification females at risk	limited options time constraint unfamiliar with disorder	(in)stability intermediate or PM sized alleles
Neonatal	newborns	1/5000 FM	+	+ (♂♂)**	early diagnosis several options for parents	possible interference child-parent bond	girls with FM
Later age	mentally retarded	1/ 100 FM	+	+ (♂♂)**	diagnosis, improved care	blood test stressful possible sibs with Fra(X) already born	

* for references see section 1.7 and chapter 6.2
** restricted to males
PM = premutation; FM = full mutation

risk (chapter 5.2). Ways of improving the intrafamilial transmission of genetic information are limited. Standardized information letters and brochures cannot remove the burden on the 'messenger' bringing news about a possibly unknown genetic cause of mental retardation, especially to female relatives. Ethical and medico-legal rules prevent clinical geneticists from approaching relatives or their physicians directly. On the other hand, the 'messengers' often express a preference for doctors informing their relatives: they find it burdensome to explain difficult and emotional facts about the risk of disorders in offspring (chapter 5.2).

The limited effectiveness of informing relatives after the identification of index patients is just one argument for considering fragile X (pre)mutation screening among young women prior to reproduction or in early pregnancy (see table). The other argument in favour of screening females is that there may be many more premutation carriers at risk for affected offspring than can possibly be identified through affected males. Some data indicate that 1 in 250 women in the Canadian and Finnish population are carriers of a premutation sized allele[45] (Ryynänen, *personal communication*). They have a risk of approximately 1 in 20 in each pregnancy of transmitting a full mutation to a male or female child. Ideally, such testing of adult women should be offered prior to conception. Once pregnant, the options for a carrier are limited: decisions about acceptance of the risk or prenatal testing have to be made within days. However, preconceptional testing is more difficult to organise in a health-care system where non-pregnant woman rarely have contacts with either GPs, gynaecologists or midwives.

The third option in testing would be screening of newborns (see table). Testing all newborns for mutations in the *FMR1* gene might result in logistical and ethical dilemmas.[46-48] Proponents of an early diagnosis will point to the possibility for parents to anticipate problems in their child and to make a timely informed decision about further offspring. However, the child will not yet show obvious signs of developmental delay. Knowing beforehand about the future mental handicap might interfere with initial child-parent bonding. Using molecular analysis, newborns with an intermediate or premutation allele will also be identified. Counselling families about future reproductive risks in their

healthy new-borns may increase anxiety. The carrier status of the new-born will not be detected or revealed when the FMRP antibody test is used. At present, there are no operational neonatal screening programmes for the fragile X syndrome.
A full discussion about the conditions for implementation of screening programmes can be found in two reports on genetic screening, one from the Health Council of the Netherlands[47] and the other from the Nuffield Council on Bioethics[48].

Epilogue: the future

A child with an intellectual disability has a major impact on parents and its family. In the process of adjustment, parents are often confronted with uncertainty and incomprehension by the environment, professionals included. A diagnosis assists in coping with problems and eventually accepting their mentally handicapped child. In contrast to many other mental retardation disorders, a molecular diagnosis is possible in the fragile X syndrome.
The idea that 'DNA' or 'molecular' diagnosis might induce fear or resistance in parents/relatives of mentally handicapped individuals was not confirmed. In the life of a parent of a mentally retarded child, the most important thing is a diagnosis as a starting point towards acceptance, expectations about the future, possible therapy etc. For the purposes of an early diagnosis and counselling of the family, the test may be done when the symptoms of the disorder become manifest. For boys with the fragile X syndrome, this will be between the ages of 1 and 2. This diagnosis should also be considered during the medical work-up in the course of admission to a school for the learning disabled (at age 5-6 in the Netherlands).
The fragile X diagnosis in a child will generally result in parents looking for treatment. Some parents ask for pharmacotherapy to suppress the behavioral problems. However, its value is still a subject of debate. Most parents hope for a cure, either through protein replacement and/or gene therapy. However, before studying such future therapy strategies, one should consider that protein production probably needs to be restored in at least ≈ 50% of the cells (based on studies in blood) to achieve normal cognitive function. Prior to this long-term aim, one should now concentrate on improving conventional support and

intervention for the fragile X syndrome, including physical, speech and occupational therapy. In the meantime, (basic) research can concentrate on increasing our understanding of the actual mechanism(s) of the neuronal dysfunction at the root of the mental retardation.

Conclusion

This study provided an extension of the data on the phenotype of the fragile X gene mutation in males and in females. It also demonstrated the acceptance and feasibility of diagnostic studies among mentally retarded individuals in the Netherlands.

In view of the problems and burden of transmitting the genetic knowledge to relatives, preconceptional or prenatal screening for (pre)mutation carrier status among women might be an option. It is for ethical, legal, patients' / parents' organisations and health-care bodies in different countries to decide whether this route should be taken and which screening option or combination of options is the most or least desirable: neonatal / mentally retarded / young adult (pregnant) female individuals. Clinical genetics has at least the obligation to provide a clear view of the available options.

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Summary

Summary

This thesis presents studies on the clinical and genetic heterogeneity of the fragile X syndrome, including an epidemiological study of the prevalence of this syndrome among a representative sample of mentally retarded individuals in the Netherlands.

The fragile X syndrome is characterized by mental retardation, behavioral features and physical features such as a long face with large protruding ears and macro-orchidism. Affected males and most affected females have a fragile site at Xq27.3 in a percentage of the cells tested under special culture conditions. In 1991, after identification of the Fragile X Mental Retardation (*FMR1*) gene, this cytogenetic marker became replaced by molecular diagnosis. The fragile X syndrome was the first example of a 'novel' class of disorders caused by a trinucleotide repeat expansion. Affected individuals have expanded CGG repeats (>200) in the first exon of the *FMR1* gene (the full mutation). This expansion is accompanied by hypermethylation of the repeat and its flanking region resulting in silencing of transcription and absence of the FMR protein (FMRP). In the normal population, the CGG repeat varies from 6 to 54 units. Phenotypically normal carriers of a repeat in the 43 to 200 range are called premutation carriers. Female carriers of a premutation or full mutation have an increased risk of affected offspring. Male carriers of a premutation will usually transmit it unaltered to their daughters.

The cloning of the *FMR1* gene led to the characterisation of its protein product FMRP (the functions of which are as yet unknown), encouraged further clinical studies and opened up the possibility of more accurate fragile X screening programmes.

The aims of the study (chapter 2) were threefold:

- Further delineation of the clinical phenotype.
- Assessment of the relation between the genetic defect and cognitive functioning.
- Determination of the prevalence of the fragile X syndrome in a genetic epidemiological study of mentally retarded individuals in the Netherlands and analysis of the feasibility and acceptability of such screening.

Phenotypic variability

The variability of the phenotype in the fragile X syndrome was studied in eight males with a special clinical presentation of the fragile X syndrome (chapter 3.1). There is a certain similarity with the Prader-Willi syndrome in terms of mental retardation, extreme obesity with a full round face and small, broad hands/feet. This phenotype in fragile X patients has become known as the 'Prader-Willi-like' subphenotype and appeared to be inherited in one family. General overgrowth was observed in four sporadic cases (chapter 3.2), resembling in some aspects the Sotos syndrome ('Sotos-like subphenotype'). Endocrine studies in these four cases revealed raised IGF-I and IGFBP-3 in one patient.

These two subphenotypes demonstrate clinical variability and the necessity for analysis of the *FMR1* gene in mentally retarded individuals with symptoms of the Prader-Willi or the Sotos syndrome.

The effects of variability in FMR1 gene methylation on the phenotype

Fragile X patients with a full mutation in the *FMR1* gene (>200 CGG repeats) lack the FMR protein. The absence of FMRP leads to the mental retardation and additional phenotypic features. In rare cases, the full mutation is incompletely methylated in a proportion of cells ('methylation mosaics'). This may result in various levels of protein production and a less severe or even normal phenotype. A mentally normal male with an *FMR1* gene trinucleotide repeat expansion in the full mutation range was found to have unmethylated *FMR1* alleles in the majority of his lymphocytes (chapter 3.3). This individual had two mentally retarded cousins with a corresponding *FMR1* gene expansion but a higher degree of methylation and lower percentage of FMRP-producing lymphocytes. Apparently, methylation of the promoter region rather than the length of the repeat is the primary determinant of reduced FMR1 protein production and the associated level of mental impairment.

The effect of the full mutation on mental development

There is another type of mosaicism which is more common in fragile X patients than variation in *FMR1* gene methylation: in addition to a proportion of cells with the methylated full mutation, there are other cells with a premutation ('size mosaicism'). Since cells with a premutation can produce FMRP, one might expect 'size mosaicism' to be associated with a mitigated clinical presentation. However, in 35 fragile X male patients tested by standardized IQ tests, there was no sign of any influence of size mosaicism on IQ (chapter 4.1).

The relation between the mental retardation and the *FMR1* gene mutation was analyzed in affected males and females. In 52 males, the cytogenetic expression of the fragile site at Xq27.3 was positively correlated with the mean size of the full mutation in the *FMR1* gene (chapter 4.1). However, the degree of mental retardation was not related to the size of the full mutation in 35 fragile X males. This is explained by the absence of FMRP production as a consequence of the methylation of the promoter region in the full mutation.

The mental status of 33 adult females with an *FMR1* gene full mutation was compared to a control group consisting of their first-degree adult female relatives (n=28) without a full mutation (chapter 4.2). Fifty percent of the females with a full mutation were mentally retarded whereas none of the controls had an IQ below 70. The proportion of normal *FMR1* alleles on the active X chromosome was significantly and positively related to IQ. The X inactivation pattern in leucocytes seemed to reflect the distribution of X inactivation in brain cells (as far as the latter could be extrapolated from IQ data).

Prevalence of the fragile X syndrome in a representative group of mentally retarded individuals

A genetic epidemiological study of the fragile X syndrome was conducted in a representative sample of mentally retarded individuals in the Netherlands (chapter 5). Five institutions (1869 individuals) and sixteen special schools (1483 individuals) for mentally retarded individuals in the South-West of the Netherlands participated. To date, this is the

largest study to establish the prevalence of the fragile X syndrome using molecular diagnosis. Parents/guardians and medical, teaching and nursing staffs of schools/institutes received oral and written information prior to giving written consent. The test involved a brief physical examination and blood sampling for the DNA test. Perceptions and reactions of parents/guardians were assessed before and after receiving the test result. The majority (70%) of the parent/guardians of eligible patients gave consent for participation of their relative, reflecting acceptance of the programme. Some of the main reasons for participation were: the wish to obtain a diagnosis, the hereditary implications and support for research into mental retardation. The main reasons for refusal were the idea that the cause of the mental handicap was already known and that the test (blood sample) was too stressful for the patient. Generally, those who refused did not oppose the principle of genetic testing.

In addition to 32 known fragile X patients, 11 new cases were diagnosed. This reflects an under-diagnosis of $\approx$ 25% in schools/institutes. The physical examination was highly sensitive and specific (0.91 and 0.92 respectively), demonstrating the feasibility and utility of clinical pre-selection. When a scale was used beforehand for the physical features of the fragile X syndrome, it was possible to raise the detection rate using molecular diagnosis in males tenfold.

The prevalence of fragile X syndrome established for the Netherlands is 1/6,045 for males. This is considerably lower than the previous estimate of 1/1,000 - 1/2,600 based upon cytogenetic studies. The figures agree with recent results from smaller studies in Australia and the United Kingdom. The high acceptance and realistic appraisal of the screening programme by the parents/guardians are important results that may lead to consideration of large scale diagnostic programmes for this syndrome and other causes of mental retardation.

Implications of the diagnosis

The impact of the fragile X diagnosis in 19 families was studied by means of interviews with parents/guardians. There was a positive attitude towards obtaining certainty about the

diagnosis of the retardation (chapter 5.2). However, information about the genetic aspects of the syndrome is poorly disseminated to more distant relatives (third and fourth degree). Only 34% (124/366) of the relatives at risk of being a carrier have been informed (100% of first- and 59% of second-degree relatives). 27% (94/366) have actually been tested. In each family with an index patient, two new female carriers and one new fragile X patient were identified. Actual policies relating to the dissemination of information in genetic disorders require improvement both in terms of technologies for screening in early adulthood and in terms of consideration of the ethical and psychological restrictions of the transmission of knowledge by relatives.

Samenvatting

Samenvatting

Een verstandelijke handicap (zwakzinnigheid) komt bij mannen vaker voor dan bij vrouwen. Eén van de vormen van zwakzinnigheid die vooral bij mannen voorkomt, is het fragiele X syndroom. Vroeger herkende men deze aandoening aan een breekbare (fragiele) plek op het X-chromosoom, zichtbaar bij onderzoek van de chromosomen. Onderzoek van de erfelijke eigenschap zelf, mogelijk sinds 1991, laat nauwkeuriger diagnostiek van de aandoening toe bij mannen en vrouwen, en nauwkeurig onderzoek van dragers en draagsters die nageslacht met deze aandoening kunnen krijgen.
Dit proefschrift beschrijft de klinische en genetische verschillende uitingsvormen van het fragiele X syndroom. In een genetisch epidemiologisch onderzoek werd het vóórkomen van deze aandoening (prevalentie) vastgesteld in een representatieve steekproef van mensen met een verstandelijke handicap in Nederland.

Fragiele X syndroom en erfelijkheid
Het fragiele X syndroom is een verstandelijke handicap veroorzaakt door een afwijkende erfelijke eigenschap op het X-chromosoom. Bijkomende lichamelijke en gedragskenmerken zijn ondermeer een lang gelaat met vergrote, afstaande oren, vergrote testikels en verminderd oogcontact.
Vrouwen hebben twee X-chromosomen en mannen één X- en één Y-chromosoom. Bij een afwijking op het X-chromosoom zullen mannen daar in het algemeen last van hebben, terwijl vrouwen draagster kunnen zijn, soms zonder verschijnselen. Bij vrouwen compenseert (veelal) het tweede, normale, X-chromosoom voor de afwijking op het andere X-chromosoom. Echter, vrouwen die draagster zijn hebben wel een verhoogd risico op het krijgen van zowel jongens als meisjes met de aandoening.
Bij mannen en de meeste vrouwen met het fragiele X syndroom ziet men een breekbare (fragiele) plek op het X-chromosoom (Xq27.3) in een gedeelte van cellen gekweekt onder speciale omstandigheden. Het vinden van de oorzakelijke fout in de erfelijke code, het 'Fragile X Mental Retardation' (*FMR1*) gen in 1991, maakte nauwkeuriger diagnose van

mannelijke/vrouwelijke patiënten en gezonde dragers/draagsters mogelijk dan eerder met chromosoom onderzoek (hoofdstuk 1.2). In de erfelijke boodschap voor deze eigenschap ziet men bij patiënten dat een kort stukje code een afwijkend aantal keren wordt herhaald (meer dan 200 maal). Deze verandering (mutatie) leidt tot een verstoring van de functie van de erfelijke eigenschap, met als gevolg zwakzinnigheid bij de betrokken man en bij een deel van de betrokken vrouwen (50-70%).

Het aantal herhalingen in de erfelijke eigenschap kan geleidelijk toenemen bij overdracht van generatie op generatie (hoofdstuk 1.2). Bij normale personen is het aantal herhalingen tot 50 eenheden en dat aantal is stabiel bij overdracht naar de volgende generatie. Bij ± 60 tot 200 herhalingen is er een licht onstabiele verandering (premutatie of kleine verandering). De betrokken mannen en vrouwen zijn echter verstandelijk normaal. Een vrouw kan de kleine verandering onveranderd of in sterk vergrote vorm aan zoons of dochters doorgeven. De kinderen hebben dan de grote of volledige verandering en zullen verstandelijk gehandicapt zijn, jongens veelal ernstiger dan meisjes. De grote of volledige verandering leidt tot de toevoeging van methylgroepen (CH_3) aan de DNA-base cytosine in zowel het promotor gedeelte van het gen (het gedeelte van het gen dat de aflezing regelt) als de CGG herhaling ('hypermethylering'). Het gen wordt daardoor uitgeschakeld en de eiwitaanmaak (van het FMRP, fragile X mental retardation protein) wordt stilgezet (hoofdstuk 1.2). De verstandelijke handicap ontstaat door tekort aan het eiwit FMRP.

Hebben alle mannen met het fragiele X syndroom dezelfde verschijnselen?

De meerderheid van de mannen met een verstandelijke handicap door het fragiele X syndroom heeft de bovenstaand beschreven kenmerken.

Een kleine groep heeft echter een overmatig lichaamsgewicht met een vol, rond gelaat, korte brede handen/voeten, en plaatselijk toegenomen pigmentatie van de huid (zoals bij het syndroom van Prader-Willi). Vanwege die overeenkomst werd die verschijningsvorm het 'Prader-Willi-like' subfenotype genoemd (hoofdstuk 3.1). Voor het eerst wordt hier beschreven dat deze verschijningsvorm met overmatig lichaamsgewicht als erfelijke overdraagbare vorm in een familie met het fragiele X syndroom werd doorgegeven.

Overmatige groei zowel van gewicht als van lengte is een andere zeldzame verschijningsvorm van het fragiele X syndroom. In hoofdstuk 3.2 worden 4 nieuwe patiënten, allen enkele gevallen in hun familie, beschreven. Zij lijken in sommige aspecten op mensen met het Sotos syndroom ('Sotos-like subphenotype'). Bij één van deze 4 patiënten werd een verhoogd 'insulin-like growth factor-I' (IGF-1) en 'insulin-like growth factor binding protein-3' (IGFBP-3) vastgesteld: een verhoogd gehalte van factoren die de celstofwisseling positief beïnvloedden. Deze bijzondere vormen van het fragiele X syndroom geven de vraag of de afwijking in het fragiele X gen ook andere erfelijke eigenschappen kan beïnvloeden, of dat er bij deze patiënten een toevallig samengaan was van het fragiele X syndroom en een bepaalde vorm van overgroei van lengte en/of gewicht. Wel is duidelijk dat bij verstandelijk gehandicapten met overmatig gewicht en/of lengte groei ('Prader-Willi-like' en 'Sotos-like') het gewenst is aanvullend op andere diagnostiek (zoals voor het Prader-Willi syndroom) óók het fragiele X gen te onderzoeken.

De herhaling in de erfelijke code (CGG) bij het fragiele X syndroom

Hierover kunnen we ons de volgende vragen stellen:

1) Is het effect van de grote verandering in het gen steeds hetzelfde?

Veranderingen van de chemische samenstelling door toevoeging van methylgroepen (methylering) kan de effecten van een afwijkende herhaling in de erfelijke code beïnvloeden. Dat werd duidelijk bij een aantal mannen die weliswaar een grote verandering in het fragiele X gen hadden maar verstandelijk normaal waren, terwijl andere mannen in hun familie het volledige beeld van het fragiele X syndroom hadden (hoofdstuk 3.3). Bij deze mannen werd een onvolledige methylering van het gen vastgesteld. Deze onvolledige methylering leidt er toe dat soms toch het betrokken eiwit wordt gemaakt, en dat bijvoorbeeld de betrokken hersencel normaal zal functioneren. De aanwezigheid van cellen met een verschillende graad van methylering van de herhaling in de code wordt methylerings-mozaïek genoemd (hoofdstukken 1.2 en 3.3).

2) Is de lengte van de herhaling steeds hetzelfde?
In 20 tot 40% van de fragiele X patiënten ziet men naast cellen met een grote verandering ook cellen met een kleine verandering. Men noemt dat 'mozaïek voor de grootte'. Cellen met de kleine verandering maken het eiwit en zouden zo voor mildere verschijnselen kunnen zorgen. Echter, bij 35 mannen met het fragiele X syndroom bleek een dergelijk mozaïek geen invloed op de verstandelijke ontwikkeling (het IQ) te hebben (hoofdstuk 4.1).

3) Is er een verband tussen de grootte van de grote verandering en de mate van verstandelijke handicap?
De verandering in het *FMR1* gen bleek samen te hangen met het al of niet zichtbaar zijn van de breekbare plek op het X-chromosoom: deze is vaker zichtbaar naarmate er een grotere verlenging (herhaling) aanwezig is. Belangrijker is echter, dat een grote verandering van > 200 herhalingen steeds eenzelfde ernstige verstandelijke handicap gaf bij 35 fragiele X mannen (hoofdstuk 4.1). De verklaring is, dat steeds het promotor (besturings) gedeelte van het gen gemethyleerd is waardoor eiwit produktie ontbreekt.
Het verstandelijk niveau bij 33 vrouwen met een grote verandering in hun *FMR1* gen werd vergeleken met 28 eerste-graads vrouwelijke familieleden (moeders of dochters) zonder een grote verandering (hoofdstuk 4.2). Vijftig procent van de vrouwen met een grote verandering waren verstandelijk gehandicapt (IQ < 70) waartegen géén van de controle vrouwen. Vrouwen hebben in iedere cel twee X-chromosomen, waarvan er per cel telkens één is uitgeschakeld. Dit wordt X-inactivatie of Lyonisatie genoemd. Het IQ van de vrouwen bleek in het algemeen hoger wanneer het normale X-chromosoom vaker actief was. Het X-inactivatie patroon in witte bloedcellen lijkt een weergave van de verdeling van de X-inactivatie in hersen cellen (in zoverre dit geconcludeerd mag worden uit IQ gegevens).

Onderzoek van een representatieve groep verstandelijk gehandicapten

In vijf instellingen voor verstandelijke gehandicapten (1869 individuen) en zestien scholen voor speciaal onderwijs (1483 individuen) in Zuid-West Nederland werd een voor Nederland representatieve groep van mannelijk verstandelijk gehandicapten onderzocht voor het fragiele X syndroom. Ouders/voogden en medisch-, onderwijs- en verplegend personeel van de deelnemende scholen/instellingen ontvingen mondelinge en schriftelijke informatie vooraf aan het onderzoek. Het is het grootste onderzoek tot nu, en het eerste representatieve over het voorkomen van het fragiele X syndroom. Ervaringen en verwachtingen van ouders/voogden werden onderzocht voor en na ontvangst van de DNA-test uitslag. Er was een grote deelname: 70% van de ouders/voogden gaven toestemming voor een kort lichamelijk onderzoek en bloedafname voor de DNA-test bij hun verstandelijk gehandicapte familieleden. Redenen voor deelname waren het willen weten van een diagnose, weten over gevolgen i.v.m. erfelijkheid voor overige familieleden en het bevorderen van wetenschappelijk onderzoek in de zwakzinnigen zorg. Afzien van het onderzoek berustte vaak op de veronderstelling dat de oorzaak van de verstandelijke handicap bij het familielid al bekend zou zijn (hoe onduidelijk die diagnose vaak ook was) en het gevoel dat de DNA-test (bloedafname) te belastend zou zijn voor het betrokken familielid. Overigens waren de niet-deelnemenden géén principieel tegenstander van genetisch onderzoek.

Het vóórkomen van het fragiele X syndroom

Bij het grootschalig onderzoek van totaal 3352 verstandelijk gehandicapte mensen werden naast 32 reeds bekende fragiele X patiënten, 11 nieuwe gevallen gediagnostiseerd. Veel fragiele X patiënten zijn dus nog niet gediagnostiseerd. Het lichamelijk onderzoek is een goede methode voor de selectie van patiënten voor wie de test van belang is (sensitiviteit 0.91 en specificiteit 0.92). Met behulp van een lijst lichamelijk kenmerken van het fragiele X syndroom kan men de kans op het vaststellen van deze diagnose bij mannen tienvoudig te vergroten.

In Nederland komt het fragiele X syndroom voor bij 1 op 6045 mannen. Dat is lager dan

vroeger op grond van chromosoom onderzoek werd geschat: 1 op 1000 tot 1 op 2600. Recent werden in kleinere onderzoeken in Australië en het Verenigd Koninkrijk vergelijkbare waarnemingen gedaan.
De hoge acceptatie en realistische verwachtingen door ouders en voogden over het diagnostisch program is een belangrijke overweging om eventueel moleculaire diagnostiek te verrichten voor het fragiele X syndroom in de zwakzinnigen zorg.

Betekenis van de diagnose fragiele X syndroom en overdracht van erfelijkheidsinformatie binnen de families
De betekenis van de diagnose fragiele X syndroom werd onderzocht in 19 families tijdens interviews met ouders. De beoordeling is in het algemeen positief. Er komt een einde aan de onduidelijkheid (hoofdstuk 5.2). Echter de informatie over de erfelijke aspecten van de aandoening wordt beperkt doorgegeven met name aan verder verwijderde familieleden (39% van de derde- en 3% van de vierde-graads familieleden). In totaal worden slechts 34% (124/366) van de familieleden met een risico om drager te zijn geïnformeerd en 27% (94/366) laat zich uiteindelijk testen. Per patiënt/familie worden twee nieuwe vrouwelijke dragers en één nieuwe fragiele X patiënt gediagnostiseerd.
Het beleid ten aanzien van de doorgave van de informatie in families voor erfelijke aandoeningen dient te worden verbeterd. Onderzoek naar dragerschap op jong volwassen leeftijd gevolgd door informatie over kansen voor het nageslacht verdient overweging. De ethisch en psychologisch beperkingen bij de overdracht van de kennis door familieleden dienen onderkend te worden.

Appendix: fragile X checklist

Fragile X checklist A

score	Absent 0	Borderline 1	Present 2
Family history of intellectual handicap[a]			
Personality[b]			
Ears[c]			
Face[d]			
Body habitus[e]			
Total score: _____			

(adapted from Laing et al. Am J Med Genet 1991;256-9)

Score:

a 2: an affected sib, an affected maternal uncle, aunt, nephew, niece or first cousin
1: for any other affected relative (compatible with X-linked inheritance)

b 2: initial shyness and lack of eye contact followed by friendliness and verbosity with echolalic speech patterns
1: only some of these characteristics are present

c 2: large (by measurement) and protruding from the side of the head
1: large only

d 2: long jaw and high, wide forehead
1: only one of these findings are present

e *for males*
2: a slim physique with tall stature, rounded shoulders, hyperextensible finger joints, and lack of body hair
or
obese physique with feminine distribution of body fat, striae, soft skin and lack of body hair
1: only some of these findings are present

for females
2: slim with hyperextensible joints
or
obese and well proportioned
1: some of these findings are present

Fragile X checklist B

score	Absent 0	Borderline/ Present in the past 1	Present 2
Mental retardation			
Hyperactivity			
Short attention span			
Tactile defensiveness			
Hand-flapping			
Hand-biting			
Poor eye contact			
Perseverative speech			
Hyperextensible MP* joints			
Large or prominent ears			
Large testicles			
Single palmar crease			
Family history of mental retardation			
Total score: _____			

* metacarpophalangeal

(adapted from Hagerman et al. Am J Med Genet 1991;38:283-7)

Dankwoord

Het hier beschreven onderzoek is met de steun en de medewerking van velen tot stand gekomen. Zonder anderen tekort te willen doen, zijn er echter een aantal personen die een onmiskenbare rol hebben gespeeld.

Allereerst Prof. dr. M.F. Niermeijer, zowel als promotor en opleider heeft u mij telkens gestimuleerd kritisch en helder te denken. Uw onophoudelijke inzet en relativerende gedachten op gepaste momenten, hebben zeer inspirerend gewerkt.

Mijn grote dank gaat uit naar Prof. dr. H. Galjaard en de Stichting Klinische Genetica voor de voortdurende steun bij de opzet en afronding van dit onderzoek. Dr. B.A. Oostra als 'moleculair mind' achter het fragiele X onderzoek. Prof. dr. P.J. Willems en beide voorgenoemde leden van de promotiecommssie, ben ik zeer erkentelijk voor hun inhoudelijke suggesties. Mevr. dr. D.J.J. Halley en mevr. dr. ir. A.M.W. van den Ouweland voor hun creatieve hulp bij mutatiedetectie en bij het uitvoeren van hoogst accurate DNA diagnostiek in het onderzoekscohort. Prof. dr. D. Lindhout voor de geboden mogelijkheid om al tijdens mijn studie kennis te maken met de Klinische Genetica. Dr. J.O. van Hemel voor het diagnostiseren van de patienten in de 'pre-FMR1 gen' periode. Dr. A. Hoogeveen, dr. A. Reuser en dr. R. Willemsen voor de gedachtenwisselingen tijdens de wekelijkse werkbesprekingen.

Een arts-onderzoeker vervult een brugfunctie tussen het laboratorium met de analisten en onderzoekers, patiënten, de families, en artsen/hulpverleners. Aan alle zijden was het plezierig werken. Allereerst met de mensen met een verstandelijke handicap en hun families, zonder wier medewerking het onderzoek niet had kunnen plaatsvinden. Daarnaast de inzet van zowel bovengenoemde staf en hun vele medewerkers op de 24ste verdieping van de Medische Faculteit Rotterdam, als de collega's op de 'Westzeedijk' (Aad, Danielle, Eveline, Grazia, Hanne, Jeanette, Martijn, Petra) en in de vele instellingen (zie addendum), die deelnamen aan het fragiele X program. Ook de steun en het geduld van de secretaresses, met name Marina en Rieneke, als 'ik weer eens onvindbaar was', was bewonderswaardig.

De onverstoorbare inzet van Serieta Mohkamsing (analyse van > 1600 bloed monsters) was onontbeerlijk voor het grootschalig fragiele X project. De daaruit voortkomende stroom van data zijn door een aantal mensen succesvol geordend. Allereerst, L.A. Sandkuijl met zijn

gewaardeerde expertise op het genetisch statistisch gebied. Verder dr. A. Tibben voor de opzet van de psychologische evaluatie en dr. H.J. Duivenvoorden voor de daaropvolgende analyses. Deze analyses waren niet mogelijk geweest zonder het zorgvuldige beheer van bestanden door Peter van Vuuren. De onderzoeksresultaten werden uiteindelijk pas toonbaar na de inzet van de 'fotografen' Tom, Mirko en Ruud. De vele 'assistenten': Annemie, Annemieke, Anja, Bettina, Carola, Cathy, Coleta, Esther, Filippo, Marjon, Marja en de kamergenoten Julia, Senno en Robert-Jan dienen zeker niet onvermeld te blijven.

Prof. dr. J.P. Fryns, dr. L.M.G. Curfs, dr. A.P.T. Smits en drs. A.M. Wiegers ben ik erkentelijk voor hun medewerking bij het tot stand komen van enkele onderzoeksprojecten.
Prof. dr. Gillian Turner and Hazel Robinson, it has been a pleasure working with you and your colleagues on the topic of general overgrowth. My visit to your Fragile X Programme, at The Prince of Wales Children's Hospital in Sydney, enabled implementation of the fragile X screening programme in the Netherlands.
Prof. Randi Hagerman, you gave me the great opportunity to get acquainted with your extended expertise on the care and treatment of the fragile X patients at The Child Development Center of The Children's Hospital, Denver.

Buiten de werk situatie heb ik telkens op de juiste momenten de juiste steun ontvangen van King Han Gan (computer-technische hoogstandjes) en Ed Kalkman.
Jolijn, promoveren doe je samen. In ons geval het laatste jaar eigenlijk met zijn drieën. Leonie, je realiseert je grote bijdrage nog niet, maar alleen al je stabiele nachtrust was van onschatbare waarde.

Addendum

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M. de Groot, J. v/d Berg, P. Deman, J. van Grinsven, H. Veere (Craeyenburch, Nootdorp);
A. Idzinga, A. Trappenburg, W. Soeters, C. Clement (Het Westerhonk, Monster);
E. Weijers, C. de Leeuw (SVVGR Rotterdam); L. Imschoot, J. den Hartigh, M. Heijkoop, M. Dekker (De Merwebolder, Sliedrecht); H. Hoogeveen, A. Vossenaar, M. de Jager, C. Ferero (GGD Rotterdam); S. Mosterd (GGD Nieuwe Waterweg Noord); E. Gelsema-

Mudde, B. Becker, J. Akos, T. de Jong (GGD Zuid-Holland Zuid); L. van Elderen (GGD Zuid Hollandse Eilanden); J. de Wijs (GGD Stadsgewest Breda); H. Franken (GGD Streekgewest Westelijk Noord Brabant); J. de Ru (GGD Zeeland); M. Bommezijn (GGD Midden-Holland); N. de Vries- van Waert (GGD Delftland); J. Wijnmaalen, L. Vorselen (Gorkum)

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