Molecular genetics of fragile X: a cytogenetics viewpoint. Report of the Fifth International Symposium on X Linked Mental Retardation, Strasbourg, France, 12 to 16 August 1991 (organiser Dr J-L Mandel)

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The recent and rapid expansion of knowledge concerning the structure and function of the gene for fragile X mental retardation has been facilitated by two technical advances. Firstly the construction (by Steve Warren's group) of cell hybrids containing translocation chromosomes with breakpoints in the Xq26-Xqter region, so that the hybrid cell contains DNA from that region as its only human contribution, and, secondly the ability to clone this DNA, which spans the Xq27.3 FRAXA site, into yeast artificial chromosomes (YACS). Once the DNA had been successfully inserted into YACS then the closest flanking probes developed for linkage studies were used to select clones containing the fragile site. Subcloning into cosmids then permitted the isolation of smaller fragments that still contained the area of interest, which led to the isolation of probes which could identify alterations within the FRAXA gene itself.

Using these methods several groups were able to isolate such probes almost simultaneously. First came the identification of an HTF island lying proximal and very close to the putative FRAXA gene which had become hypermethylated in affected males but remained unmethylated (and therefore presumably functional) in normal males.

An important step in the understanding of gene function and also in offering the possibility of accurate diagnosis came with the detection of an unstable region of DNA lying distal to the HTF island. This unstable region contained an amplification or insertion which varied in size between affected and unaffected males permitting unequivocal identification of the former. Males who manifested the syndrome had a large amplification or insert when compared to controls, while unaffected carriers of either sex were found to have inserts of smaller size. This corre-

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amplification of the sequence. Unaffected carriers have up to approximately 200 copies but once the repeat becomes greater than 200 it appears to become unstable particularly when it is transmitted by a female. Therefore when it reaches this certain critical size the repeated sequence becomes unstable and methylated and so the syndrome manifests itself in males and the probability of females being affected is raised.

The so called small repeat, which does not express itself phenotypically, can pass through several generations becoming gradually longer until the instability point is reached resulting in the syndrome. Generally, when passed on by a transmitting male (TM), it does not alter much in size; it may become slightly larger but there has been at least one case where it decreased in size. This provides an explanation of why daughters of transmitting males are clinically unaffected. When transmitted by a female the insert often becomes larger.

Using probe pfxa3, Sutherland reported that normal X chromosomes carried a fragment of up to 1 kb in size with microheterogeneity, transmitting males had band sizes of up to 1.6 kb, and affected males had a larger band or a series of smears. Approximately 15 to 65 copies (average 43) of a repeated sequence represented a stable polymorphism. Above this it amplifies unstably to fragility.

The small insert (<600 bp) has been called the premutation as those who carry it are not retarded. Males with the full mutation or large insert have an almost 100% risk of mental retardation. Mosaics who have somatic variation may be more mildly affected. Females with the full mutation have an approximately 50% risk of mental retardation.

The transition from pre- to full mutation only occurs by transmission through a female and there has not yet been a new mutation reported. As no case of a normal insert moving directly to the full mutation has been found then all mothers of affected subjects are carriers. There has been one case of reversion from the full to the premutation but this is not frequent.

To try to determine the point at which female carriers were at risk of having affected children several types of correlation were attempted. Suther-

### Table 1 Cumulative data presented at X Linked Mental Retardation 5. Numbers of subjects who show an increase in band size using the 5-1 kb system compared to their FRAXA status and their mental status.

<table>
<thead>
<tr>
<th></th>
<th>Males</th>
<th></th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No increase</td>
<td>Increase</td>
<td>No increase</td>
</tr>
<tr>
<td>Fragile X</td>
<td>Mental status</td>
<td>Fragile X</td>
<td>Mental status</td>
</tr>
<tr>
<td>− ve</td>
<td>+ ve</td>
<td>Normal</td>
<td>MR</td>
</tr>
<tr>
<td>31</td>
<td>1</td>
<td>195</td>
<td>7</td>
</tr>
<tr>
<td>(TM)</td>
<td></td>
<td></td>
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</tbody>
</table>

MR = mentally retarded. TM = transmitting male. (Data from Schwartz, Thibodeau, Gillespie, and Van Oost.)

### Table 2 Cumulative data presented at X Linked Mental Retardation 5. Numbers of subjects who show no, small, or large increase in band size (Δ) compared to their FRAXA status and their mental status.

<table>
<thead>
<tr>
<th></th>
<th>Males</th>
<th></th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No Δ</td>
<td>Small Δ</td>
<td>Large Δ</td>
</tr>
<tr>
<td>Fragile X</td>
<td>Mental status</td>
<td>Fragile X</td>
<td>Mental status</td>
</tr>
<tr>
<td>− ve</td>
<td>+ ve</td>
<td>Normal</td>
<td>MR</td>
</tr>
<tr>
<td>123</td>
<td>0</td>
<td>139</td>
<td>3</td>
</tr>
</tbody>
</table>

(Data from Rousseau, Hirst, and Jacobs.)
land studied the size of the insert in carrier mothers and found that if her \( \Delta \) size = 0.1 then all of her children will be normal, if \( \Delta = 0.2 \) then about half will be carriers, but if \( \Delta = 0.3 \) then the majority of her children will have the large amplification. If this is so then all the males will be handicapped and there is an increased risk for the females.

Several groups reported a marked clustering in the mutation class of the offspring of women with the premutation. The offspring carrying the mutation either all had a premutation or all had a full mutation. However, this clustering was not absolute as both small and large inserts were reported within the same sibship.

A relationship was also detected between the increase in band size and the percentage of FRAXA detected. This should be interpreted with caution as estimations of FRAXA are performed in different laboratories at different times using a variety of culture conditions. There is no correlation in males between FRAXA level and degree of retardation.

Van Oost related the risk of carrying the fragile X gene as estimated from linkage data to the increase in band size. He found linkage to be a good predictor of genotype as in males a calculated risk from linkage of 1% yielded 40 with normal bands and none with an amplification, whereas a predicted risk of 99% led to none with normal bands and six with amplifications. Prediction in females was equally accurate as a 1% risk gave 46 with normal bands and none with amplified sequences, whereas in those with a 99% risk from linkage data these numbers were 0 and 14 respectively.

**Somatic variation**

Affected subjects may have not one but a series of bands owing to the great instability of the DNA insert. In all such subjects the band varies somatically as when an insert of 6 kb is reached different tissues all have differently sized bands. Mosaics which constitute about 15% of the total are also the result of somatic change. Individual variation is present in all tissues and there is no relationship between methylation and the numbers of detectable bands or fragments.

**The FMR-1 gene**

The cDNA corresponding to the FMR-1 gene was found to hybridise to almost all tissues examined and was found to be conserved in many species. Sequencing showed no homology with any known protein but along with the 30 arginine residues the gene also contained a nuclear transduction signal indicating that it may code for a nuclear protein. To elucidate protein function, antibodies were raised against a synthetic peptide corresponding to the FMR-1 gene product. Immunoprecipitation yielded a 70 kd protein which when localised under EM was present in the nucleus, RER, cytoplasm, and mitochondria from a liver cell line.

The FMR-1 gene contains three exons, one containing the CGG repeat whose change in size alters the function and expression of the gene. There is no FMR-1 expression in some affected males but the range in normal subjects of either sex is variable and Nelson reported that four of 20 fragile X patients had normal expression. Expression differences may be ascribed to methylation differences and the altered phenotype to tissue differences, as there was correlation between total CpG methylation and FMR-1.

**Methylation**

As always methylation status, inactivation, and imprinting were topics for extended discussion.

Prenatal diagnosis of a male fetus using probe pfx3 indicated that he would be clinically affected as the insert size had a \( \Delta = 0.6 \). Investigation of methylation status using SacII showed that all tissues were methylated with the exception of chorionic villus. So methylation is not a good marker of phenotype in CVS cells.

According to Laird there is persistent hypermethylation of the CpG site which is usually present on that of the inactive X chromosome. The epigenetic change is this persistent state of hypermethylation and it is less stable than a genetic change. It serves to silence the function of the FMR-1 gene as it confers instability on the repeated region. Expansion then occurs after imprinting and is the result of somatic instability.

An increase in size of the CGG repeat in either a male or a female leads to a premutation state. In the female embryo X inactivation leads to methylation of the CpG doublet associated with expression of FMR-1. At oogenesis the female carrier should reactivate the inactive X but the complex alteration of the mutated FMR-1 leads to a failure in demethylation and structural instability of FMR-1 at the fragile site.

Gillespie showed that the IDS gene which lies 1 cm distal to the FMR-1 gene has a significant reduction in activity in fragile X males when compared to controls.

Steinbach claimed that methylation occurs before band size increase as an \( HpaII \) band was found to be absent in an affected boy with a large insert. Neither the boy’s mother who carried a small insert nor his TM maternal grandfather had \( HpaII \) band loss.

**Summary**

A normal genotype has microheterogeneity of the
amplification/insertion but the average was found to be 43 CGG repeats. The premutation which has no clinical manifestation is not methylated and has an increased size of between 50 to 100 bp and 500 bp; the full mutation exists above 500 to 700 bp with methylation of CpG. Males with $\Delta = 0.6$ to 0.7 are all affected while of females with $\Delta = 0.6$ to 0.7 about half are affected. In females if the premutation remains on the active X chromosome it stays small at $<500$ bp and unmethylated, but if it lies on an X chromosome which is inactivated then it may not demethylate correctly leading to a large $>500$ bp insert plus methylation (or imprinting) and an affected phenotype in offspring.

**Cytogenetic and molecular technique**

The take home message was of improved predictive power for mental retardation in males. Females were once again more difficult to predict, as carriers can have either small or large $\Delta$ and those with large $\Delta$ can be either clinically affected or not in approximately equal numbers (table 2).

The molecular techniques now available for diagnosis of the fragile X syndrome are cheaper, faster, and more accurate than cytogenetic techniques. If the new techniques are used for screening high risk populations they will identify the approximately 10% who will be expected to have the fragile X, but will fail to detect all other cytogenetic abnormalities. Thus, at least in diagnostic laboratories, it appears more cost effective to continue to use cytogenetic techniques when testing patients referred with developmental delay.

Once the fragile X syndrome has been detected in a proband, then the greater accuracy and lower costs indicate that family members should be studied by molecular methods rather than cytogenetically. Services should be extended to include these techniques.

Genetic counselling is more expensive than either laboratory method (Pembrey) and this too should be considered as families want to have healthy children and counselling is still very difficult especially for female carriers.

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