

Is skewed X inactivation responsible for symptoms in female carriers for adrenoleucodystrophy?

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Abstract

A study of X inactivation in 12 female carriers for adrenoleucodystrophy showed no evidence that skewed patterns are related to clinical manifestation. Other possible mechanisms to explain manifestation in females are considered.

(J Med Genet 1993;30:651-4)

The X linked condition of adrenoleucodystrophy/adrenomyeloneuropathy (ALD) is characterised by marked variability in clinical manifestation. The condition in hemizygous males ranges from an acute lethal degenerative disorder of the first decade, to spastic paraplegia and adrenocortical failure in adult life, and possibly to no manifestation in adults.¹ There is also variability in female carriers, some of whom develop progressive adult onset spastic paraplegia, accompanied by sensory symptoms, but rarely by adrenal hypofunction.¹ The clinical variability in both males and females does not correlate with the extent to which very long chain fatty acids (VLCFA) are raised. Why do some carriers develop clinical problems and why do others escape?

There are several possible explanations. Firstly, is adrenoleucodystrophy an X linked dominant, so that all, or nearly all, obligatory heterozygotes will develop a spastic paraplegia in time? Secondly, does clinical manifestation depend upon whether the carrier has inherited the mutant gene from her mother or from her father? In investigating this possibility it is important to know whether a symptom free but dead father could have been a gene carrier. Thirdly, could manifestation depend upon whether it is the mutant or wild type gene that is predominantly inactivated? In this respect it is interesting to note the *in vitro* studies of Migeon *et al*² in which clones from cultured fibroblasts of carrier mothers showed a preponderance with the mutant gene active.

Fourthly, could secondary phenomena, such as an autoimmune response, account for the variability in manifestation, as suggested by Moser *et al*?¹ Fifthly, could autosomal modifier genes influence the expression of the X linked mutant gene?³ Finally, could it be that the ALD gene is associated with an insert, so that, like the fragile X syndrome and myotonic dystrophy, the size of the insert correlates with the severity of the clinical picture?

In order to consider the third of these possibilities, namely the role played by X inactivation patterns, we carried out an investigation into X inactivation in manifesting and non-manifesting female carriers.

The probe M27 β detects locus DXS225 which contains a VNTR sequence within it.⁴ The degree of polymorphism shown by the sequence indicates that there is >90% heterozygosity in females. In addition the locus also contains *MspI* sites which are methylated on the active X chromosome but are unmethylated on the inactive X chromosome. Such sites are vulnerable to digestion by the isoschizomer *HpaII* only when they are unmethylated, that is, when they lie on the inactive X chromosome. Therefore, M27 β can be used to differentiate between the active and inactive X chromosomes, and to analyse the pattern of X inactivation in any particular female. Correlations between skewed X inactivation and phenotypic expression of X linked disease have been documented using both PGK and HPRT probes.⁵⁻⁹ The PGK and HPRT probes are more limited in their application, however, owing to the low level of heterozygosity shown by females.

Methods

PATIENTS

We studied 12 women who were carriers for ALD; their genetic and clinical features are

Clinical features and results on 12 female carriers for adrenoleucodystrophy.

No in pedigree*	Age	Reason for considering her to be a carrier	Whether clinically affected and age of onset	VLCFA C26:0†	Densitometry results‡
A II-1	52	2 sons affected	No	+0.5	75:25
A III-3	24	2 bro affected, shares their DXS52 allele	No	+0.2	60:40
B II-1	73	Fa, son, & grandson affected	Yes (58)	+4.0	75:25
B III-2	46	Mo, bro, & son affected	No	+2.2	50:50
B IV-6	18	Bro affected, shares his DXS52 allele	No	+2.8	65:35
D I-2	53	Son affected, high VLCFA	Yes (44)	+1.6	60:40
E II-1	46	Affected nephews, shares their DXS52 allele	No	+0.7	75:25
E II-3	45	Son & nephews affected	No	+0.3	50:50
E II-5	44	Sons & nephew affected	No	+0.6	65:35
E II-7	23	Affected nephews, shares their DXS52 allele	No	+1.1	60:40
F II-2	76	Son & daughter affected	No	+1.4	65:35
F III-4	44	Brother affected	Yes (40)	+0.8	55:45

* From Del Mastro *et al*¹⁰ and fig 1.

† Expressed in terms of 1 SD from the mean.

‡ Ratio of relative band intensity in the two X chromosomes.

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Received 17 December 1992.
Revised version accepted 22 April 1993.

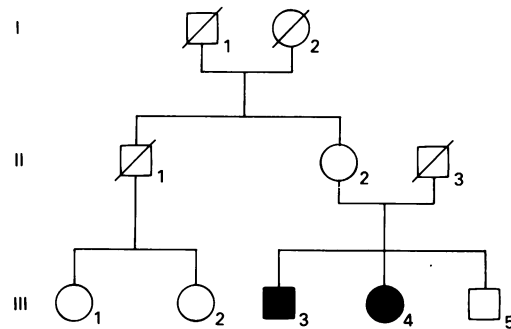


Figure 1 Pedigree of family F.

listed in the table. They belonged to five families, of whom four were described by Del Mastro *et al*¹⁰ (families A, B, D, and E); the pedigree of the fifth family (family F) is presented in fig 1. All women in these families were examined; three had a progressive spastic paraplegia, which was accompanied by sensory symptoms in one (FIII-4), thus raising the diagnostic suspicion in her of multiple sclerosis.

LABORATORY METHODS

Blood samples of 20 ml were collected in potassium EDTA tubes from each female under study. DNA was extracted from lymphocyte pellets using a 340A Nucleic Acid Extractor (Applied Biosystems). Parallel DNA samples (5 µg) were restricted with *PstI*/*MspI* and *PstI*/*HpaII* or, if uninformative, *AvaII*/*MspI* and *AvaII*/*HpaII* following the method of Boyd and Fraser.¹¹ The samples were then electrophoresed on 0.7% agarose at 65 V for 24 to 36 hours. Gels were Southern blotted under standard conditions using Hybond N+ (Amersham International Ltd). The probe

M27β was labelled with [32P] dCTP using a Random Primed DNA labelling kit (Boehringer Mannheim Diagnostics Ltd). Filters were hybridised overnight in a hybridisation oven (Techne Hybridiser HB-2). Washes were performed under standard conditions. The Southern blots were first looked at by two independent observers and then subjected to densitometry to determine whether or not there was skewing of the X inactivation process in these subjects. The relative band intensities were measured using a densitometer (LKB Ultrascan XL Laser densitometer).

Owing to the presence of extraneous bands which sometimes appear after digestion with *HpaII*, more detailed studies have recently been made of the DXS255 locus.¹² The 5'CpG island on the active X chromosome was found to be invariably fully methylated, but variable methylation patterns could sometimes be detected on the inactive X. This was found to be because of additional *MspI* sites located in the region.¹³ In all samples a majority of cells had site 2 unmethylated on the inactive X. Although patterns of methylation were heterogeneous between subjects and even between different tissues from the same subject, at least one of the *MspI* sites was found to be unmethylated on the inactive X chromosome.^{12,13} For this reason, in this study comparisons were made between the residual *PstI* bands, that is, material uncut by *HpaII*, as this represented the percentage of fully methylated or active X chromosome. If any or all of the *MspI* sites remained unmethylated on the inactive X chromosome, then the CpG island would be cut by *HpaII* and the band would alter in size and change position in the gel. Comparison of intensities of the bands corresponding to the original *PstI* alleles thus represented the more accurate determination of the percentages of active and inactive X present (fig 2).

We considered how to define 'skewedness' using three approaches. Firstly, there is the statistical approach, based on the premise that there are 20 primordial stem cells that contribute to the bone marrow,¹⁴ and that initially these should be randomly allocated to one of two groups (maternal X active, or paternal X active) with a 50% probability for both. On this basis, a distribution of 75:25 would be significantly different from that expected at the 5% level, and one of 80:20 would be significantly different from that expected at the 1% level. Secondly, results from a control series of women can indicate a 'normal distribution'. In the series of Harris *et al*,¹⁵ four of 42 women (9.5%) displayed skewing of 80:20 or greater. This is a higher proportion than would be expected on theoretical grounds and indicates that any pattern between 80:20 and 50:50 should not be considered to be abnormal. Lastly we could ignore the degree of skewedness and just compare any change from 50:50 with clinical manifestation; thus a ratio of 65:35 might well be significant when related to clinical symptoms. However, as our experiments could not distinguish between the mu-

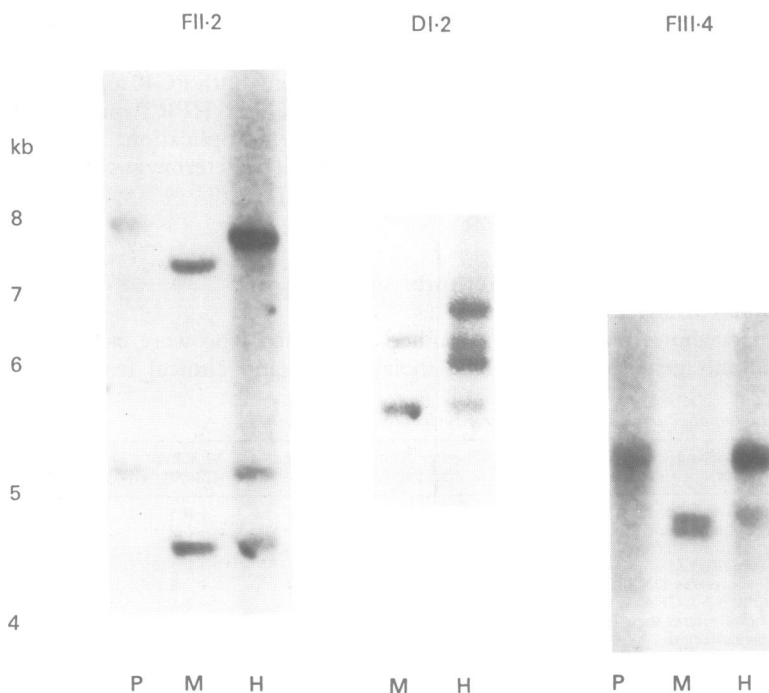


Figure 2 Methylation analysis of three carriers of ALD using the probe M27β. P = *PstI* digest, M = *MspI* + *PstI* double digest, H = *HpaII* + *PstI* double digest. DI-2 and FIII-4 show random X inactivation with ratios of 60:40 and 55:45 respectively. Bands used for densitometric studies were those uncut by *HpaII*.

tant X and the normal X, such conclusions would be prone to error.

Results

Three of the women tested were manifesting carriers and nine were free of symptoms and signs (table). Of the three manifesting carriers tested, only one (BII-1) showed skewedness with a ratio of 75:25; the other two, both of whom had a lower age of onset, had ratios of 60:40, (DI-2) and 55:45 (FIII-4) respectively (fig 2). Of the nine non-manifesting carriers, skewed X inactivation of 75:25 or worse was shown by two, namely AII-1 and EII-1; three further women had ratios of 65:35.

Discussion

It is not yet clear what proportion of female carriers of ALD are symptomatic, since their presence in a pedigree increases the family's chances of being diagnosed or ascertained. All three manifesting carriers in our five families had presented to a neurologist independently of the illness in affected males, and in one of the three cases it was the presence of both a male with Addison's disease and his sister with a spastic paraplegia that led to the correct diagnosis. Ignoring such biases in ascertainment, three of our nine carriers aged 40 or over had symptoms of ALD, while the remainder had no signs. However, had brain stem evoked responses been performed it is possible that we might have recognised abnormalities in some of the asymptomatic women.¹

Densitometry proved useful in our investigations in giving an accurate and objective documentation of X inactivation. We considered that a ratio of 75:25 of the two X alleles indicated 'skewedness'. Unfortunately, it was not possible to identify whether the X chromosome bearing the mutant ALD allele was predominantly active or vice versa, since the ALD locus in Xq28 is far from the DXS255 locus in Xp11, and nine of the 12 fathers of carriers were dead. In family E, however, of the four non-manifesting carrier sisters, three had random X inactivation and the fourth had skewed inactivation favouring activity of the maternal X chromosome.

Our results on X inactivation patterns in female carriers show no association of skewed inactivation with manifestation, as only one of the three manifesting carriers showed skewing, and so did two of nine non-manifesting carriers. There was also no obvious association of skewing with age and no familial consistency of pattern, since in families A and B mothers with skewed patterns had daughters with random X inactivation. It has been suggested that these features indicate a lack of association between a specific mutation and the tendency to inactivate.¹⁵

Despite the finding that some cases of female manifestation of certain severe X linked diseases could be attributed at least in part to the disease gene being over-represented on the active X chromosome,^{7,15} such events are usually driven by mechanisms such as X;auto-

some translocations,¹⁶ X chromosome deletions,¹⁷ or even twinning.¹⁸ Indeed there is such marked selection in favour of cells carrying the non-mutant allele in active form in some forms of X linked immunological disease that it can be used to determine carrier status in unaffected females.^{5,7,9,19} In female carriers of Alport's syndrome, however, no correlation between clinical manifestation and skewing of X inactivation could be detected.²⁰ It may be that in some X linked diseases, such as ALD, extrapolation from fibroblasts or lymphocytes does not indicate the percentage of normal to mutant alleles present in more relevant tissues. The percentage of active mutation-bearing X chromosome homologues might be influenced by selection, although in one case of incontinentia pigmenti in a female both fibroblasts and lymphocytes showed a symmetrical inactivation pattern.¹⁵ It is also not possible to determine the direction of skewing in the majority of our subjects as their parents are dead.

In the present study, there is a lack of any association between the pattern of X inactivation and clinical manifestation. We have to conclude that X inactivation patterns in DNA derived from white cells does not accord with clinical manifestation and that the other possible causes mentioned in the Introduction should be considered. If ALD is a dominant disorder, then FII-2 should be manifesting symptoms at her age of 76. However, she is not an obligatory heterozygote and could be an example of a gonadal (or gonosomal) mosaic, a situation which has been shown in another family²¹ where three unaffected males possessed the same allele at DXS52 as did their carrier sister and affected brother. Another possibility we considered is that the effect of the mutant allele is greater when transmitted from a father to his daughters, as in the two girls described by O'Neill *et al*,²² who developed spastic paraplegia in the first year of life. However, our patient BII-1, who inherited the mutant gene from her affected father did not develop symptoms until aged 58. The remaining three possibilities are those of secondary immunological phenomena, or of modification by autosomal genes, or of a gene that readily changes its structure and effect.

This work is supported by a grant from Action Research. We are also grateful to Dr Yvonne Boyd for the gift of probe M27 β and for helpful discussion.

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