European Journal of Human Genetics (2000) 8, 449–454

www.nature.com/ejhg

ARTICLE

X inactivation phenotype in carriers of Pelizaeus-Merzbacher disease: skewed in carriers of a duplication and random in carriers of point mutations

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Pelizaeus-Merzbacher disease (PMD) is an X-linked recessive disease caused by coding sequence mutations in the *PLP* gene, sub-microscopic duplications of variable sizes including the *PLP* gene or very rarely deletions of the *PLP* gene. We analysed the X inactivation pattern in blood of PMD female carriers with duplications and with point mutations. In the majority of duplication carriers (7/11), the X chromosome bearing the duplication was preferentially inactivated, whereas a random pattern of X inactivation was detected in point mutation carriers (3/3), a deletion carrier (1/1), affected females (4/4) who did not have a recognised mutation and normal control females. However 2/5 non-carrier female relatives of patients with a duplication, had skewed X inactivation. The skewed pattern of inactivation observed in most duplication carriers and not in mutation carriers suggests a) that there is selection against those cells in which the duplicated X chromosome is active and b) other expressed sequences within the duplicated region rather than mutant PLP may be responsible. Since the skewed X inactivation did not segregate with the disease in two families and the pattern of X inactivation was variable among the duplication carriers, the pattern X inactivation is an unsuitable diagnostic tool for female carriers of PMD *European Journal of Human Genetics* (2000) **8**, 449–454.

Keywords: Pelizaeus-Merzbacher disease; PMD; PLP; X inactivation; duplication; deletion

Introduction

Pelizaeus-Merzbacher disease (PMD; MIM 312080) is a recessive X-linked dysmyelinating disorder of the central nervous system (CNS). The gene implicated in PMD is *PLP* (proteolipid protein) on Xq22 encoding a major myelin protein in the CNS which, together with its splicing variant DM20, is a hydrophobic integral membrane protein.[1]

Three different types of mutation have been identified in PMD patients. The majority of mutations (approximately 50%) involve a duplication of the *PLP* gene.[2–5] The duplications are variable in size but do not affect the coding sequence of the PLP gene[5] and therefore it is an increased dosage of the *PLP* gene that is responsible for PMD. Sequence alterations in the *PLP* gene are found in 20–30% of patients.[3,4,6] Loss of PLP caused by a deletion[7] or a null allele[8,9] has also been reported in a few cases. A few female carriers of point mutations exhibit some of the clinical features of PMD.[10–12] There are also several reports of affected females with no identified mutation in the *PLP* gene; some cases were familial and others were sporadic. It has been suggested that these cases represent an autosomal recessive form of PMD.[13–16]

In several X-linked recessive disorders X inactivation is skewed in asymptomatic carrier females, so that the X bearing the mutation is preferentially inactivated.[17] This is also found in carriers of cytogenetically detectable deletions

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Received 2 September 1999; revised 20 January 2000; accepted 9 February 2000

and duplications on the X chromosome.[18,19] The skewed X inactivation, in most cases, is a result of selection against cells with growth disadvantage. Carriers of PMD with a duplication have a submicroscopic chromosomal rearrangement which includes other coding sequences in addition to the *PLP* gene. In carriers of point mutations it is only the *PLP* gene that is affected. In the present study we analysed the pattern of X inactivation in WBC of PMD carrier females with a duplication and with a point mutation in the *PLP* gene.

Material and methods

The study group

A total of 36 females was analysed:

(a) carrier females with duplications (n = 13) were all mothers of PMD affected sons. The duplication mutations were identified by FISH using the cosmid probe cU125A1 from the *PLP* gene.[5] Some of the patients with duplications (PMD1,2,4,9) have been previously described.[5] In the majority of carriers the duplication was tandem; families PMD1 and PMD5 were exceptional in that the duplicated segment was transposed to Xq26 and Xp22.1, respectively.[20] In families PMD22 and PMD23 the duplication was identified by quantitative PCR[5] and we were unable to determine the size of the duplication;

(b) a female carrier of a deletion mutation;[7]

(c) carrier females with point mutations (n = 3) were all mothers of affected sons. In these three families there was a substitution mutation, T181P in PMD12, L223P in PMD13[6] and P173S (C→T nucleotide 519) in PMD14 (C Schwartz, unpublished results, 1997);

(d) affected females (n = 4) with no duplications or identified point mutations;

(e) a group of normal control females comprised of non-carrier female relatives of patients with a duplication (n = 5), including a maternal grandmother and aunt of the patients PMD1 and PMD3, and a maternal aunt of patient PMD24 (we did not have a DNA sample from the mother of patient PMD24), and unrelated females (n = 10).

X inactivation analysis

For X inactivation studies, DNA was obtained from lymphoblastoid cell lines or uncultured WBC. The androgen-receptor (*AR*) gene X-inactivation assay using HpaII-sensitive methylation was performed according to Allen *et al*.[21] A VNTR polymorphism in the monoamine oxidase A gene (*MAOA3*), with adjacent HpaII-sensitive methylation sites on Xp11.4–11.23,[22] was evaluated in subjects who were homozygous for AR. DNAs from males were used as control for complete HpaII digestion. Products were analysed on an ABI 377 sequencer and the peak heights were analysed using

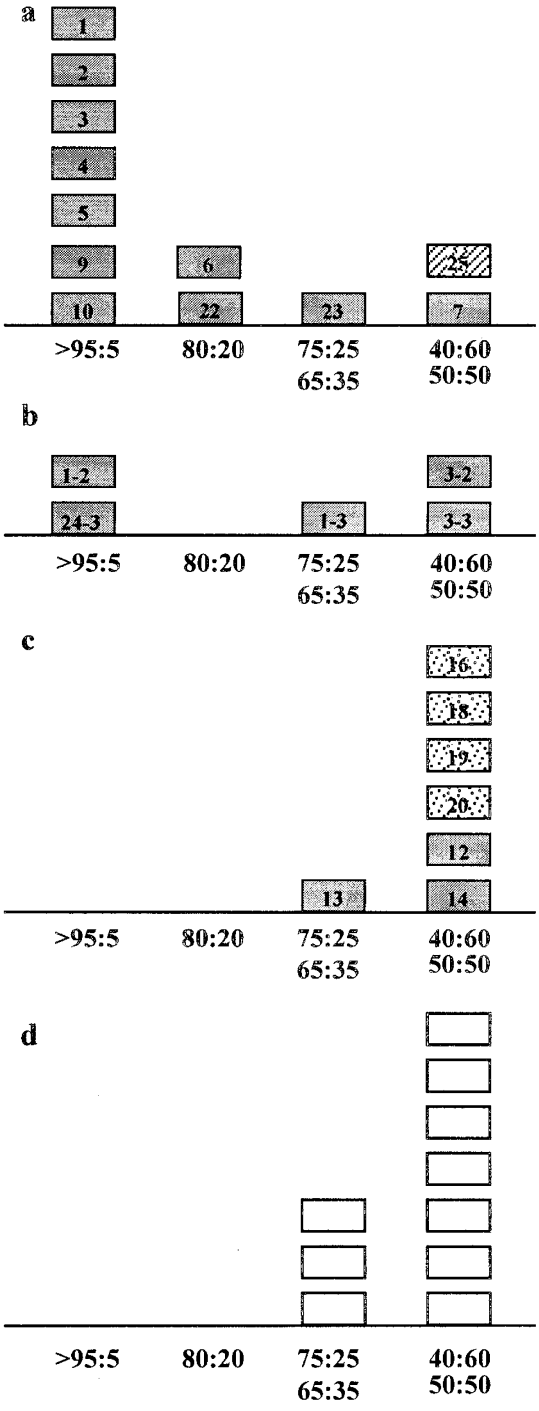

Figure 1 The pattern of X inactivation, **a** duplication carriers and the deletion carrier (PMD25); **b** non-carrier relatives of duplication carriers; **c** affected females (PMD16,18,19,20) and point mutation carriers PMD12,13,14); **d** normal female controls. The number in boxes refer to PMD family number.

GeneScan and Genotyper software (PE Biosystems, Warrington, Cheshire, UK). Each assay was repeated 2–5 times and the average values were calculated.

Analysis of X inactivation on metaphase chromosomes

This assay was performed using a culture of skin fibroblasts. BrdU was applied 7 h before harvesting the cells.[23] FISH using the cosmid probe cU125A1 including the PLP gene was followed by mouse anti-BrdU antibody (6 μg/ml) (Roche Diagnostics Ltd, Lewes, East Sussex, UK) and texas-red anti-mouse IgG (150 μg/ml) (Vector Laboratories Ltd, Peterborough, UK). Cells were viewed using a Zeiss Axiophot fluorescent microscope with a triple-band pass filter.

Characterisation of the size of the duplication

The size of the duplications was determined by FISH using PAC and cosmid clones from the region around the *PLP* gene as previously described.[5]

Results

The pattern of X inactivation

Thirty-one females were informative for the AR-$(CAG)_n$ assay. Three females homozygous for AR were found to be heterozygous for the MAOA3 VNTR. Two females were not informative for either the *AR* or the *MAOA3* gene. The pattern of X inactivation for the 34 females is presented in Figure 1. Two duplication carriers with skewed X inactivation were analysed for both AR and MAOA3 and the results were identical. In one carrier (PMD1), the X inactivation was completely skewed in DNA from a lymphoblastoid cell line, uncultured WBC and skin fibroblasts.

In 6/7 mother–son pairs with a duplication, the AR-$(CAG)_n$ or the MAOA3-VNTR allele on the preferentially inactivated X was the allele that their affected sons carry. In family PMD1, the maternal allele on the inactive X was different from that of her PMD affected son due to recombination between the *PLP* genes on Xq22 and Xq26, which was confirmed by polymorphic markers from the X chromosome (not shown). In the other carriers the alleles of the sons were unknown.

Cytogenetic analysis of X inactivation

The carrier PMD1 had the duplicated segment transposed to Xq26.[20] In her case, it was possible to directly determine which of her two X chromosomes was preferentially inactivated using FISH combined with BrdU replication banding on metaphase chromosomes. In all 24 metaphase cells analysed, the late replicating (inactive) X chromosome had two FISH signals, corresponding to Xq22 and Xq26 (Figure 2).

Characterisation of the duplication in female carriers with skewed and random X inactivation

To investigate the possibility that the genetic content of the duplication determines the pattern of X inactivation, we compared their sizes and location. Lymphoblastoid cell lines were available from 9 of the 11 carriers, PMD1,2,3,4,5,9,10 with a completely skewed pattern of inactivation, PMD6 with a mildly skewed pattern and PMD7 with a random pattern.

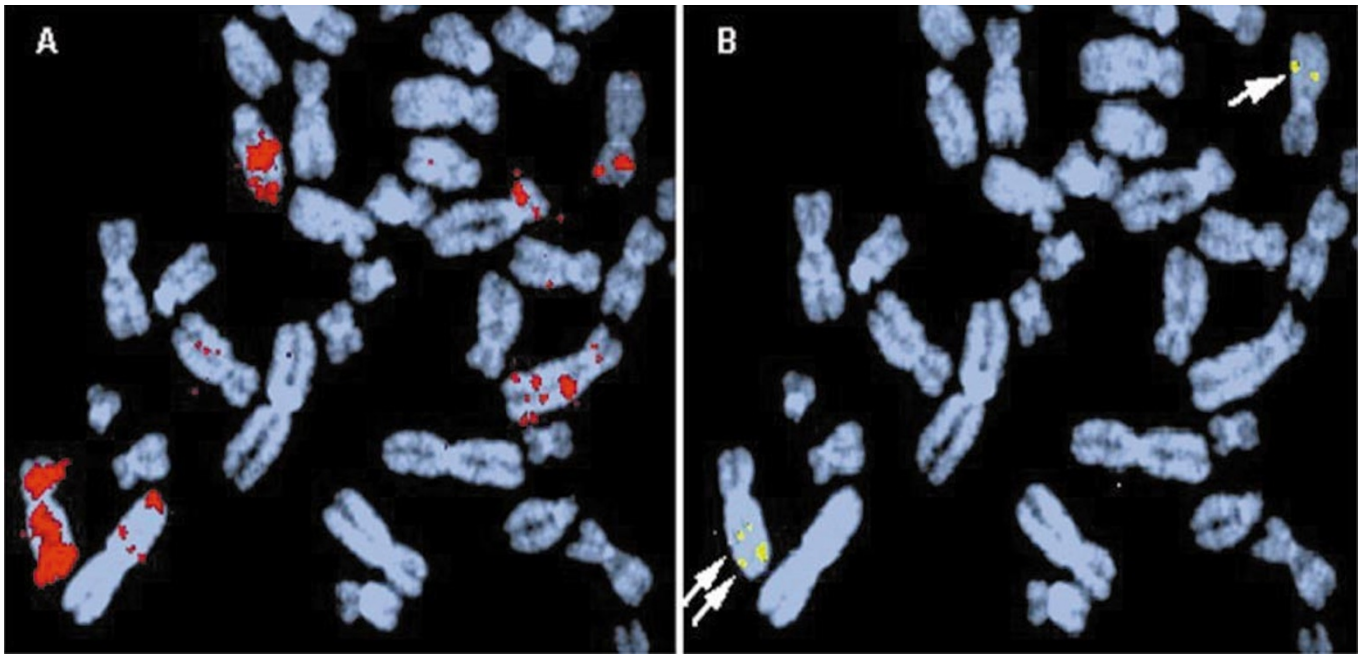

Figure 2 Metaphase chromosome from fibroblasts of PMD1 female carrier. **A** BrdU staining is shown in red and identifies the late replicating regions of DNA; **B** FISH of a PLP cosmid probe is shown by a yellow signal. The *PLP* gene duplication on Xq22 and Xq26 is shown on the late replicating (inactive) X chromosome.

The duplications were classified into groups according to their putative breakpoints (Figure 3).

Discussion

Among the PMD carrier females, a marked difference between the two types of carrier emerged; female carriers with point mutations had random X inactivation, whereas 7/11 of duplication carriers had completely skewed X inactivation and 2/11 had a suggestively skewed pattern. In all cases the X bearing the duplication was preferentially inactive. A random pattern of X inactivation was observed in the carrier of the deletion mutation. In several large studies of normal females, fewer than 10% showed skewing greater than 85%.[24–27] The excess of duplication carriers with completely skewed X inactivation as compared to the published control groups is highly significant.

Skewed X inactivation may be a result of a stochastic event, a defect in the primary control of X inactivation or a secondary effect due to cell selection after X inactivation is established.[17,28,29] A selection against cells carrying the mutation on the active X might be the basis for the discrepancy between the carriers of point mutations and duplications. The *PLP* gene is not expressed in WBC,[30] therefore it is not expected that point mutations in PLP would be a selective factor. The duplications, on the other hand, might affect the growth rate of PLP-non expressing cells due to a dosage effect of other genes within the duplicated segment. It is interesting to note that although the duplicated segments contain sequences that affect the pattern of X inactivation, there is currently no indication that they have a major effect on the PMD phenotype of the affected boys. It is possible that in PLP-expressing cells such as those in the CNS, the X inactivation pattern in asymptomatic carriers of PMD might be skewed regardless of type of mutation. A skewed X-inactivation pattern in the CNS has been suggested to occur in heterozygote females of the shaking (sh) pup, an animal model of PMD[31] with a point mutation in the *PLP* gene.

A variable X inactivation phenotype was found in the WBC of duplication carriers. This has been noted in other X-linked recessive genes[27,32,33] and it has been suggested to reflect incomplete penetrance due to other factors, genetic or stochastic. In PMD one such factor might be the genetic content of the duplicating segment. The duplication in PMD7 was among the largest (> 1.2 Mb), the duplications in PMD1 and PMD5 were the smallest (< 0.6 Mb), and they were overlapped by the duplication of PMD7. Yet, the carrier

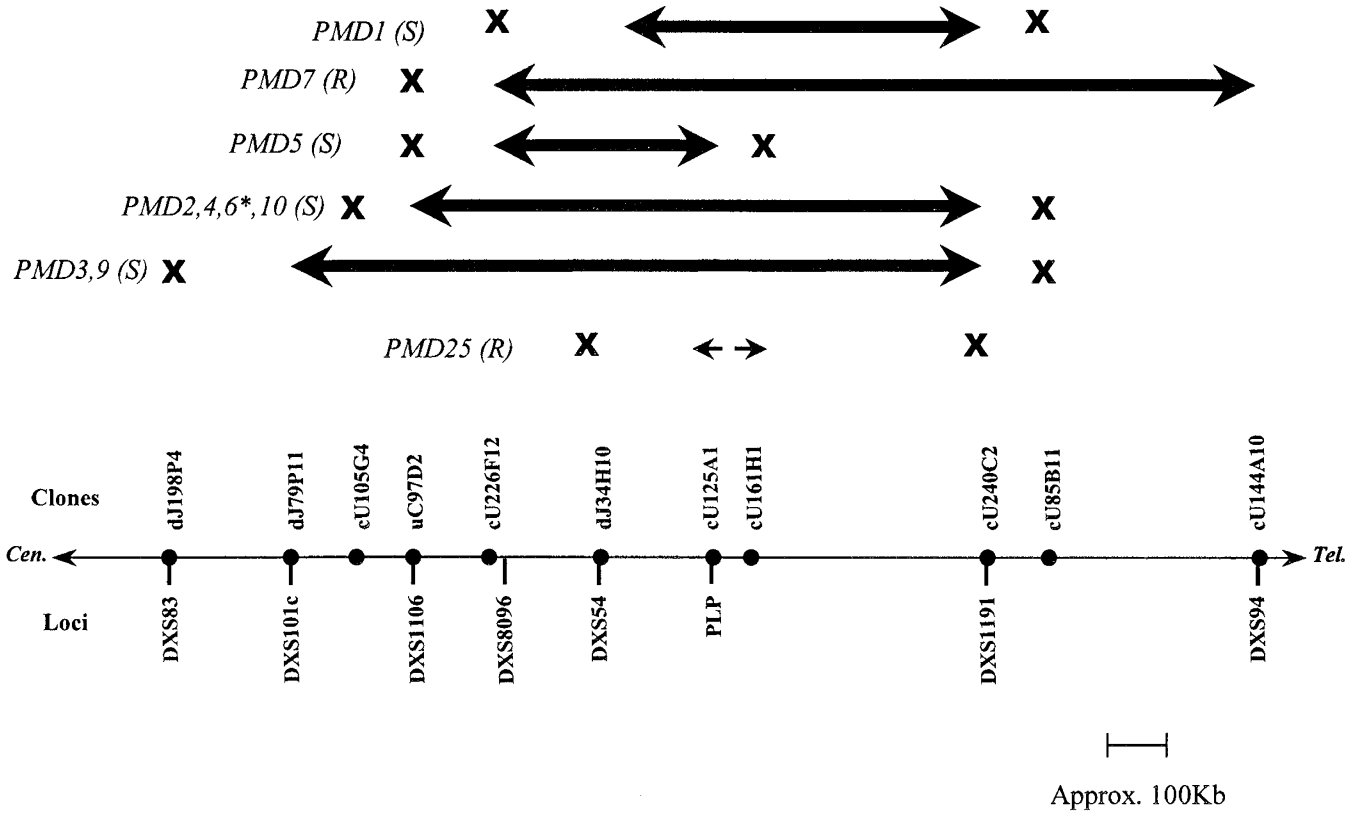

Figure 3 Map of the PLP region showing the extent of the duplication in nine patients and the deletion in patient PMD25. The minimum size of the duplication/deletion is shown by the arrowed line and the boundaries are marked by X. R indicates a random pattern of X-inactivation and S indicates a skewed pattern with * being mildly skewed and the rest completely skewed.

female PMD7 had a random X inactivation pattern and PMD1 and PMD5 had a completely skewed pattern. Although the duplication size and position of PMD6 is consistent with that of PMD2, 4 and 10, which have completely skewed inactivation, only a mildly skewed pattern of X inactivation was observed in his carrier mother. These observations suggest that the increased dosage of a unique gene within the duplication is unlikely to be responsible for the skewing effect, unless the determinant factor is the breakpoints. The only possible location for a single gene would be beyond the proximal breakpoint of PMD7 but within the duplication of PMD2,3,4,5,6,9,10 and then the skewing in PMD1 may be due to the insertion into Xq26 and the mild skewing in PMD6 would have to be caused by another factor. An alternative explanation would be that the PMD duplication mutations might belong to the category of cytogenetically unbalanced X chromosomes which are preferentially inactive, regardless of the map position of the unbalanced segment.[34–38] In contrast to the duplication mutation the PMD deletion mutation in patient PMD25 did not result in a skewed X inactivation pattern. The most likely explanation would be that this deletion of about 100 Kb does not affect cell growth, as has been described in other deletions, such as those involving the *FMRI* and *DMD* genes,[39,40] and unlike the 800 kb deletion in Xq28.[26]

The PMD affected females have as yet unknown etiology. In these patients skewed X inactivation would have suggested the involvement of an X-linked gene. A random pattern of X inactivation, however, does not exclude it, since a skewed pattern in the CNS is a valid possibility.

A skewed pattern of X inactivation was observed in two of five non-carrier females relatives. Although it may be due to a stochastic or age-related event,[41] we suspected that in these families there could be another mutation that primarily affected the pattern of X inactivation. To date, at least three loci have been suggested to affect the process of X inactivation,[25,26,42] but XIST is the only gene in which a mutation was shown to affect the choice of the inactive X.[25] The recurrent mutation in the minimal promoter region of XIST[24] was not found in any of the females with skewed X inactivation including the non-carrier female relatives of PMD1 and PMD24 (not shown). However, we did not rule out another mutation in XIST.

We evaluated non-carrier female relatives in the study primarily to assess the X inactivation assay as a possible diagnostic tool for identifying PMD carriers. However, since not all the proven duplication carriers had skewed X inactivation, and in two out of five non-carrier relatives the X inactivation was skewed, the pattern of X inactivation is not reliable as a diagnosis of the carrier status in PMD.

Acknowledgements

We are grateful to the Wellcome Trust and Great Ormond Street Hospital Trustees for support (KW and KK). TB and WR were supported by funds from the Department of Veterans Affairs. We thank ME Hodes for providing samples and Graeme Suthers, Charles Schwartz and Melanie May for providing the DNA and mutation information for PMD14. Rodger Palmer and Tom Spencer are acknowledged for providing the BrdU incorporated fibroblast cell suspension for PMD1. John Wolff assisted with the studies on the deletion family.

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