ELECTRONIC LETTER

Position effect on *PLP1* may cause a subset of Pelizaeus-Merzbacher disease symptoms

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Pelizaeus-Merzbacher disease (PMD, MIM 312080) is an X linked disorder characterised by dysmyelination of the central nervous system (CNS)^{1,2} (see review by Koeppen and Robitaille³). Two main forms of the disease, a connatal and a classical type, are recognised. The connatal type has a severe course with feeding problems, progressive pyramidal and extrapyramidal symptoms, laryngeal stridor, microcephaly, very little development, and a natural disease duration of 5–7 years. The classical type evolves into spastic tetraparesis, ataxia, choreoathetoid movements of the limbs, and cognitive impairment. The disorder progresses at a variable rate and some children are able to sit with support or occasionally walk, before slow regression sets in. Seizures may occur in both types (table 1). PMD has been shown to be caused by mutations in the proteolipid protein (*PLP1*) gene, encoding the major myelin component in the CNS.^{4–5} *PLP1* is also involved in the aetiology of X linked spastic paraplegia (SPG2), which manifests as progressive weakness and spasticity of the lower extremities with or without CNS involvement.^{6–7} Mutations causing PMD can be divided into two main categories: duplications of *PLP1*, which account for the majority of cases (60–70%), and sequence variations within the gene (15–20%).⁸ The remaining 10–20% of patients have no detectable mutation within *PLP1*, suggesting that mutations in regulatory regions or other gene loci can also cause PMD. Disease severity ranges from severe connatal PMD to mild PMD/SPG2 with an intermediate classical form. Patients with *PLP1* duplications have a predominantly milder classical phenotype, whereas patients with missense mutations show a much wider spectrum, ranging from more severely affected to SPG2.⁸ Duplication sizes vary significantly.^{9–10} Absence of the PLP protein caused either by gene deletions or null mutations causes a specific mild form of PMD that includes length dependent axonal degeneration.^{11–12}

We have mapped and analysed the chromosomal breakpoint regions of a male patient with an inv(X) (p22.3; q22) suffering from a subset of PMD symptoms including moderate mental retardation (Wechsler Intelligence Scale for Children, revised Dutch edition (WISC-RN) IQ 55–59) and cerebellar ataxia associated with dysmyelination. The breakage event in Xq22 affects *GLRA4*, a putative pseudogene of the glycine receptor gene family, approximately 70 kb apart from the *PLP1* gene. We discuss the fact that a position effect on *PLP1*, rather than a duplication or missense mutation, is considered to be causative for a subset of clinical PMD symptoms.

METHODS

Fluorescence in situ hybridisation (FISH) analysis

Biotinylated cosmid, PAC, or YAC DNA was hybridised to metaphase chromosomes of lymphocytes of patients as described.¹³ Hybridisation signals were detected via avidin conjugated FITC. Chromosomes were counterstained with

Key points

- Pelizaeus-Merzbacher disease (PMD, MIM 312080) is an X linked recessive dysmyelination disorder of the central nervous system caused by mutations in the proteolipid protein gene (*PLP1*). The clinical spectrum varies widely. In the two main forms of the disease, the classical and connatal types, onset of symptoms is in the first few months of life with nystagmus and hypotonia, but rate of progression and severity of the clinical picture differ.
- We report on a patient with an inverted X chromosome displaying a subset of PMD symptoms including a PMD typical dysmyelination pattern. Physical mapping revealed that the Xq22 breakpoint, residing in close vicinity to the *PLP1* gene, interrupts *GLRA4*, a putative pseudogene similar to glycine receptor alpha-2 chain. Mutation screening of *GLRA4* in patients with ataxia (with or without dysmyelination) and in patients with spastic paraplegia failed to identify patient specific mutations.
- Therefore, we do not consider *GLRA4* to be involved in the phenotype, but suggest that a position effect on *PLP1* ranging over 70 kb is responsible for the pathogenesis. This is the first case where a position effect on *PLP1* rather than a duplication or missense mutation is considered to be causative for a subset of PMD symptoms.

DAPI. Images of FITC and DAPI stained chromosomes were taken separately by using a cooled charge coupled device camera system (Photometrics, Tucson, AZ, USA). A Macintosh Quadra 900 was used for camera control and digital image acquisition in "TIFF" format using the software package Nu200 2.0 (Photometrics). Separate grey scale fluorescence images were recorded for each fluorochrome. Images were overlaid electronically and further processed with Adobe Photoshop software.

Polymerase chain reaction (PCR) amplification

All PCR amplifications were carried out in a final volume of 50 µl with 100–200 ng template, 20 pmol of each primer, 200 µM dNTPs (MBI Fermentas, Vilnius, Lithuania), 1.5 mM MgCl₂, 75 mM Tris-HCl pH 9.0, 20 mM (NH₄)₂SO₄, 0.01%

Abbreviations: CNS, central nervous system; FISH, fluorescence in situ hybridisation; PMD, Pelizaeus-Merzbacher disease; SPG2, X linked spastic paraplegia; SSCP, single strand conformation polymorphism; WISC-RN, Wechsler Intelligence Scale for Children, revised Dutch edition

Tween 20, and 2 U of Goldstar DNA Polymerase (Eurogentec, Seraing, Belgium). Cycling was carried out in a GeneE Thermocycler (Techne, Burkhardtshof, Germany) under the following conditions: 94°C for 3 min; 94°C for 20 s, T_A for 20 s, 72°C for 20 s (for 38 cycles), and a final extension of 5 min at 72°C (T_A indicates annealing temperature).

Single strand conformation polymorphism (SSCP) analysis

SSCP analysis was performed on amplified genomic DNA from patients as described.¹⁴ The PCR products (1–5 µl) were mixed with 5 µl of denaturation solution containing 95% formamide and 10 mM EDTA pH 8.0 and denatured at 95°C for 10 min. Samples were immediately chilled on ice and loaded on a 10% polyacrylamide gel (acrylamide:bisacrylamide = 37.5:1 and 29:1; multi slot gel, TGGE base, Qiagen, Venlo, The Netherlands) containing 2% glycerol and 1×TBE. Gels were run at 15°C, in 1×TBE, at 500 V for 3–5 h and silver stained as described in the TGGE handbook (Qiagen).

Cloning and sequencing of PCR products

PCR products were cloned into the pCR2.1-TOPO vector (Invitrogen Carlsbad, CA, USA). The clones were sequenced with Cy5 labelled vector primers M13, universal and reverse, by the cycle sequencing method described by the manufacturer (ThermoSequenase Kit, Amersham; merged with General Electrics Healthcare, Slough, UK), and analysed on an ALF express automated sequencer (Pharmacia; merged with General Electrics Healthcare, Slough, UK).

RESULTS

Clinical report

Investigations were carried out on a male patient from the Erasmus MC/Sophia Children's Hospital and his unaffected mother. The pregnancy, delivery, and perinatal period of the patient had been uneventful. He was an inactive baby with delayed motor development, walking with a broadly based ataxic gait from the age of 3 years. His mental development was retarded. At the age of 23 and 36 months his scores on the Denver test were 12 and 24 months, respectively. At the age of 3.9 years he was dysarthric with a slow and monotonous speech and hoarse and soft voice. Complex movements of mouth and tongue were disturbed. Eye

movements were full, but saccadic and there was no nystagmus. Optic discs were pale and vision was moderately impaired. In addition mild spasticity and ataxia were present in all extremities. At the age of 8.6 years neurological examination was unchanged and he attended a school for physically disabled children. Mental handicap was moderately severe: WISC-RN IQ 55–59 (verbal IQ 68–79, performal IQ 45–49). From the age of 10 years vision started to deteriorate due to progressive optic atrophy. His speech became unintelligible and truncal ataxia as well as ataxia and spasticity of extremities progressively increased until he was wheelchair bound and helpless at the age of 15 years. At 13 years of age frequent tonic-clonic epileptic seizures occurred, which were controlled with lamotrigine monotherapy.

CT scan at the age of 4 years was normal. Brain MRI taken at 5.3, 8.6, and 12.6 years of age all showed the same severe abnormalities of myelination. On T1 weighted images, high signal intensity was only present in the white matter of the brainstem, cerebellum, optic radiation, and internal capsule. On T2 weighted and inversion recovery images, there was nearly complete absence of low signal intensity in the supratentorial white matter (fig 1). Proton MRS at the age of 11.7 years (CSI 135 and 40 ms) showed normal concentrations of *N*-acetyl aspartate, choline, and creatine levels in 48 white and grey matter 2 ml voxels (TR 135 ms, TE 40 ms).

Neurophysiological studies showed severe postchiasmatic slowing of the visual evoked responses on both sides and severe slowing of the brainstem auditory evoked responses, with normal sensitivity of both ears to sounds. Sensory and motor nerve conduction studies at 3.10 and 9.3 years of age were normal. MRI abnormalities are identical to those described in Pelizaeus-Merzbacher disease (PMD), showing severe dysmyelination without evidence of myelin destruction.^{15 16} No involvement of the peripheral nervous system could be demonstrated. Development in our patient slowly progressed until the age of 10 years. At that age regression started and from the age of 15 years onwards he neurologically deteriorated quite rapidly.

Analysis of the X chromosomal breakpoint regions

Cytogenetically, both the patient and his unaffected mother presented an apparently balanced inverted X chromosome

Figure 1 Axial T2 weighted NMR images at the age of 6.3 years (A–D) and 9.3 years (E–H). The characteristic hypointense signals indicating the presence of myelinated fibres are absent in the pons and the medial cerebellar peduncles (A and E) and in the internal capsules (B and F) and are sparse in the basal areas of the corona radiata (C–D and F–H). This pattern remained the same from 6.3 to 9.3 years of age. In addition, no brain atrophy is present.

Table 1 Clinical presentation of connatal and classic type PMD versus the analysed patient

Symptoms	PMD type		Patient
	Connatal	Classic	
Nystagmus	+	+	—
Stridor	+	+	—
“Head bobbing”	+	+	—
Developmental delay	+	+	+
Developmental deterioration	~3 months	~4 years	~10 years
Optic atrophy	+	+	+
Extrapyramidal movements	+	+	+/-
Spasticity	+	+	+
Ataxia	+	+	+
Epilepsy	+/-	—	+
VEP	Abnormal	Abnormal	Abnormal
BAER	Abnormal	Abnormal	Abnormal
NMR	Severe dysmyelination, progressive cortical atrophy	Severe dysmyelination, progressive cortical atrophy	Severe dysmyelination, no cortical atrophy

BAER, brainstem auditory evoked responses; NMR, nuclear magnetic resonance; VEP, visual evoked potential.

with the breakpoints in Xp22 and Xq22 (fig 2A). To characterise the breakpoint regions in more detail, fluorescence in situ hybridisation (FISH) analysis was carried out. As regards the breakpoint region in Xp22, Rao and colleagues¹⁷ had previously shown that the breakpoint on the short arm of the X chromosome is close to the *SHOX* gene in the pseudoautosomal region, but does not interrupt the gene. As the patient's unaffected mother shows the identical X chromosomal rearrangement, the breakpoint within the pseudoautosomal region is not causative for the phenotype of the patient. Therefore, the second breakpoint in Xq22, which can not be compensated for in the male patient, is likely to be involved in the pathology.

The YAC clone CEPHy904G09923 was shown by in situ hybridisation to span the breakpoint in Xq22. This clone

contains the *PLP1* gene, encoding the proteolipid protein, a major constituent of myelin sheaths. To further narrow down the breakpoint region, a progressive hierarchy of YAC, PAC, and cosmid clones covering the region of CEPHy904G09223 was used for FISH analysis on metaphase spreads of the patient.¹⁸ We can show that the PAC clone RPCIP7041055C14 (insert size 100 kb) bridges the breakpoint (fig 2B). Using *EcoRV* fragments from this PAC as FISH probes, we were able to demonstrate that the breakpoint lies in the genomic region of the *GLRA4* gene. *GLRA4* encodes a gene with high homology to the human glycine receptor subunit *GLRA2* and the murine *glra4* and is approximately 70 kb away from the *PLP1* gene (fig 2C). Southern blot analysis mapped the breakpoint between exons 8 and 9 of *GLRA4* (data not shown).

Possible implications of *GLRA4* and *PLP1* in patients with ataxia, dysmyelination, or spastic paraplegia

Although we could show expression of *GLRA4* by using reverse transcription polymerase chain reaction (RT-PCR) in adult and fetal brain (data not shown), we considered it to be a pseudogene as the gene encodes a truncated protein lacking a fourth transmembranic domain typical for other glycine receptor subunits. To analyse whether the interruption of *GLRA4* nevertheless contributes to the pathogenesis or whether a position effect on *PLP1* is the more likely reason for the phenotype, we investigated the DNA samples of an additional 24 patients. These patients presented with ataxia and a hypomyelination pattern comparable with that seen in PMD patients, and displayed a clinical phenotype similar to that of the patient with the inverted X chromosome, but did not have a duplication or mutation within the *PLP1* gene. We performed SSCP analysis on the genomic DNA of nine patients with isolated ataxia and dysmyelination followed by sequencing, but we did not find any gene variations. We also screened 15 patients with isolated ataxia for mutations in *GLRA4*. However, no change in SSCP pattern was observed. Spastic paraplegia has been shown to be allelic to PMD in some entities,^{6,7} although there are families with spastic paraplegia and linkage to Xq22, but no mutation in the *PLP1*

Figure 2 (A) Schematic representation of the normal (left) versus the inverted (right) X chromosome. For better visibility the short and long arms of the chromosomes are shaded differently. The Xp22.3 region at the tip of Xp is not drawn to scale. (B) FISH of PAC RPCIP7041055C14 to metaphase spreads of the patient. The PAC spans the breakpoint in the patient (white arrowhead). (C) Scheme showing orientation and distance between *PLP1* and *GLRA4*, and localisation of the breakpoint 5' of *PLP1*. Distances and genes are not drawn to scale.

gene.¹⁹ We analysed two members of the reported family with spastic paraplegia¹⁹ by sequencing the coding region of *GLRA4*. At position 555 of the cDNA a C to G transition was detected. Analysis of unrelated controls identified this change as a frequent polymorphism that occurs in approximately 40–50% of all individuals tested.

As we did not consider *GLRA4* to be causative in the patients with an inverted X chromosome, we addressed the possibility that the *PLP1* gene itself, which is located only 70 kb away, was affected (fig 2C). However, metaphase as well as interphase FISH analysis using a cosmid containing *PLP1* revealed no inversion, deletion, or duplication. Nor did fine mapping PCRs reveal any aberration. Sequencing of the entire coding region of *PLP1* did not show any nucleotide alteration. Therefore, the most likely mechanism underlying the specific and PMD related phenotype in this patient is a position effect due to the close proximity of the *PLP1* gene.

DISCUSSION

PMD is a rare X linked central nervous system leukodystrophy characterised by dysmyelination of the CNS with key clinical features of early onset nystagmus and hypotonia and, depending on whether the clinical type is either connatal or classic, relatively rapid or slow progression of cerebellar, pyramidal, extrapyramidal, and cognitive symptoms.^{1,2} Additionally, cerebellar ataxia and mental retardation can be observed in some patients. Point mutations in *PLP1* have been detected in 10–25% of PMD patients, but duplications of the gene account for the majority of cases.^{9,10,20,21} Deletions of *PLP1*^{22,23} and splice site²⁴ and intronic region mutations²⁵ have also been described. In 10–20% of patients there is no detectable duplication or mutation within the *PLP1* gene. Patients with a duplication commonly exhibit a milder PMD phenotype than those with missense or other mutations. However, much of the clinical variability is not yet understood. Gene dosage of *PLP1*, possibly also affected by modifier genes either within the duplicated region or elsewhere in the genome, seems to be critical as regards the phenotype.^{8,26,27}

We have analysed the breakpoints of an X inversion in a patient with a subset of PMD symptoms. One breakpoint, in the pseudoautosomal region, could be excluded as the unaffected mother carries the same inversion.¹⁷ The breakpoint in Xq22 disrupts the genomic sequence of *GLRA4*, encoding a putative glycine receptor α subunit. Variants of such ligand binding α and structural β subunits form different isoforms of the glycine receptor.^{28,29} These subunits share a similar membrane topology with a large extracellular N-terminal domain being followed by four membrane spanning segments. Mutations in several of the corresponding genes, both in humans and mice, have been involved in neurological disorders such as hyperekplexia.^{30–32} However, unlike the other human glycine receptor subunit genes, *GLRA4* harbours a premature stop codon within exon 9, leading to the translation of only three transmembrane domains. As such a subunit is unlikely to form a functional receptor, we support the classification of *GLRA4* as a pseudogene as annotated in the, NCBI database (AL049610). More evidence that the fourth transmembrane domain is crucial for receptor function and that *GLRA4* therefore is a non-functional pseudogene, also comes from studies of the *oscillator* mouse.³¹ A microdeletion in the gene coding for the $\alpha 1$ subunit (*Gla1*), resulting in the loss of a cytoplasmic loop and the fourth transmembrane domain, leads to a 90% reduction in binding affinity of the receptor, resembling a complete loss of function allele.³¹ It is therefore unlikely that the gene interruption contributes to the pathogenesis of our patient. This idea is also supported by the fact that we could not find any patient specific mutations. Although we are aware that the number of analysed patients is limited and

that we can not definitely rule out a putative role for *GLRA4* as a modifier gene as it is situated in the duplication fragment of some patients and could therefore contribute to the wide variations of the PMD phenotype, we do not consider it causative with respect to the dysmyelination defect seen in our patient. The main clinical features can rather be explained by a positional effect on the nearby *PLP1* gene.

Position effects have been described for a number of genetic diseases³³ and are thought to influence the expression pattern of the affected genes. Distances between breakpoints and affected genes can range from 3 kb as shown for *SRY*^{34,35} to over 1 Mb in the case of *SOX9*.³⁶ In our case one could speculate that the breakpoint may put the gene under different regulatory constraints leading to a changed expression pattern, which would alter developmental pathways in only some tissues affected in PMD and not in others. This could explain why the patient with an inverted X chromosome displays only a subset of symptoms. Another possibility would be that the degree of expression is varied: as *PLP1* silencing is expected to lead to a very different phenotype,¹² the most likely explanation is that its expression will be increased, mimicking rather a duplication of the gene. This might explain the high degree of similarity between PMD patients carrying a *PLP* duplication and the inversion patient. Our findings underline once more that gene dosage of *PLP1* seems to be the crucial factor in the pathology of the variable phenotypes.

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