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Evidence for disease penetrance relating to CNV size: Pelizaeus-Merzbacher disease and manifesting carriers with a familial 11 Mb duplication at Xq22

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Abstract

The potential causes for the incomplete penetrance of Pelizaeus-Merzbacher disease (PMD) in female carriers of *PLP1* mutations are not well understood. We present a family with a boy having PMD in association with *PLP1* duplication and three females who are apparent manifesting carriers. Custom high-resolution oligonucleotide array comparative genomic hybridization (aCGH) and breakpoint junction sequencing were performed and revealed a familial complex duplication consisting of a small duplicated genomic interval (~56 kb) and a large segmental duplication (~11 Mb) that results in a *PLP1* CNV gain. Breakpoint junction analysis implicates a replication-based mechanism underlying the rearrangement formation. X-inactivation studies showed a random to moderate advantageous skewing pattern in peripheral blood cells but a moderate to extremely skewed (~90%) pattern in buccal cells. In conclusion, our data shows that complex duplications involving *PLP1* are not uncommon, can be detected at the level of genome resolution afforded by clinical aCGH and duplication and inversion can be produced in the same event. Furthermore, the observation of three manifesting carriers with a large genomic rearrangement supports the contention that duplication size along with genomic content can be an important factor for penetrance of the PMD phenotype in females.

Keywords

complex rearrangement; FoSTeS; manifesting female carriers; MMBIR; penetrance; *PLP1*; PMD

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INTRODUCTION

Proteolipid protein (PLP1) is a highly hydrophobic tetraspan protein that constitutes the major myelin protein present in the central nervous system (CNS). Duplication copy number variation (CNV) of the Xq22 genomic region including *PLP1* is the underlying cause in 60 to 70% (1) of patients with Pelizaeus-Merzbacher disease [PMD, MIM #312080], a disorder characterized by leukodystrophy in which myelin is not properly formed in the CNS. PMD follows a progressive course clinically characterized by nystagmus, spastic quadriplegia, ataxia, and developmental delay (reviewed in (2) and (3)). The disease expression may vary among patients, in part due to the type of molecular alteration present. The clinical phenotype can generally be classified as connatal and classic; clinical forms of intermediate severity between the connatal and classical syndromes were labeled as ‘transitional’ (3). The connatal form is less frequent, but more severe; most cases are caused by missense mutations in the *PLP1* gene. Classical PMD is frequently caused by duplications of the *PLP1* whereas null point mutations of *PLP1* usually produce the allelic disorder, spastic paraplegia type 2 (SPG2) (2). Rare whole gene deletions cause PMD plus mild peripheral neuropathy (2, 4, 5), considered a complicated form of SPG2 (2). Triplications were also reported with a more severe phenotype, further emphasizing the dosage sensitive nature of *PLP1* (6).

Duplications including *PLP1* are nonrecurrent, that is, the size of the duplication and the breakpoint junction locations are unique in each patient or pedigree (1, 4). Females are generally asymptomatic carriers that transmit their duplicated segment to their affected sons (7–9), and typically the asymptomatic carrier females show a skewed X-inactivation pattern (10). The genomic rearrangements causing CNV gains at the *PLP1* locus that are associated with PMD and related phenotypes can often be more complex than anticipated (11, 12) and may occur outside coding regions (13).

Here we report a family with one male member affected with PMD in addition to three apparently manifesting carriers, mother and two daughters, each carries an 11.1 Mb duplication including *PLP1* in either the hemizygous or heterozygous state, respectively. The rearrangement size observed in this family is amongst the largest reported thus far. Due to the low frequency of female carriers manifesting the PMD phenotype, we suggest that rearrangement or CNV size might influence the disease penetrance. In addition, the presence of a complex rearrangement in this family supports previous data (12) proposing that a replication mechanism underlies many of the CNVs involving *PLP1* and flanking regions.

MATERIAL AND METHODS

Subjects

A family with PMD was ascertained and studied using protocol H-16213. This study was approved by the Institutional Review Board for research involving human subjects at Baylor College of Medicine. Clinical histories and physical examinations were obtained by one of the authors (JS). Peripheral blood and buccal smear samples from patients and family members were obtained after informed consent. DNA was extracted using standard methodology.

Array Comparative Genomic Hybridization (aCGH)

A tiling-path custom oligonucleotide microarray was designed to interrogate the copy-number of a 15.5 Mb segment encompassing *PLP1* on chromosome Xq22. Probes spanning ChrX: 97,915,511–113,400,000 (NCBI build 36) were selected using the Agilent earray website (<http://earray.chem.agilent.com/earray/>); totaling 40,208 with an average distribution of 1 probe per each 386 bp (format 4 X 44K). Protocols for labeling subject

DNA samples, hybridization of the arrays and analyses were as described (14). A gender-matched control was used for the hybridizations. Genomic copy number was defined by analysis of the normalized $\log_2$ (Cy5/Cy3) ratio average of the CGH signal. Regions that reached an average threshold of 0.6 and 1.0 were considered as gains consistent with duplication in females and males, respectively.

Breakpoint mapping and X-inactivation studies are described in details in Tables S1 and S2 under Supporting information.

RESULTS

Family clinical findings

The male proband (BAB2867) was first evaluated at 12 months of age because of global developmental delay and failure to thrive. Due to unstable social circumstances and different homecare environments, the timing of early milestones was unclear. At the initial evaluation, he demonstrated skills scattered between 3 and 6 months of age, and was able to hold his head up more than 45 degrees, reach and grasp at objects, and babble in consonants. Rotary nystagmus began perhaps before 2 months of age. His failure to thrive was somewhat responding to caloric supplementation. Physical examination showed a child at the 25th percentile for his height, 3rd percentile for his weight, 3rd percentile for his head circumference, nevus flammeus, prominent forehead with associated deep set eyes, rotary nystagmus, bilaterally short proximal 5th finger phalanges, pedal edema, and hyperextensibility to his fingers. Neurological examination showed diffuse moderate hypotonia worse peripherally but also axially, possible choreiform movement of his hands, and symmetric bilateral hyperreflexia at the patellae.

Brain MRI at 14 months of age showed diffuse increased T2 signal throughout the white matter of both cerebral hemispheres, and the corpus callosum was much smaller than expected, perhaps suggestive of delayed myelination (Fig. 1a). At 24 months of age, he continued to exhibit significant developmental delay (no rolling over, was able to transfer and also place objects in his mouth at 16 months old, no marked improvement in verbal ability), generalized hypotonia continued to dominate his neurologic examination, and his height and weight were significantly below the 3rd percentile despite gastrostomy tube placement at 21 months of age. At 36 months of age, he was noted to roll over both ways, and could army crawl but not bear weight on his legs. Currently, his diagnosis of PMD is thought to phenotypically fit in between the connatal and classic form, sometimes referred to as transitional PMD (3).

The proband's second oldest sister (BAB2869) was first seen by a medical geneticist at 16 months of age due to developmental delay and poor growth on caloric supplementation. She smiled at 3–4 months, rolled over at 6 months, sat at 10–11 months, crawled at 13 months, walked at 16–17 months, her first words were at 12 months, and she had 6 words reported at the initial visit. Physical examination showed her to be at the 25th percentile for height, 3rd percentile for weight, 25th percentile for head circumference, central forehead nevus flammeus, slight hypertelorism, somewhat deep set eyes, and mildly flattened midface. Her neurological examination exhibited very mild diffuse hypotonia and no hyperreflexia. There was no nystagmus. Brain MRI at 15 months showed mildly prominent periventricular white matter signal intensity (Fig. 1b). Now at 5 ½ years of age, her growth parameters are in the 25th to 50th percentile, she is in special education classes in kindergarten, and is summarized as having mild to moderate global developmental delay.

The proband's oldest sister (BAB2868) has not been seen previously by a geneticist, but her clinical history mirrors that of her younger sister. She sat at 11 months, crawled at 12

months, verbalized 5–10 words at 18 months. She had a history of failure to thrive but by the time of her first evaluation by a geneticist at almost 6 years old, all her growth parameters were in the 25th to 50th percentile. She also had the central forehead nevus flammeus and mildly deep set eyes. Her first grade classes were all special education.

The mother (BAB3182) has not been formally evaluated, with the only source of information regarding her being self-reported. She perhaps had failure to thrive as a child, has a history of illicit substance abuse, does not have custody of her five children with her itinerant lifestyle, and likely has some degree of developmental delay. She did not share the craniofacial features that her three affected children had in common, and has not had any neuroimaging.

High resolution CGH revealed two separate duplications

Family pedigree and CGH results for each member are shown (Fig. 2). Array CGH of genomic DNA isolated from blood cells collected from subjects BAB2867, BAB2868 and BAB2869, BAB3182 revealed duplications of two regions at chromosome Xq22 (Fig. 2 and Fig. 3a). The smaller duplication (DUP1) encompasses chrX: 102,043,198–102,098,823 and includes a 55.6 kb genomic interval; the larger one (DUP2) encompasses chrX: 102,251,730–113,341,669, includes *PLP1* and encompasses 11.08 Mb of chromosome Xq22. The approximate distance between DUP1 and DUP2 in the reference genome is 160 kb. Patient BAB2867, two sisters (BAB2868 and BAB2869) and mother (BAB3182) carry the same rearrangement, whereas another sister (BAB2870) and the brother (BAB2871) carry no visible alterations in this region.

X-inactivation studies (XCI) were performed in each female from this family. The two girls showed a random X-inactivation pattern in blood (BAB2868, 69/31; BAB2869, 57/43; BAB2870, 41/59) and a skewed to moderate skewed pattern in buccal cells (BAB2868, 93/7; BAB2869, 83/17). The mother (BAB3182) presented moderate skewing in both blood (88/12) and buccal cells (83/17) (data not shown). All of the females presented skewing towards inactivation of the X-chromosome that bears the duplication.

Sequencing the duplication breakpoint junctions revealed a complex rearrangement

Different primer pairs and PCR conditions were attempted in order to obtain the breakpoint junctions of both duplications. We successfully amplified two junctions using primers R1.1 and R2.1 corresponding to the DUP1 proximal breakpoint and DUP2 proximal breakpoint, respectively, and primers F4 and F5 corresponding to the DUP1 distal breakpoint and DUP2 distal breakpoint, respectively (Fig. 3). As anticipated, all members of the family with the duplication that were tested have the same breakpoint junctions (Fig. 3c and Fig. 3d). Sequencing of the junction fragments revealed that the smaller duplication was inserted in an inverted orientation amid the two copies of the large duplicated segments.

Our data revealed the presence of a complex rearrangement involving *PLP1* and flanking regions but a remaining question was whether the reference genomic structure was also present in this altered chromosome. To answer this question we assayed for the presence of genetic markers characteristic of the reference genomic structure flanking the duplications in patient BAB2867 and controls using the same primers (R1.1 and R2.1) previously used to obtain the breakpoint junctions plus two primers, A16AF and A16BF designed according to the reference genome (Fig. 3a). Two bands of expected sizes, 359 bp and 2.2 kb, respectively, were obtained by PCR in patient (BAB2867) and control (Fig. 3b), consistent with the presence of the reference genomic structure in addition to the complex rearrangement. Only male patient BAB2867 was used in this experiment in order to avoid cross- amplification from both X-chromosomes in females

DISCUSSION

We report here a family with a boy having PMD and three females who are apparent manifesting carriers who carry an abnormal X-chromosome with an ~11 Mb complex duplication including *PLP1*. Phenotypically, females diagnosed with PMD generally present variable expressivity of the disease (7). Carrier females of point mutations or deletions may manifest a late-onset spastic paraplegia phenotype with variable severity and may develop progressive leukodystrophy with dementia later in life (2). *PLP1* duplications usually are nonpenetrant in females; symptomatic cases are rare despite the observation that the majority of males with PMD inherited an abnormal X-chromosome from their carrier mothers (7, 10, 15, 16).

In the family reported herein the carrier female patients present a random (daughters) to moderate (mother) skewing in the X-inactivation pattern in their peripheral blood cells contrasting with the observation that most of the *PLP1* duplication carrier females exhibit skewed X-inactivation in blood (10). Woodward and colleagues hypothesized that the dosage effect of genes other than *PLP1* may cause unfavorable growth rate in peripheral blood cells expressing the X-chromosome duplication. This phenomenon, however, is not universal for all asymptomatic carrier females as showed by a few exceptions reported therein (10). Intriguingly, we observed that females carrying large duplications such as those reported here and a few others in the literature (10, 17) do not present skewing of X-inactivation in peripheral blood samples for reasons that are currently not known.

By contrast, we do detect advantageous moderate to strong skewing in X-inactivation pattern in buccal cells collected from the affected females of this family supporting the contention that cells expressing the duplicated X-chromosome may undergo differential selective processes in different tissues. This result is consistent with the hypothesis that females carriers may undergo a secondary skewing of the X-inactivation in brain cells, an hypothesis that was proposed to explain lack of clinical symptoms in carrier mothers of patients with *PLP1* duplications (18). According to that hypothesis, the oligodendrocyte cell population expressing the non-altered X-chromosome in carrier females is anticipated to predominate over those oligodendrocytes expressing the altered X-chromosome due to preferential survival. This process may eventually lead to a normal or almost normal myelination pattern in the CNS resulting in carrier females without clinical symptoms.

We hypothesize that both affected female children, and potentially the carrier mother, reported herein likely present secondary X-inactivation in their CNS as both girls have developmental delay but the clinical phenotype is milder compared to their male brother. Furthermore, MRI in one of the girls showed a mild intensity increase in contrast with the diffuse pattern observed in her brother. Because this secondary skewing process is anticipated to lead to asymptomatic carrier females, as opposed to what we observe for this family, we propose that the large size of the duplication they bear is likely playing a role in the manifestation of the disease.

Few reports of affected female patients with *PLP1* duplication are available in the literature (7, 17). Carrozzo et al. (17) reported a severely affected girl suggestive of PMD with a direct duplication of the Xq21.32-q24 sub-bands visualized by microscopy (estimated minimum size of 23.3 Mb and maximum size of 28.8 Mb). From a neurological standpoint, she is more affected than the two sisters described in our study, with significantly greater global developmental delay, presence of nystagmus, and evidence of hypomyelination on brain MRI. Despite the difference in clinical presentation from ours and Carrozzo's patient, we favor the interpretation that the genomic duplication is the cause of the disease in females of the family presented herein; an hypothesis which is further strongly supported by the fact

that the clinical phenotype segregates with the duplicated X chromosome. We speculate that reasons these females present clinical symptoms potentially include: 1) perturbed X-inactivation in brain cells; 2) the uncommon large size of the duplication may encompass genes or functional elements that alter time and/or tissue-specific expression patterns of *PLP1*; 3) the uncommon large size of the duplication encompass genes or functional elements that alter time and/or tissue-specific expression patterns of genes other than *PLP1*; 4) dosage sensitive gene(s) within the duplicated segment escape X-inactivation and contributes to the phenotype.

Thus far, there is no data to support that genes other than *PLP1* contribute to the clinical phenotype observed in our patients. For the male proband, his PMD disease is best characterized as in between the clinical spectrum of the severe/connatal and the classic type, without additional phenotypic features or known organ system involvement to suggest a contiguous gene duplication syndrome. This family's 11 Mb duplication encompasses approximately 65 protein-coding genes, of which 18 have been individually shown in literature reports to be expressed in the brain. Furthermore, our analysis of the RNA expression data of the same 65 protein-coding genes from 20 neuroanatomic human brain regions available at the Allen Human Brain Atlas (<http://human.brain-map.org/>) indicates that the majority of them display some expression in one or more brain regions. However, of those only a few genes within the duplicated segment were shown to cause mental retardation/developmental delay when mutated or deleted including *DCX* (Lissencephaly and agenesis of corpus callosum [MIM #300067]), *ACSL4* (X-linked mental retardation, MRX63 [MIM #300387]), *PRPS1* (Arts Syndrome [MIM#301835]) and *PAK3* (X-linked mental retardation, MRX30 [MIM#300558]) (Fig. 4). Three cases of smaller duplicated segments within this region have been reported in the literature (Fig. 4). Jehee et al. (19) describes a male who had severe developmental delay, trigonocephaly, and recurrent unexplained fevers that was given a clinical diagnosis of FG syndrome. He had an Xq22.3 duplication involving >15 genes (Fig. 4), of which the authors postulated that the extra copy of *MID2* was responsible for his disease.

Increased dosage of other genes in this region that potentially could lead to clinical or neurological consequences include *SERPINA7* (higher serum levels of thyroxine binding globulin and T4) and four genes known to be expressed in the brain: *IL1RAPL2*, *CLDN2*, *TSC22D3*, and *PRPS1*. A disease entity has been ascribed to increased activity of *PRPS1*, but thus far only activating mutations have been reported, rather than gene amplification (20); additionally, in post-mitotic cells, such as those of the central nervous system, lower enzyme activity has been postulated due to the heightened liability of the mutant protein (19). For our male proband, though, he had no similar head shape abnormality, and his T4 and uric acid (in instances of *PRPS1* overactivity) have been normal.

Another duplication with a breakpoint in the *IL1RAPL2* gene has been described (21) (Fig. 4). However, this female had a clinical diagnosis of Schinzel-Giedion syndrome with Hirschsprung disease; multiple organ systems were affected including her craniofacies, skeletal, and genitourinary system, along with significant developmental delay. None of the male or female family members described herein had a matching phenotype. Vetrie et al. (22) described a duplication of many exons within *COL4A5*, causative of Alport syndrome, but none of the children in this family had any clinical manifestation of Alport disease.

Nevertheless, we can not rule out a contribution of other genes within the duplicated segment to the clinical phenotype observed in our subjects. However, we favor *PLP1* overexpression as the predominant causative factor. Furthermore, our data support the hypothesis that there is a positive association between the size of the duplication and penetrance of PMD in females. This hypothesis is supported by the following observations,

i) the paucity of affected female carriers of *PLP1* duplications present in the literature in which affected boys carry duplications smaller than 8 Mb (1, 12, 23, 24); ii) symptomatic female patients with large duplications, such as in this present work (~11 Mb) and in (17), >20 Mb. Interestingly, the influence of the size of the CNV on the penetrance of craniofacial abnormalities was observed in a mouse model of Smith-Magenis Syndrome (SMS) (25, 26). The authors proposed that, while *Rai1* is the major gene responsible for the SMS-like phenotype, the penetrance of the trait seems to be modified by other genetic elements flanking the gene perhaps by means of changing *Rai1* expression levels or disturbing its developmental timing of expression.

The duplication causing PMD in the present family unveiled a complex rearrangement constituted by two separate duplicated segments, DUP1, ~56 kb in size and DUP2, ~ 11 Mb in size with a 160 kb non-duplicated, or normal copy number genomic interval in between (Fig. 3). Sequencing of the breakpoint junctions revealed that the small duplication (DUP1) was inserted amid the two copies of DUP2 in an inverted orientation. Genomic rearrangement complexities involving *PLP1* duplications were revealed by high-resolution array CGH and/or breakpoint sequencing in up to 65% of cases in our previous studies (12). In cases for which breakpoint junction sequencing was achieved, we identified insertion of small segments of variable sizes (32 bp up to 5 kb; larger sizes were also predicted) originating from nearby regions (as far as 760 kb); insertion of segments in inverted orientation regarding the reference genome were also observed (12). Therefore, identification of a complex rearrangement involving *PLP1* in the present family was not surprising, although the size of the small inserted segment is 10X larger than those seen in (12). Microhomology of 3 bp was observed in junction R1.1 + R1.2 whereas no microhomology in addition to an insertion of 4 bp was observed in F5 + F4 junction (Fig. 3c and Fig. 3d).

Lee et al. (12) proposed a replication-based mechanism, Fork Stalling and Template Switching (FoSTeS), to explain the formation of the complex rearrangements identified at the *PLP1* locus; alternatively those complexities can also be explained by the microhomology-mediated break induced replication (MMBIR) (27, 28). In all cases described therein, microhomologies were identified at the junctions, including the small insertion junctions, leading to the proposal that the observed microhomologies may reflect nucleotides used to prime DNA replication during template switching (12, 28, 29). Therefore, the presence of microhomology at one of the junctions in this present case supports FoSTeS as the mechanism for formation in at least one of the junctions, but the presence of an insertion of random bases could be consistent with the involvement of Non Homologous End Joining (NHEJ) (28, 30) underlying the formation of the second junction.

In summary, we present a family with an apparent manifesting carrier mother who has two manifesting carrier daughters and a son diagnosed with PMD; each patient carries a complex large duplication of the Xq22 chromosome including *PLP1* likely originating in part from a replication-based mechanism. Our data support the contention that duplication of *PLP1* is the causative factor of the clinical phenotype in these manifesting females. The unusually high penetrance of the mutation in females from this family, in conjunction with reports in the literature, is consistent with CNV size contributing to the penetrance of the disease in females, perhaps by perturbing X-inactivation and/or altering *PLP1* expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Brain magnetic resonance imaging (MRI). a) BAB2867: Axial T2 image at 14 months of age showing diffusely increased signal throughout the white matter and smaller than expected corpus callosum. b) BAB2869: Axial T2 image at 15 months of age showing mildly prominent periventricular white matter signal intensity.

Fig. 2.

Pedigrees of the family HOU1129 and individual array CGH results for each family member tested. To the right are the aCGH profiles (only part of the duplications is shown). Note dynamic range for males with duplications versus female manifesting carriers reflecting 2:1 male gain vs 3:2 female gain. Index patient arrow, BAB2867, two sisters (BAB2868 and BAB2869) and mother (BAB3182) carry a complex duplication DUP1-normal-DUP2 spanning genomic coordinates (Hg18) DUP1 (55.6 kb), chrX: 102,043,198–102,098,823 and DUP2 (11.1 Mb), chrX: 102,251,730–113,341,669 including *PLP1*. Filled circles and square represent affected male and females, respectively. X-inactivation (XCI) patterns observed for females are shown. Top: genomic location of *PLP1*.

Fig. 3.

Genomic structure for patients BAB2867, BAB2868, BAB2869 surmised using breakpoint sequencing analysis and array CGH. a) Patients with the complex rearrangement carry the reference genomic structure plus two duplicated segments spanning 55.6 kb (DUP1) and 11.1 Mb (DUP2), respectively. In the reference genomic sequence, these two duplicated segments are 160 kb apart; upon the rearrangement event, DUP1 segment was inserted in inverted orientation amidst the two copies of DUP2 yielding DUP1/INV-DUP2. Color matched arrows represent PCR primers used in this study. b) PCR assay results for the reference genomic segment flanked by primers A16AF + R1.1 (359 bp) and A16BF + R2.1 (2.2 kb) in index patient BAB2867. c) PCR assay result for the breakpoint junction flanked by primers F5 + F4 in all siblings. No microhomology was detected at the breakpoint but instead an insertion of four random nucleotides (CTGC) was observed. d) PCR assay result for the breakpoint junction flanked by primers R1.1 + R2.1 in all siblings. Three nucleotide microhomology (CAT) was observed at the breakpoint junction. Control samples (female: F and male: M), negative control (-).

Fig. 4. Genomic context of the complex duplication for the family reported here and a few selected cases from the literature. a) **Top:** location of the duplicated region at chromosome X is boxed in purple. **Bottom:** selected genes involved in the duplication; genes shown associated with mental retardation/developmental delay phenotypes have MIM numbers labeled (blue). b) Selected females with or without phenotype carrying interstitial duplication reported in the literature. All of them present a random X-inactivation pattern in lymphocytes and/or fibroblasts. Duplications are represented by a red rectangle; please text for details. SGS: Schinzel-Giedion syndrome; MCA: Multiple congenital anomalies; MR: Mental retardation.