Role of genomic architecture in \textit{PLP1} duplication causing Pelizaeus-Merzbacher disease

Jennifer A. Lee\textsuperscript{1}, Ken Inoue\textsuperscript{3}, Sau W. Cheung\textsuperscript{1}, Chad A. Shaw\textsuperscript{1}, Pawel Stankiewicz\textsuperscript{1} and James R. Lupski\textsuperscript{1,2,4,*}

\textsuperscript{1}Department of Molecular and Human Genetics, \textsuperscript{2}Department of Pediatrics, Baylor College of Medicine, One Baylor Plaza, Room 604B, Houston, TX 77030, USA, \textsuperscript{3}Department of Mental Retardation and Birth Defect Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira, Tokyo 187-8502, Japan and \textsuperscript{4}Texas Children’s Hospital, Houston, TX 77030, USA

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Genomic architecture, higher order structural features of the human genome, can provide molecular substrates for recurrent sub-microscopic chromosomal rearrangements, or may result in genomic instability by forming structures susceptible to DNA double-strand breaks. Pelizaeus-Merzbacher disease (PMD) is a genomic disorder most commonly arising from genomic duplications of the dosage-sensitive proteolipid protein gene (\textit{PLP1}). Unlike many other genomic disorders that result from non-allelic homologous recombination utilizing flanking low-copy repeats (LCRs) as substrates, generating a common and recurrent rearrangement, the breakpoints of \textit{PLP1} duplications have been reported not to cluster, yielding duplicated genomic segments of varying lengths. This suggests a distinct molecular mechanism underlying \textit{PLP1} duplication events. To determine whether structural features of the genome also facilitate \textit{PLP1} duplication events, we analyzed extensively the genomic architecture of the \textit{PLP1} region and defined several novel LCRs (LCR–PMDs). Array comparative genomic hybridization showed that \textit{PLP1} duplication sizes differed, but revealed a subgroup of patients with apparently similar \textit{PLP1} duplication breakpoints. Pulsed-field gel electrophoresis analysis using probes adjacent to the LCR–PMDs detected unique recombination-specific junction fragments in 12 patients, enabled us to associate the LCR–PMDs with breakpoint regions, and revealed rearrangements inconsistent with simple tandem duplications in four patients. Two-color fluorescence \textit{in situ} hybridization was consistent with directly oriented duplications. Our study provides evidence that \textit{PLP1} duplication events may be stimulated by LCRs, possibly non-homologous pairs at both the proximal and distal breakpoints in some cases, and further supports an alternative role of genomic architecture in rearrangements responsible for genomic disorders.

INTRODUCTION

Architectural features of the human genome can result in genome instability, and susceptibility to rearrangements resulting in disease traits. Such rearrangements, including deletions, duplications and inversions at specific regions may result in the loss or gain of a dosage-sensitive gene (or genes), disruption of a gene, generation of a novel gene at the breakpoints, or dysregulation of a gene in proximity to the rearrangement, causing what is termed a genomic disorder (1–3). Genomic disorders represent a wide spectrum of unrelated clinical conditions including, for example, Charcot-Marie-Tooth disease type 1A (4,5), Smith-Magenis syndrome (6,7) and Williams syndrome (8,9). Yet, the underlying molecular basis is the same: rearrangement involving specific genomic segments ranging from a few kilobases (kb) to several megabases (Mb) in length (10). Many genomic disorders share a common mechanism for their underlying rearrangement: non-allelic homologous recombination (NAHR) between low-copy repeats (LCRs) that flank the rearranged genomic segment (1). Such rearrangements yield common recombinant genomic segments. However, some

*To whom correspondence should be addressed. Tel: +1 7137983723; Fax: +1 7137985073; Email: jlupski@bcm.tmc.edu

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genomic disorders, and selected rearrangements for specific genomic disorders, that deviate from this classic model exist.

Among them is Pelizaeus-Merzbacher disease (PMD), a rare X-linked dysmyelinating disorder of the central nervous system (CNS) (11–13). PMD most commonly (60–70%) arises from genomic duplications of the dosage-sensitive proteolipid protein gene (PLP1) (14,15) located on chromosome Xq22.2 (16,17), and less commonly by PLP1 deletions (18) and point mutations (11). Both the deletions (18) and duplications (19) causing PMD appear to arise by non-homologous end joining (NHEJ) as surmised from the nucleotide sequence of the products of recombination. However, what makes the genomic region surrounding PLP1 susceptible to rearrangement remains to be elucidated. PLP1 is an integral membrane protein and the most abundant component of myelin in the CNS; an extra copy of PLP1 resulting from whole gene duplication affects the development of oligodendrocytes. Specifically, PMD caused by PLP1 duplication is characterized by oligodendrocyte cell death and abnormal CNS myelination (20).

Many genomic disorders result from homologous recombination between non-allelic LCRs, yielding recombination products with common breakpoints. Interestingly, in PMD patients with PLP1 duplication, the rearrangement breakpoints are not common, yielding duplicated genomic segments of varying lengths (14,21). This suggests that the molecular mechanism underlying PLP1 duplication events is likely distinct from the more common NAHR mechanism. We hypothesized that various LCRs in the PMD region might stimulate DNA rearrangements resulting in PLP1 gene duplication, because complex genomic architecture can cause genomic instability and predispose chromosomal rearrangements (1,2,22) and as LCRs are thought to catalyze non-recurrent rearrangements (23). We therefore performed an extensive bioinformatic analysis of this region and mapped the breakpoints in multiple PMD patients with duplication of PLP1. Interestingly, we found that although the duplications differ in size, they appear to cluster into groups of similar size. The rearrangement breakpoints in these groupings appear to be in proximity to unusual genome architecture.

RESULTS

Numerous LCRs identified in the region surrounding PLP1

Extensive in silico analysis of an ~8 Mb region (Build 35: 98 358 000–106 357 999; Xq22.1-q22.3) uncovered several novel LCRs proximal to PLP1, whereas the distal LCRs included only LCR–PMDA through D (Fig. 1). We previously defined the structures LCR–PMDA and B as two inverted repeats, each with two homologous segments: A1a and A2 for LCR–PMDA, and A1b and A3 for LCR–PMDB (18); LCR–PMDC and D were more recently defined by Woodward et al. (19) as distal LCRs which flank LCR–PMDA and B. The novel LCRs have greater than 89% identity and are at least 500 bp in length. Compared with similar analyses of the Charcot-Marie-Tooth disease type 1A (4,24), Smith-Magenis syndrome (23,25,26) and Sotos syndrome (27,28) regions, analyses of the PLP1 region revealed more complex genome architecture, with a greater diversity in the number, copies and orientations of the constituent LCRs surrounding the dosage-sensitive gene PLP1.

LCRs with shared homology were discovered only within the 3 Mb region surrounding PLP1 (Fig. 1). Inter-chromosomal LCRs with homology to regions on various autosomes and chromosome Y, and intra-chromosomal LCRs with homology to other non-adjacent regions on chromosome X were excluded from further analysis. Novel LCRs located ~1.4 Mb proximal to PLP1, which we termed LCR–PMDF1 and F2 (Figs 1 and 2, Table 1), represent a set of large LCRs in inverted orientation, each spanning ~122 kb with 99.9% shared sequence identity. LCRs identified nearest to PLP1 are situated ~276 kb proximal (LCR–PMDE2) and ~125 kb distal (LCR–PMDC) to the gene (Fig. 1, Table 1). Other LCRs in this 3 Mb region (Fig. 1), with their sizes, positions and percent identities, are as shown in Figure 2 and Table 1. There are LCRs in the vicinity of proximal and distal breakpoints of PLP1 duplications in PMD patients delineated by fluorescence in situ hybridization (FISH) mapping (14), including 11 patients reported herein; therefore, we focused on these particular genomic regions for more in-depth analyses of their potential role in PLP1 duplication events.

Duplication breakpoints are diverse but appear to cluster into groups

In all patients (Table 2) whose PLP1 duplication has been confirmed by FISH, custom array comparative genomic hybridization (CGH) analysis revealed their duplication sizes at a bacterial artificial chromosome (BAC) and P1 artificial chromosome (PAC) clone resolution (Fig. 3). We detected duplications which covered the span of one or two BAC clones (~180–250 kb), as well as larger duplications (~4–7 Mb), some of which extend distal to PLP1 beyond the detection range of the array (over 7 Mb). Of the five patients with larger duplications (Table 2), two have cytologically visible duplications by G-banding: BAB2396 [46,XY,dup(X)(q22.1q22.2)] and BAB2425 [46,XX,dup(X)(q22.1q22.3)] (Supplementary Material, Fig. S1). Siblings BAB2389 and 2390 (from family HOU893), have a duplication estimated to be ~4 Mb in size, and their duplication was not cytologically visible; BAB1327 was not analyzed by G-banding.

Interestingly, there appears to be a subgroup of 15 patients (12 families) who have similar proximal and/or distal breakpoints at a BAC/PAC resolution (Figs 3 and 4). This subgroup represents ~71% of our patient cohort (15/21), or 71% of families (12/17), and ~72% of breakpoints (23/32) for the families we report herein. For this subgroup, custom array CGH often revealed four to five BAC clones duplicated (~650 kb), generally including BACs RP11-1123D8, RP11-1144F22, RP11-832L2 (PLP1), RP11-34P3 and RP11-462K21 (Fig. 3). Some end clones appear to be partially duplicated because of their intermediate signal intensity, indicating that the breakpoint likely occurs within that particular clone (duplication end clone), whereas others are completely duplicated, suggesting that the breakpoint either occurs within that clone, and/or within the adjacent overlapping clone (duplication end-clone pair).
Breakpoint groupings are coincident with complex architecture

After determining which of the custom array CGH clones contain LCRs (Table 1), we sought to determine if the breakpoints of the above subgroup of PMD patients who appear to have similar proximal and/or distal breakpoints at a BAC/PAC resolution (Figs 3 and 4) occur within or near LCR-containing clones to test the hypothesis that LCR–PMDs are stimulating, but not necessarily mediating, these rearrangements. From this analysis, we identified the following LCR-containing clones at or near the breakpoints: RP11-1123D8 (LCR–PMDE2),
RP11-34P3 (LCR–PMDC) and RP11-462K21 (LCR–PMDA, B, and D), which range from 132 to 192 kb in length (average being 146.5 kb). The latter clone is also found at the distal breakpoint for some larger-sized PLP1 duplications. In a BLAST 2 analysis of the individual breakpoint-spanning clones (Fig. 3), we did not find any additional stretches of 200 bp that shared 85% identity.

We calculated the number of breakpoints that occur within an LCR-containing BAC or PAC duplication end clone or duplication end-clone pairs, versus the number of breakpoints that occur within non-LCR clones. Considering only families with at least one similar breakpoint, we determined that 92% of proximal breakpoints (11/12) and 92% of distal breakpoints (11/12) occur within LCR-containing duplication end clones or duplication end-clone pairs. Considering both proximal and distal breakpoints together, 92% of the breakpoints (22/24) occur within LCR-containing duplication end clones for this subgroup. Analyzing the aggregate data from all families in our cohort, 71% of proximal breakpoints (12/17) and 87% of distal breakpoints (13/15) occur in an LCR-containing end clone or end-clone pair. We included only 15 total distal breakpoints (and 32 total breakpoints) because the duplications in two patients (BAB2396 and 2425; Fig. 3) extend distally beyond the genomic region detected by our custom array.

On our array, 14 of the 68 tiled BAC and PAC clones contain LCR–PMDs (Table 1). When we compared the frequency of families with at least one breakpoint in an LCR-containing end clone or end-clone pair (12/17 = 0.7059) with the frequency of LCR-containing clones on the full-length array (14/68 = 0.2059), the observed frequency is significantly higher, with a $P$-value of $1.255 \times 10^{-5}$. The $P$-value was determined by performing a binomial test of the proportion

**Table 1. LCR–PMDs from previous and present in silico analyses**

<table>
<thead>
<tr>
<th>LCR–PMD</th>
<th>BAC(s) within</th>
<th>Size (kb)</th>
<th>Position (Build 35)</th>
<th>Orientation</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA1a</td>
<td>RP11-462K21</td>
<td>18.5</td>
<td>103,029,820-103,048,307</td>
<td>(+)</td>
</tr>
<tr>
<td>AA2</td>
<td>RP11-462K21</td>
<td>10.3</td>
<td>103,064,260-103,074,533</td>
<td>(-)</td>
</tr>
<tr>
<td>BA3</td>
<td>RP11-462K21</td>
<td>13.9</td>
<td>103,096,154-103,110,142</td>
<td>(+)</td>
</tr>
<tr>
<td>BA4b</td>
<td>RP11-462K21</td>
<td>18.5</td>
<td>103,111,984-103,130,746</td>
<td>(-)</td>
</tr>
<tr>
<td>C</td>
<td>RP11-34P3</td>
<td>34.8</td>
<td>102,978,791-103,013,644</td>
<td>(+)</td>
</tr>
<tr>
<td>D</td>
<td>RP11-462K21</td>
<td>26.9</td>
<td>103,137,452-103,164,433</td>
<td>(-)</td>
</tr>
<tr>
<td>E1</td>
<td>RP11-155E24</td>
<td>2.4</td>
<td>101,998,037-102,000,480</td>
<td>(+)</td>
</tr>
<tr>
<td>E2</td>
<td>RP11-1123D8</td>
<td>2.4</td>
<td>102,559,737-102,562,186</td>
<td>(-)</td>
</tr>
<tr>
<td>F1</td>
<td>RP11-461E24</td>
<td>121.9</td>
<td>101,276-701-101,398,620</td>
<td>(+)</td>
</tr>
<tr>
<td>F2</td>
<td>RP11-669M24</td>
<td>121.9</td>
<td>101,410,623-101,532,603</td>
<td>(-)</td>
</tr>
<tr>
<td>F3</td>
<td>CTD-2006G2</td>
<td>8.4</td>
<td>100,898,811-100,907,221</td>
<td>(-)</td>
</tr>
<tr>
<td>F4</td>
<td>CTD-2131P19</td>
<td>1.4</td>
<td>101,625,289-101,626,682</td>
<td>(+)</td>
</tr>
<tr>
<td>F5</td>
<td>RP11-106F13</td>
<td>1.3</td>
<td>102,141,879-102,143,193</td>
<td>(-)</td>
</tr>
<tr>
<td>G1</td>
<td>RP11-165B8, RP11-461C20</td>
<td>11.3</td>
<td>101,234,198-101,245,515</td>
<td>(+)</td>
</tr>
<tr>
<td>G2</td>
<td>RP11-461C20</td>
<td>4.8</td>
<td>101,282,660-101,287,458</td>
<td>(+)</td>
</tr>
<tr>
<td>H1</td>
<td>RP11-165B8</td>
<td>0.9</td>
<td>101,187,082-101,187,979</td>
<td>(+)</td>
</tr>
<tr>
<td>H2</td>
<td>RP11-165B8</td>
<td>0.9</td>
<td>101,202,419-101,203,313</td>
<td>(+)</td>
</tr>
<tr>
<td>I1</td>
<td>RP11-661I7</td>
<td>4.5</td>
<td>100,658,634-100,663,108</td>
<td>(+)</td>
</tr>
<tr>
<td>I2</td>
<td>RP11-661I7</td>
<td>4.5</td>
<td>100,672,941-100,677,415</td>
<td>(+)</td>
</tr>
<tr>
<td>J1</td>
<td>RP11-15E22</td>
<td>0.7</td>
<td>100,557,050-100,557,755</td>
<td>(+)</td>
</tr>
<tr>
<td>J2</td>
<td>RP11-15E22</td>
<td>0.7</td>
<td>100,619,928-100,616,623</td>
<td>(+)</td>
</tr>
<tr>
<td>K1</td>
<td>RP11-15E22</td>
<td>0.6</td>
<td>100,621,785-100,622,389</td>
<td>(+)</td>
</tr>
<tr>
<td>K2</td>
<td>RP11-15E22</td>
<td>0.7</td>
<td>100,689,999-100,690,668</td>
<td>(+)</td>
</tr>
</tbody>
</table>

LCR-containing BAC clones listed are from those on our CGH array. Sets of LCRs are designated by LCR-PMD letter, with the following previously published exceptions: A and B are a set of four LCRs (18), and C and D are a set of two LCRs (19). Relative orientation within each set of related LCRs assigned as (+) or (−). LCRs and BACs in italics are those which are present at/near the breakpoints in a subset of patients with similar breakpoints at a BAC/PAC resolution.

**Table 2. Cohort of patients included in the present study**

<table>
<thead>
<tr>
<th>Family (HOU#)</th>
<th>Patient (BAB#)</th>
<th>Sex</th>
</tr>
</thead>
<tbody>
<tr>
<td>485</td>
<td>1258&lt;sup&gt;d&lt;/sup&gt;</td>
<td>M</td>
</tr>
<tr>
<td>487</td>
<td>1263&lt;sup&gt;d&lt;/sup&gt;</td>
<td>M</td>
</tr>
<tr>
<td>587</td>
<td>1482&lt;sup&gt;b&lt;/sup&gt;</td>
<td>M</td>
</tr>
<tr>
<td>519</td>
<td>1334&lt;sup&gt;a&lt;/sup&gt;</td>
<td>M</td>
</tr>
<tr>
<td>674</td>
<td>1705&lt;sup&gt;a&lt;/sup&gt;</td>
<td>M</td>
</tr>
<tr>
<td>503</td>
<td>1305&lt;sup&gt;d&lt;/sup&gt;</td>
<td>M</td>
</tr>
<tr>
<td>501</td>
<td>1301&lt;sup&gt;d&lt;/sup&gt;</td>
<td>M</td>
</tr>
<tr>
<td>944</td>
<td>2448&lt;sup&gt;b&lt;/sup&gt;</td>
<td>M</td>
</tr>
<tr>
<td>486</td>
<td>1261&lt;sup&gt;c&lt;/sup&gt;</td>
<td>M</td>
</tr>
<tr>
<td>494</td>
<td>1282&lt;sup&gt;d&lt;/sup&gt;</td>
<td>M</td>
</tr>
<tr>
<td>491</td>
<td>1275&lt;sup&gt;d&lt;/sup&gt;</td>
<td>M</td>
</tr>
<tr>
<td>497</td>
<td>1290&lt;sup&gt;d&lt;/sup&gt;</td>
<td>M</td>
</tr>
<tr>
<td>561</td>
<td>1420</td>
<td>M</td>
</tr>
<tr>
<td>893</td>
<td>2389&lt;sup&gt;c&lt;/sup&gt;</td>
<td>M</td>
</tr>
<tr>
<td>924</td>
<td>2396</td>
<td>M</td>
</tr>
<tr>
<td>516</td>
<td>1327&lt;sup&gt;c&lt;/sup&gt;-&lt;sup&gt;d&lt;/sup&gt;</td>
<td>M</td>
</tr>
<tr>
<td>936</td>
<td>2425</td>
<td>F</td>
</tr>
</tbody>
</table>

<sup>a</sup>Siblings within the same family, as listed by HOU#.
<sup>b</sup>Cousins within the same family.
<sup>c</sup>Larger duplication patients (at least ~4 Mb).
<sup>d</sup>Clinical details previously published (14).
<sup>e</sup>Clinical details previously published (47).
of observed breakpoints in LCR-containing end clones or end-clone pairs, and the $P$-value is exact.

Probes adjacent to LCRs detect junction fragments

To further investigate whether these LCRs may stimulate the genomic rearrangements resulting in $PLP1$ duplication, we examined for an association between breakpoint genomic position and co-localization of an LCR. For this, we employed pulsed-field gel electrophoresis (PFGE) analysis followed by hybridization with a series of DNA probes complementary to unique sequences adjacent to LCRs neighboring $PLP1$ (Fig. 5). We examined 15 families in this analysis with two different endonuclease digestions and up to 10 different hybridization probes, which resulted in multiple independent hybridizations per family and a scan that covers a total of ~2 Mb spanning the $PLP1$ genomic region. We detected recombination-specific junction fragments in 12 patients in 10 families (Table 3). Similar sized junction fragments, within ~100 kb in size, were observed in eight of 10 families. For example, for hybridization with Distal A probe after $Pmelo$ digestion, 375 kb junction fragments were observed for families HOU587 and 519; 295 kb junction fragments were observed for families HOU674, 503, 497 and 561; 275 kb junction fragments were observed for families HOU486 and 494 (Table 3). In four patients from three families for which complete restriction mapping by PFGE was possible (Fig. 6), we mapped both the proximal and distal ends of
the \textit{PLP1} duplications. Of all the hybridization probes tested, only Proximal A, B, C, G and Distal A probes identified junction fragments. Distal Probe A is located just proximal to LCR–PMDA (Fig. 5).

For family HOU587, as anticipated, we observed segregation of the junction fragment for all affected and carrier family members, whereas the unaffected male BAB1754 showed only the expected normal band (Fig. 6). Hybridization with Proximal G probe and Distal A probe yielded junction fragments after both \textit{PmeI} and \textit{NruI} enzyme digestions, whereas hybridization with Proximal C probe yielded junction fragments after \textit{NruI} digestion. In all junction-positive members of family HOU587, the junction fragments were larger than the expected band sizes, indicating that for the junction fragment, the restriction enzyme sites flanking the hybridization probe are further apart than on non-rearranged DNA (Fig. 5). By restriction enzyme mapping and comparative analysis with reference genome sequence, the duplication is in tandem and is direct in orientation.

In families HOU486 and HOU494, hybridization with Proximal C and Distal A probes yielded junction fragments after \textit{PmeI} digestion, and with Proximal C and G and Distal A probes after \textit{NruI} digestion. We observed junction fragments of larger than normal-sized bands expected based on restriction maps (Fig. 5) after \textit{PmeI} digestion as described earlier for family HOU587, but of smaller size than the...
normal bands after NruI digestion, indicating that for the junction fragment, the NruI sites flanking the hybridization probe are closer together than on non-rearranged DNA. By restriction analysis for family HOU494, the duplication is consistent with being directly oriented, but appears to be interrupted, and inserted nearby the original reference sequence. Interestingly, we observed the absence of bands of expected sizes based on restriction maps in family HOU486, and also in families HOU503 and 561, and instead saw junction fragments only, suggesting that more complex rearrangements have occurred, and that more than one breakpoint may have occurred within the same restriction fragment. For families HOU486 (BAB1261) and HOU503 (BAB1305), the distal breakpoints determined by PFGE align with that found by array CGH analysis. However, for family HOU561 (BAB1420), array CGH analysis detected a duplication that extends three more BAC clones (~500 kb) distal to that predicted by PFGE analysis, also consistent with a complex rearrangement. We detected no bands for family member BAB1698 (HOU486) for hybridization with

Table 3. Summary of band sizes (in kb) observed for PLP1 duplication patients tested for hybridization with Proximal B, C, and G and Distal A probes

<table>
<thead>
<tr>
<th>Family</th>
<th>Patient</th>
<th>Proximal B probe</th>
<th>Proximal C probe</th>
<th>Proximal G probe</th>
<th>Distal A probe</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PmeI NruI</td>
<td>PmeI NruI</td>
<td>PmeI NruI</td>
<td>PmeI NruI</td>
</tr>
<tr>
<td>HOU587</td>
<td>BAB1482</td>
<td>197 ND</td>
<td>113,136</td>
<td>480</td>
<td>216,375 184,480</td>
</tr>
<tr>
<td>HOU519</td>
<td>BAB1334</td>
<td>197 ND</td>
<td>113,136</td>
<td>480</td>
<td>216,375 184,480</td>
</tr>
<tr>
<td>HOU674</td>
<td>BAB1705</td>
<td>197 ND</td>
<td>ND</td>
<td>ND</td>
<td>216,295 184,400</td>
</tr>
<tr>
<td>HOU503</td>
<td>BAB1305</td>
<td>197 ND</td>
<td>113,136</td>
<td>ND</td>
<td>216,295 184,400</td>
</tr>
<tr>
<td>HOU501</td>
<td>BAB1301</td>
<td>197 ND</td>
<td>113,136,190</td>
<td>ND</td>
<td>216 ND</td>
</tr>
<tr>
<td>HOU486</td>
<td>BAB1261</td>
<td>197 ND</td>
<td>113,115,127,136</td>
<td>650</td>
<td>216,275 100,184</td>
</tr>
<tr>
<td>HOU494</td>
<td>BAB1282</td>
<td>197 ND</td>
<td>90,113,115,136</td>
<td>650</td>
<td>216,250 100,184</td>
</tr>
<tr>
<td>HOU491</td>
<td>BAB1275</td>
<td>197,210 ND</td>
<td>113,136</td>
<td>ND</td>
<td>216 ND</td>
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<tr>
<td>HOU497</td>
<td>BAB1290</td>
<td>197 ND</td>
<td>113,136</td>
<td>ND</td>
<td>216,295 100,184</td>
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<tr>
<td>HOU561</td>
<td>BAB1420</td>
<td>197 ND</td>
<td>ND</td>
<td>ND</td>
<td>216,295 100,184</td>
</tr>
</tbody>
</table>

Hybridization with all other probes in the probe series yielded expected band sizes. Band sizes in italics indicate approximate junction fragment lengths; all others indicate observed bands of expected sizes. ‘ND’ signifies not determined. All band sizes listed are estimated based on migration with a yeast or lambda DNA size marker.

Figure 5. Probes adjacent to LCRs detect apparent junction fragments. Block arrows proximal to PLP1 (red circle) centromeric (cen) to telomeric (tel) represent LCR–PMDE1, F5 and E2, respectively, which were identified by large-scale genome sequence analysis as described. Block arrows distal to PLP1 include LCR–PMDA and B in dark blue and light blue, respectively, which are flanked by LCR–PMDC (yellow) and LCR–PMDD (dark green). Proximal A through G probes were designed proximal to PLP1, whereas the Distal A, B and D probes were designed distal to PLP1. The positions where probes hybridize are shown as small red squares. The upper and lower panels depict the restriction maps for enzymes PmeI, ‘P,’ and NruI, ‘N,’ respectively. The expected band sizes by Southern hybridization with a particular probe and restriction enzyme combination are specified in kilobases below each restriction map. ‘X’ indicates that NruI does not cut at the expected site, likely because of its CpG methylation sensitivity. Positions are given relative to the PLP1 gene in megabases.
the Distal A probe after \textit{NruI} digestion, which is possibly due to mutation affecting the \textit{NruI} restriction pattern in this individual, as bands are otherwise able to be visualized by hybridization with other probes. A summary of the results of our PFGE analysis of patients in families HOU587, 486 and 494 is illustrated in Figure 7, highlighting LCR–PMDE2 found adjacent to where Proximal C and G probes hybridize, and LCR–PMDA found adjacent to where Distal A probe hybridizes.

Diverse duplication breakpoints with grouping near complex genomic architecture supported by recombinant junction analysis

Earlier, we showed by array CGH that duplications have diverse breakpoints, but that they occur in groups coincident with LCRs. Furthermore, in a prospective approach, we used unique sequence probes adjacent to LCRs in Southern analysis, after separation of high molecular weight genomic DNA by PFGE, to detect junction fragments associated with the rearrangement breakpoints. To examine an independent data set to determine whether or not there was any evidence for grouping among the diverse breakpoints, we utilized the recently published PMD duplication recombinant junction sequence analysis by Woodward et al. (19). This enabled us to position \textit{PLP1} duplication breakpoints precisely on the X chromosome genomic sequence to examine for breakpoint grouping and potential association with LCRs. Indeed, we found an apparent grouping of breakpoints coincident with LCRs (Fig. 8). In fact, \~{}62\% of the proximal breakpoints (8/13) and \~{}79\% of the distal breakpoints (11/14; one patient has two sequenced distal breakpoints) occur within or near LCR-containing BAC clones. Overall, \~{}77\% of patients (10/13) in their data set had at least one breakpoint within or near an LCR-containing BAC clone. In common, with our subset of patients with at least similar breakpoints at a BAC/PAC resolution, the proximal breakpoints of their data set (6/13 patients) occurred within or near BAC clone RP11-1123D8 (LCR–PMDE2), and the distal breakpoints of their data set (9/13 patients and 3/13 patients) occurred within or near BAC clones RP11-34P3 (LCR–PMDC) and RP11-462K21 (LCR–PMDA, B, and D), respectively. In addition, two of their patients had proximal breakpoints within or near BAC clone RP11-155E24 (LCR–PMDE1).

The duplications are directly oriented

The PFGE restriction patterns we observed for families HOU486, 503 and 561 prompted us to investigate duplication orientations in all available patients. Two-color FISH analysis
for each patient revealed an alternating red-green-red-green signal order consistent with directly oriented duplications in patients BAB1305, 1301, 1261, 1282, 1275, 1290, 1420, 2389, 2390, 2396 and 2425, as had been observed previously (14). For patients BAB1482, 1597, 1334, 1705 and 1707, the furthest duplicated clones, RP11-1144F22 and RP11-34P3, had only 51 kb of genomic sequence between them, and thus the fluorescent signals could not be reliably distinguished by interphase FISH. Representative data for the direct duplications are shown in Figure 9. For all unaffected male and female control chromosomes, we observed only a red-green signal pattern (data not shown).

No evidence for an association with copy-number variation in LCR–PMDA and B region

We previously identified copy-number variation (CNV) within the LCR–PMDA and B region within a Caucasian control population by a quantitative real-time PCR assay (29). We sought to determine if our PMD patients or family members have CNV in this region in order to investigate CNV as a potential further genomic susceptibility factor for rearrangements. Using the same quantitative real-time PCR assay (29), we evaluated all available PMD patients and family members for CNV in the LCR–PMDA and B region. The results of our dosage assay (data not shown) were consistent only with either PLP1 duplication or duplication carrier status, and were consistent between patient and mother, as determined previously by FISH and PFGE analyses. Thus, there was no evidence for CNV in the interrogated region in this cohort of patients and family members. These real-time PCR results were also informative for breakpoint analysis in some patients, allowing us to either include or exclude the interrogated region as duplicated or not duplicated, respectively (data not shown).

DISCUSSION

PLP1 duplications causing PMD have been reported as varying in size with scattered breakpoints and lack of homology reported at the actual breakpoints (14,30). For both PLP1 deletions and duplications causing PMD, recombinant junction sequence analysis revealed a lack of homology and the addition of nucleotides at the breakpoints, consistent with recombination occurring by an NHEJ mechanism (18,19,31). Moreover, NHEJ has been implicated recently as an alternative mechanism responsible for a number of human genomic disorders because of non-recurrent rearrangements with scattered breakpoints (22,23,32). On the basis of the following observations, we suggest that the LCRs not only render this genomic region unstable and susceptible to
rearrangements, but that they are in fact, stimulating these genomic rearrangements.

Genomic architecture of the PLP1 region is complex

By in silico genome sequence analysis, there is no gap in sequence information between LCR–PMDA and B, unlike what we previously published based on the then current draft sequence information. According to our FISH mapping data (14), the distal breakpoint ends of PLP1 duplications in most PLP1 duplication families tested grouped at or near LCR–PMDA and B, a complex arrangement of four LCRs of varying shared sequence identities and different relative orientations (Figs 1 and 2; Table 1), whereas the proximal breakpoints were more scattered. These data prompted us to perform an extensive in silico genome sequence analysis described herein that uncovered many LCRs scattered proximal to PLP1, and fewer distal to PLP1. Interestingly, the locations of scattered LCRs within 2.3 Mb of sequence proximal to PLP1 correlates with the scattered proximal breakpoints previously reported in PLP1 duplication patients, and the clustering of LCRs distal to PLP1 correlates with the grouped distal breakpoints previously reported (Fig. 1).

Figure 8. Comparative analysis of an independent data set (19) reveals apparent grouping of breakpoints. The region detected by array CGH is shown on the top, followed by the duplications found in our patients by array CGH in condensed form (Fig. 3). Below are the duplications reported by Woodward et al., and their alignment within our ~10 Mb array detection region. For this independent data set, patients are shown on the left; duplication breakpoints are shown as vertical arrows, whereas the duplicated segments are shown as bold black lines between the arrows. Similar breakpoint regions in a subset of our patients are delineated by vertical dotted lines.

LCR–PMDs are associated with duplication breakpoints in a subset of patients

Although duplication breakpoints proximal to PLP1 have been shown to be scattered, array CGH analysis on our cohort of PLP1 duplication patients surprisingly revealed that there is a subgroup of duplication patients who have similar proximal and/or distal breakpoints at a BAC/PAC resolution (Figs 3 and 4), accounting for ~71% of our patient cohort (15/21) or 71% of families (12/17). Furthermore, when our array CGH breakpoint mapping data were aligned with LCR data generated from in silico analysis, we found that the breakpoints in 12 families aligned with the locations of LCR–PMDs with statistical significance.
results for the direct duplications we observed. cated segments are shown in each respective insert. These data are typical analyses of unrelated male duplication patients. Enlargements of the dupli-
of a male duplication patient, whereas (as shown by array CGH. (P
probe labeled in green is the most distal fully duplicated clone (if available)
is the most proximal fully duplicated clone (if available), whereas the BAC
order is shown as (P,
for two-color FISH analysis for duplication orientation are shown. The signal
red-green-red-green for inverted duplication and (P
red-green-red-green for direct duplication and (P
for PFGE analysis, we were able to confirm that the LCRs
in the PLP1 region are associated with breakpoints of duplications causing PMD. Using probes that map in prox-
imity to LCRs and target suspected proximal and distal break-
point regions, we identified junction fragments in 12 patients in 10 families; in four of them, junction fragments were ident-
ified on both the proximal and distal ends of the duplicated
segments. These four patients are included in the subgroup
of patients who have similar proximal and distal breakpoints
at a BAC/PAC resolution. The Distal A hybridization probe,
designed adjacent to LCR–PMDA and B, detected distal
junction fragments in the majority of patients tested,
whereas multiple hybridization probes mapping proximal to
PLP1 were informative, consistent with the pattern of LCRs
we find in this region. In other words, a majority of distal
breakpoints occur near or within LCR–PMDA and B in our
patients, whereas the proximal breakpoints occur near
various LCR–PMDs. Junction fragments could not be
resolved for patients with particularly large or small duplica-
tions under the separation conditions and by the restriction
enzymes used. In addition, if a duplication proximal break-
point occurred within LCR–PMDE2 or within the 9.5 kb
between the Pmel sites flanking LCR–PMDE2, the resulting
junction fragment would only be able to be detected after
NruI digestion, unless conventional Southern analysis were
performed. Our PFGE data presented herein suggest an associ-
ation between breakpoints and proximal and distal LCRs,
implying genome architecture in the rearrangement events
leading to PLP1 duplications in some PMD patients.
Moreover, when we investigated the potential grouping of
breakpoints in an independent data set published by Wood-
ward et al., in which recombinant junctions were sequenced
(19), we also found that indeed a majority (10/13) of their
patients had at least one breakpoint within or near
LCR-containing BAC clones. In particular, similar to the
findings reported herein, we found an association between
LCR–PMDE1 and E2 and proximal breakpoints, and
between LCR–PMDA, B, C, and D and distal breakpoints
in their data set.

PLP1 duplications may share a common mechanism

PLP1 duplications resulting in PMD generally occur in
tandem by an intra-chromosomal event in an ancestral male
during gametogenesis (14,21,33). Intra-chromosomal events
having occurred in an ancestral male are also responsible for
duplications causing Duchenne muscular dystrophy (34) and
PLP1 deletions (18).

In analyzing PFGE data, the placement of hybridization
probes relative to the restriction sites can be informative for
determining breakpoint locations of rearrangements (35). By
PFGE analysis, we observed similar size-junction fragments
for some unrelated patients, possibly indicating that they
share a common breakpoint region, or at least that the break-
points occur within a similar genomic vicinity. Our array CGH
analysis revealed that the breakpoints are indeed similar for a
subset of patients. Also, our restriction analysis by PFGE
showed that the rearrangements in some of our patients are
consistent with directly oriented duplications. Likewise, our
two-color FISH analysis revealed that the duplications in all
patients tested were direct in orientation. The commonalities
observed in the junction fragment sizes, breakpoint locations
and duplication orientations suggest that a common mechan-
ism may be responsible for the duplication rearrangements
in these patients.

The PLP1 region is prone to rearrangement

Not only have patients with PMD resulting from PLP1 dupli-
cation, deletion or point mutations been described, but other
DNA rearrangements have also been shown to be causative.
PMD cases in which PLP1 has behaved as a sub-microscopic
transposon have been reported (30,36), including one in which all the genetic material including the centromere was inverted between two intact PLP1 copies (30). Whether inversions are the cause of such duplications, or if duplications have led to these inversions by mispairing is difficult to determine. In addition, extra copies of PLP1 have been reported to be found on some autosomes (18,37). Interestingly, in some patients, three or more copies of PLP1 have been reported (38), including partial triplications and partial deletions as well (39). We report herein at least one patient (BAB1282) with an interrupted direct duplication, and at least three patients (BAB1305, 1261, 1420) with potentially complex duplication rearrangements as shown by the absence of normal-sized bands by PFGE analysis, one of which (BAB1420) has a duplication detected by array CGH that extends three more BAC clones (~500 kb) distal to that predicted by PFGE analysis. Considering that the region surrounding PLP1 contains numerous LCRs based on in silico analysis reported herein, and that repeat sequence confers genome instability (1,40), the genomic architecture likely renders the DNA in this region highly susceptible to duplication and other rearrangements causing PMD.

Particular LCR–PMDs may be hotspots for stimulating PLP1 duplications

It appears that the region containing LCR–PMDA and B is possibly a hotspot for stimulating rearrangements (18), including those leading to CNV of the LCR–PMDA and B region in normal individuals as we recently described (29). CNV in the LCR–PMDA and B region was excluded in this study, in all available PMID patients and family members. Besides LCR–PMDA and B, LCR–PMDE2, which is the closest LCR proximal to the PLP1 gene and is adjacent to PFGE Proximal C and G probes (Fig. 5), may also play a role in stimulating PLP1 duplication events, as we report rearrangement proximal breakpoints having occurred near this sequence. Therefore, we suggest that rearrangements stimulated by both of these particular LCR regions together, proximally and distally, in the same event result in duplications of relatively similar size in some patients. Likewise, other pairs of LCRs in this region may stimulate rearrangement together, proximally and distally, in other PMID patients, resulting in the various sizes of PLP1 duplications that we observe. The underlying genomic architecture may also be responsible for the grouping of similar duplication size superimposed on the diversity, perhaps reflecting apparent breakpoint-prone genomic regions.

Model for PLP1 duplication rearrangements

We suggest that LCR–PMDs may be hotspots for stimulating rearrangements, possibly by acting together in the same rearrangement event, and thus render the DNA surrounding the PLP1 gene unstable and susceptible to rearrangements causing PMD. Interestingly, although most PLP1 duplications have been reported to be oriented head-to-tail in tandem, we report three potentially complex rearrangements and one interrupted duplication which appears to be inserted near the original reference sequence. Similarly, PLP1 duplications have been reported to act sometimes as transposons (30,36). We propose a general model (Fig. 10), which incorporates the following: the role of LCR–PMDs in rendering the DNA susceptible to double-strand breaks (DSBs), a mechanism of coupled one-sided homologous single-strand invasion and repair by NHEJ. This model can explain duplications and deletions as well as intra- and inter-chromosomal transposition of the duplicated PLP1 segment and complex rearrangements.

In this model (Fig. 10), the rearrangement originally occurs during gametogenesis in the ancestral male (33). We propose this event occurs by the induction of a single DSB stimulated by a nearby LCR–PMD. Much like long tract gene conversion (LTGC) (41), and as recently discussed by Woodward et al. (19) in the context of PLP1 duplications, a one-sided single-strand invasion by the broken chromatid occurs in which the sister chromatid is copied. Consistent with what has been found for other PLP1 duplications (19) and deletions (18), this event is completed by a NHEJ repair mechanism (42).

However, for the cases in which the duplicated segment becomes transposed and inserted either intra-chromosomally, such as the case of interrupted direct duplication inserted close by which we report herein, or inter-chromosomally to autosomes or the Y chromosome, we propose that a second DSB may be stimulated by another nearby LCR–PMD in trans, followed by transposition of the duplicated segment to a secondary chromosomal location and repair by NHEJ. Furthermore, our model may also help to account for some complex PLP1 duplication rearrangements (19), for which more than one proximal or distal breakpoint is predicted. These rearrangements may be explained by part(s) of the duplicated segment being deleted and/or inserted, in a direct or inverted orientation, at either the primary rearrangement site or elsewhere in the genome. For such rearrangements, we hypothesize that at least two LCR–PMDs stimulate at least two DSBs, or potentially greater than two in more complex rearrangements, breaking the duplicated segment into fragments that may become either lost or integrated by NHEJ either intra- or inter-chromosomally, conceivably in inverted or direct orientations and not necessarily in tandem.

CONCLUSIONS

We illustrate that the genomic architecture of the region surrounding PLP1 is complex, as it contains numerous LCR–PMDs, especially proximal to PLP1 (Fig. 1). Our data suggest that genomic architectural features, namely LCRs, may stimulate although not necessarily mediate the genomic rearrangements responsible for the majority of PMID cases, as LCRs were identified near the breakpoint regions in a majority of our patients. Specifically, genomic architecture may play an important role in rendering DNA in the PLP1 region more susceptible to rearrangement, resulting not only in duplications of varying size, but also in the grouping of some duplications of apparently similar size. Duplication rearrangements may be quite common in the human genome; however, only when they result in ascertainable
human disease phenotypes are they likely to be discovered. PLP1 duplication rearrangements causing PMD represent one example of many genomic rearrangements causing human disease that are stimulated by complex genomic architecture in the region surrounding the causative dosage-sensitive gene.

**MATERIALS AND METHODS**

**In silico analysis of the PLP1 region**

To define the genomic architecture in the Xq22 region that potentially facilitates the variable PLP1 duplication events, we performed in silico genome sequence analysis covering 8 Mb of repeat-masked DNA sequence from NCBI draft build 35 surrounding the PLP1 region by BLAST 2 analysis using default parameters (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi) (43); DNA sequences were obtained from the UCSC Genome Browser (http://genome.ucsc.edu/) (44,45). Specifically, this analysis was performed by dividing the entire analyzed region into ∼1 Mb segments; the sequence for each ∼1 Mb segment was then compared with itself and with each of the other segments by BLAST 2 to identify LCR sequence and genomic architecture within the analyzed region. We compiled these LCR data from the present analysis together with LCR–PMDA through D (18,19). The sizes of the LCRs detected may be underestimated because each individual identity match does not include interspersed repetitive sequences and low complexity DNA sequences. Additional BLAST 2 analyses of breakpoint clones were performed to further assess for LCRs.

**Patients**

We obtained samples from 21 PMD patients with PLP1 duplication and their family members (BAB#) in 17 families.
Custom array CGH

We prepared and printed DNA from either 38 or 68 BAC and PAC clones tiling an ~5 or ~10 Mb region surrounding PLP1, respectively, in addition to control clones from chromosomes 1, 2, 5, 10, 17, X and Y, as described (47). We isolated patient and control genomic DNA either by phenol/chloroform extraction from human cell lines or from peripheral blood lymphocytes by standard methods for all patients except for patients of BAB2448 and 2390. We performed FISH to confirm PLP1 duplication status as described (14,46).

Pulsed-field gel electrophoresis

We harvested intact lymphoblastoid cells from PMD patient and control individuals and resuspended them in 1% InCert agarose (BMA). We digested DNA in agarose plugs from 5 \times 10^6 cells with PmeI or NruI restriction enzymes for 2 days at 37°C (New England Biolabs), followed by separation ranging from 10 kb to 1 Mb on a 1% agarose gel (Bio-Rad) in 0.5X Tris–Borate–EDTA buffer using a Bio-Rad CHEF Mapper. All samples co-migrated with a yeast or lambda chromosome size marker (New England Biolabs). We visualized separated DNA by ethidium bromide (Sigma) staining.

Probe design and preparation

We designed a series of DNA probes to target regions adjacent to the LCRs in the PLP1 region revealed by in situ analysis. Also, we designed additional probes to obtain better coverage. Primer sequences for DNA probes proximal (centromeric) to PLP1 include: Proximal A, 5’-AGATGATTCCCTGCCCC ACTAC-3’ and 5’-GTAGCAAGTACAGGCTTGGACTAC C-3’; Proximal B, 5’-ACATCTTACACCCACTGATAC-3’ and 5’-GAGGCAATGTATCTGCAAGCC-3’; Proximal C, 5’CCGCAAGATTTCACGCAT-3’ and 5’-CTTTTGGCT C ATGCAGAGGG-3’; Proximal D, 5’-GAGTCTACACCTCTTG TCTTTGCTT-3’ and 5’-AGTGTGTCACATTGGGA G-3’; Proximal E, 5’-GTAGTTTAAAGGAGCCAAGG TCT C-3’ and 5’-GAGGAGAGACTGCAAGGATAG; Proximal F, 5’-CCATCTGCTACTGCAACCTAC-3’ and 5’-GCT GTCACAATACCGGGGATCTAC-3’; Proximal G, 5’-CC CAGTCTTGAACCTATGGACAG-3’ and 5’-AGGAGTCA GTCTGGTGAAGATG-3’. Primer sequences for DNA probes distal (telomeric) to PLP1 include: Distal A, 5’-GAA TGTGGTCAAGTTACCTAGGATT-3’ and 5’-GCAGAGAT GAAATTGGCATGA-3’; Distal B, 5’-ACTGGAAGGCT ACTCAAACAC-3’ and 5’-GAGGTAAGCTGACATGT GTGAC-3’; and Distal D, 5’-GGTGGACCGTGTATTCT CCTC-3’ and 5’-GTCTCTGCTCAAGTGGAGTAGT-3’. We amplified these DNA probes by PCR from PAC clone templates prepared by standard alkaline lysis followed by phenol/chloroform extraction, and subsequently isolated the probe DNA by gel extraction (Qiagen).

Southern hybridization

Following the separation of PMD patient and control DNA by PFGE, we transferred DNA to positively charged Sure Blot Nylon Membranes (Intergen Company) by standard methods for 2 days. We labeled ~200–250 ng of probe DNA with 32P-dCTP (MP Biomedicals) by random priming for at least 2 h at 37°C (Roche). We pre-hybridized membranes with salmon sperm DNA (Sigma) for 4 h and hybridized overnight at 65°C with labeled probes pre-associated with human placental DNA for 1.5–2 h. Subsequently, we analyzed hybridized blots by autoradiography for the presence of bands of expected size and also for bands of varying size, representing recombination-specific junction fragments. We stripped and rehybridized blots with different probes as necessary.

Interphase and metaphase fluorescence in-situ hybridization

We harvested cultured lymphoblastoid patient and control cells after Colcemid (Sigma) treatment by standard methods, and dropped them onto glass slides. G-banding was performed by standard methods on all patients with larger duplications except for BAB1327, including BAB2389, 2390, 2396 and 2425. We based our selection of clones to be used as FISH probes for orientation analysis on their presumed full-duplication status determined by custom array CGH described herein. For interphase FISH, we selected BAC clones RP11-1144F22 and RP11-34P3 as FISH probes for duplication orientation analysis of patients BAB1482, 1597, 1343, 1705 and 1707; clones RP11-1123D8 and RP11-462K21 for analysis of patients BAB1261, 1282, 1275 and 1290; clones RP11-1123D8 and RP11-34P3 for analysis of patients BAB1482, 1597, 1334, 1705 and 1820; clones RP11-1001M23 and RP11-34P3 for analysis of patient BAB1420. For metaphase FISH analysis of larger duplications, we selected clones RP11-1001M23 and RP11-462K21 for analysis of patients BAB2389 and 2390; clones CTD-227005 and RP11-393D6 for analysis of patient BAB2396; clones RP11-1001M23 and RP11-393D6 for analysis of patient BAB2425. If available, we analyzed the carrier mothers’ cells to provide visualization of the signal pattern on the normal X chromosome as a comparison to the duplicated X chromosome.

Each probe pair above has ~51 kb, 323 kb, 314 kb, 694 kb, 3 Mb, 4.5 Mb, and 5 Mb of genomic sequence between them, respectively. We could not test patients BAB1258, 1263 and 1264 for duplication orientation because the furthest duplicated clones, RP11-1123D8 and RP11-34P3, overlap by 84 kb. Orientation analysis was also not done for patient BAB1327. We isolated BAC clone DNA by standard alkaline lysis followed by phenol/chloroform extraction. We labeled
DNA from clones for which pairs have intervening sequence by nick translation either indirectly with digoxigenin (Roche) or biotin (Invitrogen), or directly with SpectrumRed™ or SpectrumGreen™ dUTP (Vysis Inc.). We hybridized probes to interphase and metaphase nuclei on glass slides, counterstained with DAPI (Sigma) in Vectashield (Vector Laboratories Inc.), and visualized the nuclei and metaphase chromosomes by fluorescent microscopy. The order of red and green signals observed, namely red-green-red-green versus red-green-green-red, is indicative of either a direct or inverted duplication, respectively, having occurred in the patient as previously described (48).

Real-time quantitative PCR

As previously described, we performed multiplex real-time quantitative PCR Taqman assays (Applied Biosystems) using a custom probe and primer set designed to ascertain the copy number of a unique region between LCR-PMDA and B (FAM) (29). Assays designed to test the EIF1AX gene (FAM) and the RNaseP RNA component RPPH1 (VIC) were used as X chromosome and loading controls, respectively (Applied Biosystems). Using the following reaction conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min, we amplified in triplicate 10 ng of genomic DNA. We determined DNA copy number as 2 x (dA/dT) for patients versus a male control.

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