Metachromatic leucodystrophy (MLD) in a patient with a constitutional ring chromosome 22

M B Coulter-Mackie, J Rip, M D Ludman, J Beis, D E C Cole

Abstract

Metachromatic leucodystrophy (MLD) is an autosomal recessive lysosomal storage disease resulting from a severe deficiency of arylsulphatase A. The arylsulphatase A gene is located on chromosome 22 at q13.3. An MLD patient is described who carries a common splicing mutation ("I" allele) and a de novo ring 22 deleted for the arylsulphatase A gene. The father was determined to be a heterozygous carrier of the "I" allele and the mother a heterozygous carrier of the arylsulphatase A pseudodeficiency allele. The ring 22 was shown by Southern blotting to be deleted in one copy of the arylsulphatase A gene. Minisatellite analysis showed the extent of the deletion and confirmed the biparental inheritance of chromosome 22 sequences. The carrier status of the parents and the child's 46,XX,r(22) karyotype complicated the initial diagnosis in this family. However, the causal relationship of the ring 22 and MLD have implications for the recurrence risk in this family.

Metachromatic leucodystrophy (MLD) is a neurodegenerative lysosomal storage disease resulting from a deficiency of arylsulphatase A (ASA, EC 3.1.6.1), an enzyme involved in the normal catabolism of cerebrosides sulphatide. MLD is characterised by accumulation of cerebrosides sulphatide especially in the myelin forming cells of the CNS.

The ASA gene has been mapped to chromosome 22q13.3 and its complete sequence determined. A variety of mutations have been reported. In the present context the most significant are the "I" allele and the pseudodeficiency allele. The infantile onset "I" allele consists of a composite of a G→A transition at the +1 position of intron 2 ("I-1"), a trp193cys mutation in exon 3 ("I-2"), and a thr391ser in exon 7 ("I-3"). The splicing mutation is the deleterious mutation. The exon 3 "I-2" mutation is usually found in association with "I-1" but has no effect on enzyme activity. The "I-3" is a common polymorphism found in up to 54.4% of the general population. ASA pseudodeficiency is a situation where ASA activity is significantly reduced without any clinical or biochemical evidence of disease. Pseudodeficiency carriers occur at 10 to 20% in the general population. ASA activity can be reduced to about 15% of normal in homozygotes. A complex of two mutations has been implicated in the pseudodeficiency, a glycosylation site mutation (asp350ser) affecting the third glycosylation site of the enzyme and an A→G in the first poly A addition signal. The former results in loss of a glycosylation site but appears to have little effect on ASA production or activity. The latter results in up to 80% reduction in ASA activity.

The map location of the ASA gene renders it vulnerable to deletion in chromosome 22 terminal deletions and ring formations. Indeed, several cases of such deletion of the ASA gene have been documented. However, the concomitant occurrence of ring 22 and a severe MLD allele has not previously been reported. We report such a case here. The occurrence of this chromosome rearrangement as one of the alleles in this MLD patient practically eliminates the risk of MLD in future offspring of this family.

Materials and methods

CASE REPORT

This female child was born to non-consanguineous parents. She showed delayed development in her first year and was investigated for deafness at 14 months. Unusual physical features including large ears, high forehead, upward slanting eyes, micrognathia, large extremities, and broad hands with short phalanges were noted at that time. She also had spina bifida occulta with a sacral dimple. Karyotype investigation showed a possible ring chromosome 22 in all cells examined. This was later confirmed in two subsequent karyotype studies. A partial spread of metaphase chromosomes from cultured fibroblasts is shown in fig 1. Neither parent showed the ring 22 formation. Because the child's condition was worsening more rapidly than would be expected from the ring chromosome alone, she was investigated for the possibility of metabolic errors. Enzymatic assay of arylsulphatase A in fibroblast extracts gave a diagnosis of MLD when the child was 2 years 3 months. She deteriorated rapidly becoming decerebrate by the age of 2 years 4 months and died at 2 years 10 months.

MATERIALS

Tag polymerase was purchased from Promega. ID Pol, a heat stable DNA polymerase from Thermus flavus, was obtained from ID Labs Inc (London, Ontario). Restriction enzymes were
from Pharmacia Biotech (Baie d’Urfé, Quebec), New England Biolabs (Mississauga, Ontario), or Boehringer Mannheim Canada (Laval, Quebec). [α-32P]dCTP (specific radioactivity 3000 Ci/mmol) was from Amersham Canada. MapPairs™ for minisatellite analysis were purchased from Research Genetics (Huntsville, Alabama). A USB Random Primed DNA Labelling kit was purchased from Amersham Canada.

Determination of ASA activity in fibroblast extracts

Fibroblast cultures were grown under standard conditions and harvested at about 75% confluency for assay. Cells were homogenised and cell free supernatants were used in assays with a p-nitrocatechol sulphate substrate in an assay specific for ASA (J Rip and B A Gordon, manuscript in preparation) modified from Baum et al.12

Polymerase chain reaction (PCR) amplification

The following oligonucleotide primers are from a series designed for screening for common mutations in the ASA gene and were used in the PCR amplifications described below and in table 1:

(3) 5’ CGGGATCTTCTGTGAGCAATCTGAC3’
(4) 5’ GCTCTCTGAGCTGCTCAAGGCTGC3’
(5) 5’ CTTAGGCAATTCTGGACAGGGCAAG3’
(6) 5’ CAGAGATCCAGGAGCTCTGTA3’
(7) 5’ TGTACAGGCTGCAATCTGACAGGGCTGC3’
(8) 5’ GGGCGAGGGTGCAAGGGATCCAGGCTGC3’

Restriction enzyme recognition sites were incorporated into primers 3, 4, 6, and 10 at the 5’ ends to facilitate cloning. Mismatched bases introduced in primers 11 and 12 to generate new restriction sites in the PCR product are underlined. All primers were synthesised using phosphoramidite chemistry on an automated DNA synthesiser.

PCR amplifications were performed using published procedures.13 Primers were added to a final concentration of 1 μmol/l each. Substrate DNA was added at 1 μg/100 μl. Taq and ID Pol polymerases were used interchangeably at 2-5 U/100 μl and 0-75 to 1-25/100 μl respectively. MgCl₂ concentration was at 1-5 mmol/l unless otherwise stated. Cycling parameters were: denaturation – 94°C, two minutes; annealing temperature as specified in table 1, 1-5 minutes; extension – 72°C, two minutes; 30 cycles followed by a seven minute extension at 72°C. All reaction mixtures were overlaid with mineral oil and amplification initiated by addition of polymerase to a hot (94°C) reaction mixture ("hot start"). RT-PCR was performed using fibroblast mRNA MMuLV reverse transcriptase as previously described.14

Amplification for minisatellite analysis was performed as above with incubation times shortened to 45 seconds for denaturation and 30 seconds for annealing and extension and using MapPairs™ oligonucleotides specific for chromosome 22. An annealing temperature of 60°C was used for all primer pairs except TO-PIP2 where 50°C was used. Products were labelled during the amplification with [α-32P]dCTP (approximately 0-5 μCi per 10 μl reaction volume), separated in a sequencing type polyacrylamide gel at 1500 V, and detected by autoradiography.

Southern blot analysis

DNA probes were excised from plasmids and labelled with [α-32P]dCTP by random primed labelling. Genomic DNA samples (5 μg each) were digested with HindIII, and subjected to electrophoresis in 0-8% agarose, capillary blotting in neutral buffer, and hybridisation with probe by standard methods.17

Results

ASA activity in fibroblast extracts

Fibroblast extracts from the patient, her parents, and controls were analysed for ASA ac-

![Image](https://via.placeholder.com/150)

**Figure 1** Partial spread of metaphase chromosomes. Tryptsin-giemsa chromosomes were prepared by standard cytogenetic techniques. The ring 22 is indicated by an arrow. All other chromosomes were accounted for and normal.

<p>| Table 1 | PCR primers and restriction enzymes used to detect &quot;I-1&quot; and pseudodeficiency mutations in the ASA gene |
|---|---|---|---|---|---|---|---|</p>
<table>
<thead>
<tr>
<th>Site</th>
<th>Primers</th>
<th>PCR product size (bp)</th>
<th>Annelling temperature</th>
<th>Restriction enzyme</th>
<th>Restriction site in normal sequence*</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;I-1&quot;</td>
<td>15/16</td>
<td>220</td>
<td>60°C</td>
<td>MvaI</td>
<td>+</td>
</tr>
<tr>
<td>&quot;I-1-2.7&quot;‡</td>
<td>10/4</td>
<td>738</td>
<td>65°C</td>
<td>MvaI</td>
<td>+/+</td>
</tr>
<tr>
<td>&quot;I-3&quot;glycosylation†</td>
<td>3/19</td>
<td>860</td>
<td>68°C</td>
<td>BglII</td>
<td>+/–</td>
</tr>
<tr>
<td>Poly A</td>
<td>17/11</td>
<td>147</td>
<td>65°C</td>
<td>RsaI</td>
<td>–</td>
</tr>
<tr>
<td>Glycosylation</td>
<td>3/12</td>
<td>157</td>
<td>60°C</td>
<td>MauIII</td>
<td>–</td>
</tr>
</tbody>
</table>

*Normal sequence as per accession number X52150 (EMBL).
†Simultaneous detection of the two sites.
Further support for this comes from PCR based analyses of the “I-2” and “I-3” polymorphisms and the pseudodeficiency mutations as outlined in table 1. The “I-1” and “I-2”, and the “I-3” and glycosylation mutations can be detected simultaneously (data not shown). The glycosylation mutation can also be identified independently of the “I-3”. Results for the poly A signal mutations are shown in fig 2B. The samples from the father and daughter were not cut by RsaI indicating that they were both homozygous negative for the mutation. The mother’s sample was partly cut by RsaI indicating that she is a carrier of the mutation. In summary, the father is heterozygous for all three “I” mutations and homozygous negative for the two pseudodeficiency mutations (glycosylation and poly A). The mother is homozygous negative for all three “I” mutations and a heterozygous carrier for the two pseudodeficiency mutations. The patient carries the three “I” mutations but not the two pseudodeficiency mutations. The finding of a pseudodeficiency allele in the mother accounts for her decreased level of ASA enzyme activity. Clearly none of the mother’s ASA markers, neither her pseudodeficiency mutations, nor her normal sequence at the sites of the “I” mutations, have been passed to her daughter, consistent with a complete deletion of the gene.

SOUTHERN BLOT ANALYSIS
DNA from family members was cut with restriction enzymes and subjected to agarose gel electrophoresis and Southern blot analysis. Results of HindIII digestion and hybridisation with a probe for the ASA cDNA are shown in fig 3. Visually the patient’s sample appears to be about half the density of that of the parents,

Table 2 ASA enzyme activity in fibroblast extracts*

<table>
<thead>
<tr>
<th>Sample</th>
<th>ASA activity (mmol/day/mg protein)</th>
<th>% control activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mother</td>
<td>17.01</td>
<td>44.9</td>
</tr>
<tr>
<td>Father</td>
<td>10.14</td>
<td>26.8</td>
</tr>
<tr>
<td>Patient</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.009</td>
<td>0-02</td>
</tr>
<tr>
<td>Average</td>
<td>37.9</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>22-3-49-6</td>
<td></td>
</tr>
</tbody>
</table>

*Activity measured with p-nitroacetol sulphate as described in Methods.

PCR/RESTRICTION ENZYME ANALYSIS
DNA from family members was tested for the “I-1” mutation using primers 15 and 16 and the restriction enzyme MvaI as outlined in table 1. Results shown in fig 2A clearly indicated that the father’s PCR product was partly cut by MvaI and thus he is a heterozygous carrier of this mutation. The patient’s sample appears to be homozygous for the “I-1” mutation; it was totally uncut. However, the mother’s sample was completely cut and therefore she does not carry the “I-1” mutation. This suggests that her daughter is probably deleted in one ASA allele and is actually hemizygous for the “I-1” mutation.

Figure 2 PCR/restriction enzyme analysis. Genomic DNA was amplified by PCR and subjected to MvaI digestion. (A) “I-1” allele: lane 1, father (F); lane 2, patient (P); lane 3, mother (M). A 220 bp standard is indicated to the left. The 220 bp abnormal product is not cut by MvaI. The 175 bp product results from cutting of a normal 220 bp PCR product by MvaI. (B) Poly A pseudodeficiency mutations: samples are shown + additions of RsaI. Sample 1, pseudodeficiency homozygote control (PD); sample 2, father (F); sample 3, patient (P); sample 4, mother (M). The 147 bp normal product is not cut by RsaI. The 117 bp product results from cutting of an abnormal 147 bp product by RsaI. A 154 bp standard is indicated on the left.

Figure 3 Hybridisation with an ASA cDNA probe. Lane 1, father (F); lane 2, patient (P); lane 3, mother (M); lane 4, control (C). Sizes of the restriction fragments are given in kb.
suggested of deletion of one allele. Analysis of the same blot with a probe for the proximally located D22S55 site, pH9118 (data not shown), gave a similar result with a single band of 5.5 kb, again consistent with a deletion and suggesting that the loss of material from C22q extends proximal to the ASA gene at least as far as D22S55. The approximate location of D22S55 relative to the ASA gene is shown in fig 4.

**MINISATELLITE ANALYSIS**

Minisatellite analysis was performed on this family and the results are shown in fig 5. Approximate locations of all markers used are shown in fig 4. Amplification with the primers for the markers D22S315 and TOPIP2 clearly indicated inheritance of unique alleles from both parents in the child. The markers D22S283 and CYP2D also showed probable inheritance from both parents; however a common allele is shared by all three family members. Thus, these markers are proximal to the breakpoints involved in the ring formation. These data, in conjunction with the "I" allele inheritance pattern, confirm that there is biparental inheritance of chromosome 22 sequences and that the ring with the deletion is of maternal origin.

**ASA SPECIFIC mRNA TRANSCRIPTION**

The production of ASA specific mRNA in the patient was tested using RT-PCR as described in Methods. Virtually no product was detectable in the child's sample although appropriate sized products were detected when primers for an unrelated gene (aspartyl-glucosaminidase) on another chromosome were used (data not shown). These results are consistent with the expectation of essentially no functional ASA specific mRNA where one allele is deleted and the other carries a major splicing mutation.

**Discussion**

We have reported here a case of a female patient with MLD who has inherited a paternal chromosome 22 carrying the infantile onset "I" allele and has a de novo constitutional ring chromosome 22 of maternal origin, deleted for the ASA gene. The deleterious "I-1" mutation is a splicing defect\(^6\) and is not expected to produce any active enzyme of functional mRNA. The deletion of the other ASA allele would also preclude production of functional mRNA. This was indeed the case, as no RT-PCR products of appropriate size were detectable in this child although the mRNA was able to support transcription of an unrelated cDNA and PCR product.

A typical clinical picture of a patient with a constitutional ring 22 includes significant mental retardation, microcephaly, growth failure, hypotonia,\(^7\) and perhaps a predisposition to meningioma formation.\(^8\)-\(^10\) In this particular case the child was referred for cytogenetic analysis because of dysmorphic features, several of which have been noted in other cases of ring 22. De novo ring chromosomes affecting chromosome 22 are relatively rare, comprising approximately 9 to 10% of all constitutional autosomal rings.\(^5\)\(^11\) Autosomal rings occur in about 1/50 000 live births although they have also been detected as isolated findings in men-

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**Figure 4** An ideogram of chromosome 22 showing the approximate locations of the markers described.\(^7\)\(^8\)\(^16\)

**Figure 5** Minisatellite analysis: 2 μl aliquots of 10 μl reactions were loaded onto the gel. The samples are from the father (F), patient (P), and mother (M) respectively in each set of three. Alleles are designated a, b, c, and d.
Metachromatic leukodystrophy (MLD) in a patient with a constitutional ring chromosome 22

ingioma tissue. The formation of the ring is thought to involve deletion of material from the telomer of the chromosome and rejoining. Loss of the ASA gene is not an unexpected event in such circumstances given its location at 22q13.3. Indeed, loss of one ASA allele has been documented in at least three other cases of terminal deletion or ring formation of chromosome 22 using either reduced enzyme activity9,11 or molecular detection11 as the criterion. In the case presented here, the co-inheritance of a severe allele from the father resulted in infantile onset MLD.

Children with infantile onset MLD usually develop clinical signs between the ages of 15 months and 2 years. They lose the ability to walk or stand and show ataxia, weakness, and progressive spastic quadriplegia. Blindness, seizures, and loss of speech are commonly seen. Neurological function declines rapidly to a decerebrate state and the disease culminates in death usually within one to seven years of onset. There are not usually striking dysmorphic features associated with MLD.

A metabolic error was not initially suspected in this child. It was not until it became obvious that she was deteriorating much more rapidly than would have been expected for ring 22 alone that she was investigated for possible metabolic errors. Subjects with documented ring 22 alone9,20,21 have usually survived longer. Those with one deleted arylsulphatase A gene would not have been expected to display any signs of MLD since they would have had a normal copy of the ASA gene on the other chromosome and therefore would have had heterozygote carrier levels of ASA enzyme activity and none of the clinical problems associated with MLD.

In considering this family’s risk for future pregnancies, there is a 25% chance of having a child with a combination of the father’s “t” allele and the mother’s pseudodeficiency allele but this would not result in MLD. Compound heterozygotes for MLD and pseudodeficiency alleles have been identified in other laboratories28 as well as our own and are usually without any clinical symptoms. However, the risk for late onset or neuropsychiatric disorders associated with this combination of alleles remains a contentious issue. Such a combination of alleles would also complicate prenatal diagnosis by enzymological means alone since it is not always possible to distinguish unequivocally a compound heterozygote fetus from one that is affected with MLD. DNA analysis would be necessary to provide an unambiguous result. The risk for a second ring chromosome event in this family is very small and thus there is essentially no risk of a second MLD child.

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