Metachromatic leucodystrophy in three families from Nova Scotia, Canada: a recurring mutation in the arylsulphatase A gene

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Abstract
Metachromatic leucodystrophy (MLD) is a lysosomal storage disease resulting from a deficiency of arylsulphatase A. We have identified a child with infantile onset MLD who is homozygous for an A212V mutation, a mutation previously reported but not further characterised. We have introduced this mutation into an arylsulphatase A expression vector by site directed mutagenesis. Transient expression of this mutant plasmid in COS cells yields very low levels of arylsulphatase A activity consistent with the patient's phenotype. The arylsulphatase A pseudodeficiency also segregates in this family causing difficulty in interpreting enzyme levels in the absence of DNA data. Two other patients from the same province, also carrying the A212V allele, have juvenile and adult onset MLD and are heterozygous for P426L ("A" allele) and I179S alleles respectively, known late onset alleles.

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Metachromatic leucodystrophy (MLD) is a neurodegenerative lysosomal storage disease resulting from a deficiency of activity of arylsulphatase A (EC 3.1.6.1), an enzyme involved in the normal catabolism of cerebroside sulphatide.¹ A characteristic feature of MLD is the accumulation of cerebroside sulphatide particularly in myelin forming cells of the CNS.

The arylsulphatase A gene is a small gene whose sequence and structure are well documented.² A number of mutations have been identified in this gene.³ Among these are two common alleles, the "I" (IVS2+1) and "A" (P426L) alleles,⁴ associated with infantile and later onset MLD respectively. These two alleles together account for 54% of MLD alleles. There is also a pseudodeficiency associated with arylsulphatase A in which enzyme levels are significantly reduced without any clinical or biochemical evidence of disease.⁵ The pseudodeficiency allele (Pd) occurs at 10-20% in the general population. This Pd allele consists of a complex of two mutations, a glycosylation site mutation (N350S) in exon 6 affecting the third glycosylation site of the enzyme, and an A→G

in the first poly A addition signal⁶ (AATAAC→AGTAAC). The glycosylation site mutation apparently has little effect on enzyme activity whereas the poly A site mutation reduces arylsulphatase A by up to 80%.

Although the arylsulphatase A gene has a few common mutations which occur in a significant proportion of cases, there are also a large number of mutations which recur to a limited extent or are private. Identification of such mutations has limited diagnostic usefulness beyond particular families or ethnic groups, although it may provide useful information for population genetics studies. Knowledge of these mutations can, however, sometimes provide valuable clues to functional domains within the enzyme and will be of interest later in correlations with phenotype and with structural analyses of the protein.

We describe here three unrelated families from the Nova Scotia province of Canada in whom a missense mutation in exon 3 causes a A212V alteration. This mutation was previously reported in a family of British origin,⁷ but no expression studies were reported nor did the mutation recur among 40 normal chromosomes and 32 MLD chromosomes in that study. In the Nova Scotia families, the phenotypes are infantile, juvenile, or adult onset depending on the other allele present.

Materials and methods

PATIENTS

Patient 1 (family 1) was born to consanguineous parents of predominantly Acadian background and was noted at about 2½ years to have difficulties with gait. She was clinically assessed at the age of 3 years and tested for arylsulphatase A because of a family history of MLD. Her fibroblast arylsulphatase A level (table 1) was consistent with infantile onset MLD. She deteriorated rapidly in her third year, developing seizures and losing speech, walking ability, and head control. At the age of 4 she was no longer able to sit, had swallowing problems, and had no reflexes in her legs. At 4½ she was unresponsive to pain and required tube feeding. She died at the age of 4 years 8 months. The pedigree for family 1 is shown in fig 1 where the proband is VII.3. This pedigree represents a highly consanguineous kindred extended through seven generations.

Patient 2 (family 2), of French or Acadian and British background, was well until about the age of 10 when he began tripping and was

Table 1 Arylsulphatase A activity in extracts of patient leucocytes or fibroblasts

Family		Cell type	Aryl A*	Control*	
				Average	Range
1	Patient	Fibroblast	0.303	37.9	22.3–49.6
	Father	Fibroblast	17.07	37.9	22.3–49.6
	Aunt VI.16	Leucocyte	0.2	10.6	4.1–17.1
	Uncle VI.17	Leucocyte	4.2	10.6	4.1–17.1
	Cousin VI.13	Leucocyte	6.2	9.8	4.7–14.8
2	Patient	Fibroblast	0	37.9	22.3–49.6
	Mother	Leucocyte	6.4	7.9	2.8–17.9
	Father	Leucocyte	10.3	7.9	2.8–17.9
	Normal sister	Leucocyte	16.1	7.9	2.8–17.9
3	Patient	Fibroblast	32.8	211	106–264
		Leucocyte	8.7	118.5	55–182
	Sister	Leucocyte	1.7	118.5	55–182
	Mother	Leucocyte	20.3	118.5	55–182
	Father	Leucocyte	27.8	118.5	55–182

*Activities are given in nmol catechol released/h/mg protein. Control ranges differ because assays were performed at different times and in different laboratories.
Patients 1 and 2 were assayed as described in Materials and methods.
Patient 3 and family were assayed according to Baum et al.¹⁰

slow to respond to questions. He was found to have an intention tremor and early signs of spasticity. Neuropsychological studies showed impaired memory but preservation of intellect. CT and MRI scans showed diffuse demyelination. Measurement of arylsulphatase A (table 1) in the patient (cultured fibroblasts) and his parents (leucocytes) were consistent with juvenile onset MLD.

Patient 3 (family 3), of British and Dutch background, was diagnosed with adult onset MLD and has been previously described.⁸ At about the age of 40 he displayed progressive confusion and lack of concentration. Psychiatric evaluations had suggested schizophrenia or severe personality disturbance. Assessment as possible early onset Alzheimer's disease was inconclusive, although CT scan showed white matter changes consistent with a demyelinating disease. At the age of 47, following further deterioration in social behaviour, he was screened for metabolic disorders. A deficiency of arylsulphatase A was identified in both white

cells and cultured fibroblasts (table 1). His older sister, also assessed for Alzheimer's disease, similarly showed a deficiency of arylsulphatase A.

These studies were approved by the University of British Columbia Clinical Screening Committee for Research Involving Human Subjects and the BC Children's Hospital In Hospital Research Review Committee.

MATERIALS

ID Pol, a heat stable DNA polymerase from *Thermus flavus*, was purchased from ID Labs Inc (London, Ontario). Restriction enzymes were obtained from New England BioLabs (Mississauga, Ontario) or Boehringer-Mannheim Canada (Laval, Quebec). ³⁵S-dATP for sequencing and Sequenase (USB) kits were obtained from Amersham Canada Ltd (Oakville, Ontario). Kits for site directed mutagenesis (Unique Site Elimination), as well as the expression vector pSVL, were obtained from Pharmacia Canada (Baie d'Urfe, Quebec). The TA cloning kit was purchased from Invitrogen (San Diego, California). The pHEXB43 cDNA clone was obtained from the ATCC (Rockville, MD). A cloned cDNA copy of the arylsulphatase A gene was kindly provided by Dr V Gieselmann (Germany).

DETERMINATION OF ARYLSULPHATASE A ACTIVITY IN CELL EXTRACTS

Fibroblast cultures were grown under standard conditions and harvested at about 75% confluence for assay. Cells were homogenised and cell free supernatants were used in assays with a p-nitrocatechol sulphate substrate in an assay specific for arylsulphatase A⁹ modified from Baum et al.¹⁰ Extracts from transfected COS cells were prepared and assayed in the same way. The COS cell extracts were diluted until a linear response to volume added was obtained.

Figure 1 Pedigree of family 1, the infantile onset MLD patient's family. The proband is VII.3. P, Q, R, and S indicate entry points of four major family lines involved in this pedigree. Symbols with dots indicate obligate heterozygotes. Filled symbols indicate MLD affected subjects. A diamond indicates a fetus. Alleles are indicated for those people whose DNA was tested. N indicates a normal sequence at the sites tested, Pd, a pseudodeficiency allele, A212V, the MLD mutation. Pd* (VI.17) indicates that only the glycosylation site mutation of the Pd allele was present. This pedigree, as shown, dates back to the early 19th century. The P family had settled in the area more than 50 years earlier. It is unknown how many MLD affected subjects were born into this family before generation VI when presumably the disease would not have been diagnosed.

Table 2 Sequences of oligonucleotides used in PCR amplifications

Sequence (5'-3')	Nucleotide number (5'-3')
1 cgggcgaagcTTCCTCATTTCGTACACAGG	2816-2797
2 gctcgaattCTGCTGGAGCCAAGTAGCCCT	-56- -36
3 cgcggaattCTTGATGGCGAACTGAGTGAC	1671-1691
4 cgcgctcaGAACGCTCTGCAAAGCTCTGCC	1062-1041
5 gctaagcCTTGCCCTGTGCACAGAATTGGC	2275-2297
6 ctatGGCCAATTCTGTGCACAGGGCAAG	2298-2275
7 atatgaaTTCGGCCACGGTCACCTCTCCA	476-454
8 gagaggatCCAGTGCTAACTCCAGTCTTTG	987-1008
9 cgtggaattCAGTCACTCAGTTCGCCATCAAG	1693-1671
10 cacagaatTCAGGGACTCTGTGACTTGTC	341-361
15 ATCAGGGCTTCCATCGATTTCTAG	562-585
20 CTTGGACAGGTCATAGAGC“T”GC	2403-2382
23 AGAAGCGGTGCACGTCCAG	1991-2009

Nucleotide numbers refer to the genomic sequence in GenBank (X52150) where position 1 is the first base of exon 1. Lower case letters indicate residues not present in the genomic template sequence and are not numbered. The underlined bases represent restriction enzyme recognition sequences which were introduced to facilitate cloning. “T” (primer 20) represents a mismatch inserted into the sequence to create a *Pst*I site when the “A” allele mutation is present.

Figure 2 SSCP analysis of segment D. (A) patient 1, A212V homozygote, and (B) patient 3, A212V/I179S heterozygote. In both cases, the PCR product was digested with *Apal* and analysed as described in Materials and methods. The results shown are for a gel run at 20°C without glycerol. S indicates a lane with a pBR322/*Hinf*I size marker. Panel A shows a control sample in lane 1, the homozygous A212V/A212V proband in lane 2, and an unrelated homozygous “I” allele (IVS2+1) sample in lane 3 for comparison. Panel B shows the A212V/I179S heterozygous sample in lane 1 and a control sample in lane 2. In both A and B the bands of interest are in the top quarter of the gel. Double stranded bands are in the lower half and correspond to the appropriate bands in the size marker lanes. (C) PCR amplified segments used for SSCP analysis. Exons 1-8 and the intervening and non-coding sequence is shown approximately to scale. Segments A-E amplified for PCR are indicated by black bars with numbers designating the primers on the ends. The restriction sites used to generate fragments suitable for PCR are shown as * (segment A, *Sma*I; B, *Hinf*I; C, *Hind*III/*Pst*I; D, *Apal*; E, *Pvu*II). The locations of the pertinent mutations are shown above the gene. Pd-1 and Pd-2 represent the two mutations associated with pseudodeficiency.

POLYMERASE CHAIN REACTION (PCR)
AMPLIFICATION

The oligonucleotide primers used in PCR reactions are listed in table 2. PCR amplifications were performed using standard procedures¹¹ using DNA extracted either from cultured fibroblasts or leucocytes. Primers were added to a final concentration of 1 μmol/l each. Substrate DNA was added at 1 μg/100 μl. ID Pol polymerase was used at 0.75-1.25 units/100 μl. MgCl₂ concentration was at 1.5 mmol/l unless otherwise stated. Cycling parameters were: denaturation at 94°C for two minutes, annealing at 60 or 65°C as indicated for 1.5 minutes, extension at 72°C for two minutes for 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.

Genomic DNA was amplified in five overlapping segments for preliminary screening for single strand conformational polymorphisms (SSCP) to detect probable sites of mutations (fig 2C). The following primer combinations and annealing temperatures were used: segment A, primers 5/1, 60°C; segment B, primers 3/6, 65°C; segment C, primers 8/9, 60°C; segment D, 10/4, 65°C; segment E, 2/7, 65°C.

PCR amplification followed by restriction enzyme digestion was used to test for the Pd allele, the “I” allele, the “A” (P426L) adult onset allele, and the A212V mutation. Primers and restriction enzymes for Pd and “I” alleles have already been described.⁹ Primers 15 and 4 (annealing temperature 65°C) were used to test for the A212V mutation. A new site for *Aat*II (GACGTC) is created by the mutation. Primers 20 and 23 (annealing temperature 65°C) were used to test for the P426L allele. A new site for *Pst*I (CTGCAG) is created by the mutation in conjunction with the mismatched base in primer 20.

SINGLE STRAND CONFORMATIONAL
POLYMORPHISM (SSCP)

PCR segments A-E were screened for alterations affecting the mobility of single strands by a modification of a previously described method.¹² PCR segments were initially digested with restriction enzymes to generate fragments smaller than 300 base pairs appropriate for analysis (A, *Sma*I; B, *Hinf*I; C, *Hind*III/*Pst*I; D, *Apal*; E, *Pvu*II). Approximate locations for primers and restriction sites are shown in fig 2C. The samples were analysed under four sets of gel and temperature conditions (±10% glycerol, 20°C or 4°C) using a 2.5% cross linked gel to optimise sensitivity.¹³ Control and patient samples were matched for known polymorphisms in the segment. Results were visualised by silver staining.

SEQUENCING

Dideoxy sequencing was performed on PCR products after purification with PCRapids™ (ID Labs) or after cloning into a pCRII vector for TA cloning. Sequencing products were labelled with ³⁵S-dATP and visualised by autoradiography. All nucleotide numbering is based

on the genomic DNA sequence in GenBank X52150 unless otherwise specified. Nucleotide 1 is the first coding nucleotide of the sequence which is position 630 in X52150. This follows the pattern of mutation position numbering used in a recent review.³

SITE DIRECTED MUTAGENESIS

An expression vector, pSVL-arylA, was created by inserting a full length arylsulphatase A cDNA into the pSVL vector. The A212V mutation was generated directly in the expression plasmids using a kit based on the "unique site elimination" method.¹⁴ Plasmids were purified with QIAGEN (Studio City, California) columns and the sequence confirmed before use.

TRANSFECTIONS

Transient expression studies were performed using the normal and mutant pSVL-arylA expression vectors. The vector was introduced into COS cells by CaPO₄ precipitation with a 20% DMSO shock six hours after addition of DNA.¹⁵ Cells were harvested for protein determination¹⁶ and enzyme assay three days after addition of the DNA. A second plasmid, (pHEXB43) coding for β -hexosaminidase B, was cotransfected with the pSVL-arylA in order to provide a control for efficiency of transfection and expression. Cell extracts were assayed for total β -hexosaminidase activity (A plus B) by standard methods¹⁷ using a 4-methyl umbelliferyl- β -N-acetylglucosamide substrate and measuring release of 4-methyl umbelliferone (4MU). Transfections were carried out in duplicate and repeated at least once.

Results

PRELIMINARY SCREENING FOR COMMON MUTATIONS

DNA samples from leucocytes or cultured fibroblasts from the three probands were initially screened for the common "T" and "A" alleles and Pd mutations by PCR amplification and restriction enzyme digestion. With this screen, patient 2 was found to be a heterozygous carrier of the "A" allele, P426L (data not shown).

SSCP ANALYSIS

Segments A-E of the arylsulphatase A gene were amplified from each of the probands and subjected to SSCP analysis as outlined in Materials and methods. Both patients 1 and 3 showed alterations in segment D, as shown in fig 2. This segment became the focus of subsequent sequencing studies.

SEQUENCE ANALYSIS

Sequencing of segment D in patient 1 indicated that she was homozygous for a C→T transition at position 898 of the gene in exon 3 which would generate an A212V alteration (fig 3A). This mutation has previously been identified in a heterozygous state in one patient of British origin.⁷ The mutation creates a new restriction site for the enzyme *AatII*. Use of this enzyme confirmed that the mutation was homozygous in this patient (fig 3B).

Figure 3 (A) Sequence analysis of A212V mutation. Cloned PCR product showing the A212V mutation (C898T) is shown on the left and normal sequence on the right. (B) *AatII* digestion of PCR product with A212V mutation. The A212V mutation creates a new *AatII* site such that the 510 bp PCR product is cut to 335 bp and 175 bp fragments. Samples are shown +/- *AatII*. Sample 1, control; sample 2, A212V homozygote, the proband from family 1 (VII.3 in fig 1); sample 3, A212V heterozygote, the father of the proband (VI.14 in fig 1). DNA standards (pBR322/*HinfI*) are shown on the left.

The *AatII* digestion was also used for PCR products from patients 2 and 3 and showed that each of them was heterozygous for the A212V mutation (data not shown). Where DNA was available, family members were also tested. The results for family 1 are noted in the pedigree in fig 1. In family 2, the A212V mutation was inherited through the mother who was of French or Acadian descent and was inherited by two of six sibs (data not shown). Subsequent sequence analysis of segment D in patient 3 identified a second mutation in exon 3. This mutation, a T→G at position 799, would cause an I179S amino acid alteration. This mutation has previously been described¹⁸ in an adult onset patient of Swiss origin. In summary, the infantile onset MLD was associated with A212V/A212V, the juvenile with A212V/P426L, and the adult onset with A212V/I179S. The locations of these muta-

tions within the arylsulphatase A gene are shown in fig 2C.

SITE DIRECTED MUTAGENESIS AND TRANSIENT EXPRESSION STUDIES

Expression studies were performed to confirm that the A212V mutation did indeed have the potential to cause late infantile MLD seen in patient 1 where it is homozygous. The mutation was introduced into the pSVL-arylA vector by unique site elimination. COS cells were cotransfected with the normal pSVL-arylA or the A212V mutant and the pHEXB43 plasmid as a control for the transfection efficiency and expression capability of the cells. The total β -hexosaminidase activity generated in each case was 22.8 and 32.1×10^3 nmol 4 MU released/h/mg protein respectively for the normal and mutant pSVL, combinations indicating that there was comparable or better transfection efficiency for the mutant plasmid case and that the cells were fully competent to express cDNAs for lysosomal enzymes. The range of arylsulphatase A activity generated with the normal pSVL-arylA plasmid was 4.83-16.53 nmol nitrocatechol released/h/mg protein whereas the A212V mutant plasmid generated on average 1.1% as much arylsulphatase A activity as the normal plasmid. This is consistent with the extremely low enzyme activity found in fibroblast extracts from patient 1 (table 1).

ARYLSULPHATASE A PSEUDODEFICIENCY IN RELATIVES OF PATIENT 1

Patient 1 came from a highly consanguineous family (fig 1) with a strong Acadian background. Because of the family history of MLD, several family members had been tested enzymatically to determine carrier status. Specifically, subjects VI.13, 16, and 17 had shown low normal or below normal arylsulphatase A activities and were considered possible MLD carriers. DNA testing for the A212V and the Pd mutations in these and other family members indicated that the Pd allele was also segregating in this family (fig 1) and had resulted in errors in previous carrier designations based on enzyme activity alone. Subject VI.16 had shown a very low arylsulphatase A activity (table 1) and was shown to carry both the A212V and the Pd alleles.

Discussion

We have identified a mutation which is present in three families from Nova Scotia, Canada. This mutation, a C898T missense alteration in exon 3 of the arylsulphatase A gene, would result in substitution of valine for alanine at amino acid 212. The A212V mutation was found in a homozygous state in one family and a heterozygous state with a P426L and an I179S mutation in the other two families.

The A212V mutation has been previously reported in one instance in a patient of British origin.⁷ In that case, the mutation was heterozygous with an A224V in an adult onset MLD patient. Expression studies for these mutations were not reported so the severity of the mutation could not be accurately determined.

We have found the A212V mutation now in three patients, one of whom, an infantile onset case, is homozygous. We have also completed transient expression studies in heterologous COS cells. The alanine at position 212 is not conserved among the various sulphatases¹ and the A212V is a relatively minor alteration between two non-polar amino acids. However, the mutation virtually eliminates production of arylsulphatase A activity from the transfected plasmid. Although we do not know at what level the mutation exerts its effect, for instance, processing, substrate binding, etc, the effects of such a mutation on enzyme activity would be consistent with the extremely low enzyme activity found in fibroblast extracts from the homozygous, affected child and her severe early onset course. The results of the expression studies and the infantile onset of the disease in the homozygous affected child clearly indicate that this mutation causes a severe phenotype. The second mutations (P426L and I179S) found in the juvenile and adult onset patients respectively are known to be associated with somewhat milder forms of disease^{4,18} and are consistent with the phenotypes in these patients.

DNA testing of family members for the A212V mutation as well as other common mutations indicated that the Pd allele was also segregating in this family. In several cases, the presence of the Pd allele had resulted in people mistakenly designated as MLD carriers when assessment was based on arylsulphatase A activity alone. For such family members, genotyping has altered their apparent risk of producing a child with MLD.

The "A" allele mutation, P426L, has been calculated to occur in approximately 30% of MLD genes. The I179S mutation has a calculated frequency of 2%.¹ Until now recurrence of the A212V has not been reported. We have found the A212V mutation in three families with homozygosity in only one. In that case the kindred is highly consanguineous and a single allele from a common ancestor is the most likely explanation. In family 1, the subjects confirmed to have the A212V mutation are direct descendants of I.1, I.5, and II.6 (fig 1), all of whom are cousins within family 1-P. Although we have not established a genealogical connection between families 1 and 2, the mother in family 2, the carrier of the A212V, also has French or Acadian heritage. Family 3 has a mixed western European background. Recurrence of the A212V allele is therefore unlikely to be the result of a recent founder effect, as has been observed for other disorders in specific Nova Scotian demes, including the prevalence of Fabry disease in Acadian Nova Scotians¹⁹ and the clustering of neonatal hyperparathyroidism in south western Nova Scotians of British origin.²⁰ For pseudo-vitamin D deficient rickets, linkage disequilibrium mapping of flanking polymorphic markers provides a genetic confirmation of the hypothesis that the prevalence of this recessive disorder in Acadians of Nova Scotia and the other Maritime Canadian provinces is the result of a

founder effect that dates back to the earliest generations of Acadian settlement.²¹

The original A212V mutation in the arylsulphatase A gene was reported in a person of British background,⁷ while British, Dutch, and French or Acadian origins are claimed by the three families reported here. Family 1-P had been settled in the Nova Scotia area before the British takeover in 1763. We suggest that the A212V mutation is likely to predate the founding settlements of Maritime Canada (1604-1800), but identification and mapping of flanking markers should allow for linkage disequilibrium analysis that could strengthen this hypothesis and yield a preliminary estimate of the date of origin.

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1 Kolodny EH, Fluharty AL. Metachromatic leukodystrophy and multiple sulfatase deficiency: sulfatide lipidosis. In: Scriver CR, Beaudet AL, Sly WS, et al, eds. *The metabolic and molecular bases of inherited disease*. 7th ed. Vol 2. New York: McGraw-Hill, 1995:2693-739.

2 Kreysing J, Gieselmann V. Structure of the arylsulfatase A gene. *Eur J Biochem* 1990;191:627-31.

3 Gieselmann V, Zlotogora J, Harris A, Wenger DA, Morris CP. Molecular genetics of metachromatic leukodystrophy. *Hum Mutat* 1994;4:233-42.

4 Polten A, Fluharty AL, Fluharty CB, Kappler J, von Figura K, Gieselmann V. Molecular basis of different forms of metachromatic leukodystrophy. *N Engl J Med* 1991;324:18-22.

5 Thomas GH. "Pseudodeficiencies" of lysosomal hydrolases. *Am J Hum Genet* 1994;54:934-40.

6 Gieselmann V, Polten A, Kreysing J, von Figura K. Arylsulfatase A pseudodeficiency: loss of a polyadenylation signal and N-glycosylation site. *Proc Natl Acad Sci USA* 1989;86:9436-40.

7 Barth ML, Fensom A, Harris A. Missense mutations in the arylsulphatase A genes of metachromatic leukodystrophy patients. *Hum Mol Genet* 1993;2:2117-21.

8 Sadovnick AD, Tuokko H, Applegarth DA, Toone JR, Hadjivavropoulos T, Beattie BL. The differential diagnosis of adult onset metachromatic leukodystrophy and early onset familial Alzheimer disease in an Alzheimer clinic population. *Can J Neurol Sci* 1993;20:312-18.

9 Coulter-Mackie MB, Rip J, Ludman MD, Beis J, Cole DEC. Metachromatic leucodystrophy (MLD) in a patient with a constitutional ring chromosome 22. *J Med Genet* 1995;32:787-91.

10 Baum H, Dodgson KS, Spencer B. The assay of arylsulfatases A and B in human urine. *Clin Chim Acta* 1959;4:453-5.

11 Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. *PCR protocols. A guide to methods and applications*. 1st ed. San Diego: Academic Press, 1990.

12 Ainsworth PJ, Coulter-Mackie MB. A double mutation in exon 6 of the β -hexosaminidase α subunit in a patient with the B1 variant of Tay-Sachs disease. *Am J Hum Genet* 1992;51:802-9.

13 Hayashi K, Yandell DW. How sensitive is PCR-SSCP? *Hum Mutat* 1993;2:338-46.

14 Deng WP, Nickoloff JA. Site-directed mutagenesis of virtually any plasmid by eliminating a unique site. *Anal Biochem* 1992;200:81-8.

15 Sambrook J, Fritsch EF, Maniatis T, eds. *Molecular cloning: a laboratory manual*. 2nd ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press, 1989.

16 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. *J Biol Chem* 1951;193:265-75.

17 Lowden JA. Evidence for a hybrid hexosaminidase isoenzyme in heterozygotes for Sandhoff disease. *Am J Hum Genet* 1979;31:281-9.

18 Fluharty AL, Fluharty CB, Bohne W, von Figura K, Gieselmann V. Two new arylsulfatase A (ARSA) mutations in a juvenile metachromatic leukodystrophy (MLD) patient. *Am J Hum Genet* 1991;49:1340-50.

19 Kirkilionis AJ, Riddell DC, Spence MW, Fenwick RG. Fabry disease in a large Nova Scotia kindred: carrier detection using leucocyte α -galactosidase activity and an *NcoI* polymorphism detected by an α -galactosidase cDNA clone. *J Med Genet* 1991;28:232-40.

20 Janicic N, Pausova Z, Cole DEC, Hendy GN. Insertion of an Alu sequence in the Ca^{2+} -sensing receptor gene in familial hypocalciuric hypercalcemia and neonatal severe hyperparathyroidism. *Am J Hum Genet* 1995;56:880-6.

21 Labuda M, Labuda D, Korab-Laskowska M, et al. Linkage disequilibrium mapping in young populations: pseudovitamin D deficiency rickets (PDDR) in French Canadians. *Am J Hum Genet* 1996;59:633-43.