

RESEARCH ARTICLE

Coincidence of Two Novel Arylsulfatase A Alleles and Mutation 459+1G>A Within a Family With Metachromatic Leukodystrophy: Molecular Basis of Phenotypic Heterogeneity

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In a family with three siblings, one developed classical late infantile metachromatic leukodystrophy (MLD), fatal at age 5 years, with deficient arylsulfatase A (ARSA) activity and increased galactosylsulfatide (GS) excretion. The two other siblings, apparently healthy at 12½ and 15 years, respectively, and their father, apparently healthy as well, presented ARSA and GS values within the range of MLD patients. Mutation screening and sequence analysis disclosed the involvement of three different ARSA mutations being the molecular basis of intrafamilial phenotypic heterogeneity. The late infantile patient inherited from his mother the frequent O-type mutation 459+1G>A, and from his father a novel, single basepair microdeletion of guanine at nucleotide 7 in exon 1 (7delG). The two clinically unaffected siblings carried the maternal mutation 459+1G>A and, on their paternal allele, a novel cytosine to thymidine transition at nucleotide 2435 in exon 8, resulting in substitution of alanine 464 by valine (A464V). The fathers genotype thus was 7delG/A464V. Mutation A464V was not found in 18 unrelated MLD patients and 50 controls. A464V, although clearly modifying ARSA and GS levels, apparently bears little significance for clinical manifestation of MLD, mimicking the frequent ARSA pseudodeficiency allele.

Our results demonstrate that in certain genetic conditions MLD-like ARSA and GS values need not be paralleled by clinical disease, a finding with serious diagnostic and prognostic implications. Moreover, further ARSA alleles functionally similar to A464V might exist which, together with O-type mutations, may cause pathological ARSA and GS levels, but not clinical outbreak of the disease. *Hum Mutat* 13:61–68, 1999. © 1999 Wiley-Liss, Inc.

KEY WORDS: metachromatic leukodystrophy; arylsulfatase A; mutations; pseudodeficiency; genotype-phenotype correlation

INTRODUCTION

The genetic deficiency of the enzyme arylsulfatase A (ARSA) causes metachromatic leukodystrophy (MLD; MIM #250100), a neurometabolic disease inherited as an autosomal recessive trait. Three major clinical variants have been characterized: late infantile, juvenile, and adult MLD (for review see Kolodny and Fluharty, 1995). Biochemically, ARSA deficiency results in impaired degradation of the enzyme substrate galactosylsulfatide (GS), which will lead to accumulation of GS in the central and peripheral nervous system and to severe myelin breakdown. GS also accumulates in visceral organs and

is excreted in high amounts in urine. Thus, biochemical diagnosis of MLD is based on assays of ARSA activity in leukocytes or fibroblasts and GS excretion in urine. Common estimations of these

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parameters, however, do not discriminate between clinical phenotypes.

The ARSA gene maps to chromosome 22q, covers 3.2 kb of genomic DNA, and includes eight exons (Kreysing et al., 1990). So far, 59 MLD-relevant mutations have been identified including six small deletions, three splice site mutations, 48 amino acid substitutions, one nonsense mutation, and a combined missense/splice donor site mutation (Gieselmann et al., 1994; Barth et al., 1995; Heinisch et al., 1995; Luyten et al., 1995; Regis et al., 1995; Lissens et al., 1996; Tsuda et al., 1996; Draghia et al., 1997; Regis et al., 1997). Two of the mutant alleles (459+1G>A and P426L) are frequent, each accounting for about 25% of MLD alleles; the remaining 50% comprise all other mutations (Polten et al., 1991; Gieselmann et al., 1994).

Functionally, MLD mutations can be divided into two groups: alleles resulting in enzymatically inactive ARSA (0 alleles) and alleles encoding for ARSA with residual enzyme activity (R alleles). A recently described correlation between genotype and clinical phenotype suggests that homozygosity for 0 alleles results in late infantile MLD, whereas two R alleles lead either to adult or juvenile MLD, depending on the residual *in vivo* enzyme activity. In 0/R compound heterozygotes the residual enzyme activity of the R allele seems to determine the clinical outcome of the disease (Polten et al., 1991; Leinekugel et al., 1992). This concept of genotype-phenotype relations has held up fairly well for each of the additional MLD alleles subsequently identified (Bohne et al., 1991; Fluharty et al., 1991; Pastor-Soler et al., 1994; Luyten et al., 1995; Hess et al., 1996).

Though clinical heterogeneity within families is not common in MLD (Kolodny and Fluharty, 1995), Clarke et al. (1989) reported marked clinical differences in two siblings affected with late onset MLD. A hypothetical explanation was presented, however, no genotype analysis was performed at that time. In our article we present a family with MLD showing marked discrepancies between clinical observations and biochemical findings. Our investigations on the molecular basis of clinical heterogeneity in this family disclosed new ARSA mutations and the association of different clinical as well as biochemical phenotypes with particular mutation patterns of the ARSA alleles. Moreover, we demonstrate that markedly abnormal ARSA and GS values need not be paralleled by clinical outbreak of MLD.

MATERIALS AND METHODS

Case Histories

We report on a family (Fig. 1) without previous history of MLD. A girl (II-1) and male dizygotic twins (II-2, II-3) were born to unrelated parents.

Sibling II-2 developed classical late infantile MLD, diagnosed clinically and biochemically at the age of 3 years and leading to death at the age of 5. Biochemical diagnosis was based on assay of ARSA activity in leukocytes and of GS excretion in urine, both displaying values in the range of MLD patients (Table 1). Diagnosis of MLD was confirmed by post mortem neuropathological investigation.

Sibling II-3 developed normally and was 5 years old at the time of biochemical investigation. ARSA activity was severely reduced and GS excretion highly increased, both values lying within the range of MLD patients. Very similar findings were obtained at 9 years of age (Table 1). On neurological examination at age 9½, the proband showed minimal signs of diffuse encephalopathy and of a minimal distally accentuated polyneuropathy. However, now at age 12½, neurological findings are entirely normal and the proband's school reports are excellent. Computerized tomography, nuclear magnetic resonance, as well as nerve conduction velocity were normal at age 11 and 12½ years.

Sibling II-1, 12 years of age at the time of biochemical investigation, also displayed severe reduction of ARSA activity and markedly increased GS excretion within the range of MLD patients (Table 1). She is presently at age 15 and apparently healthy, but neurological examination was never done.

Both parents were never examined neurologically and appear to be healthy. Biochemically, the father (I-1) showed severe reduction of ARSA activity and markedly increased GS excretion in the MLD range (Table 1). The mother displayed moderately reduced ARSA activity and slightly increased GS excretion, both values lying in the lower range of MLD heterozygotes (Table 1).

All studies on human material were performed according to Austrian law.

ARSA and Galactosulfatide Assays

Arylsulfatase A activity was determined in leukocytes with p-nitrocatechol sulfate as described by Molzer et al., 1992.

Galactosylsulfatide excretion was determined in 24 hr urine using chloroform/methanol extraction, thin layer chromatography, and densitometry as described by Molzer et al., 1992.

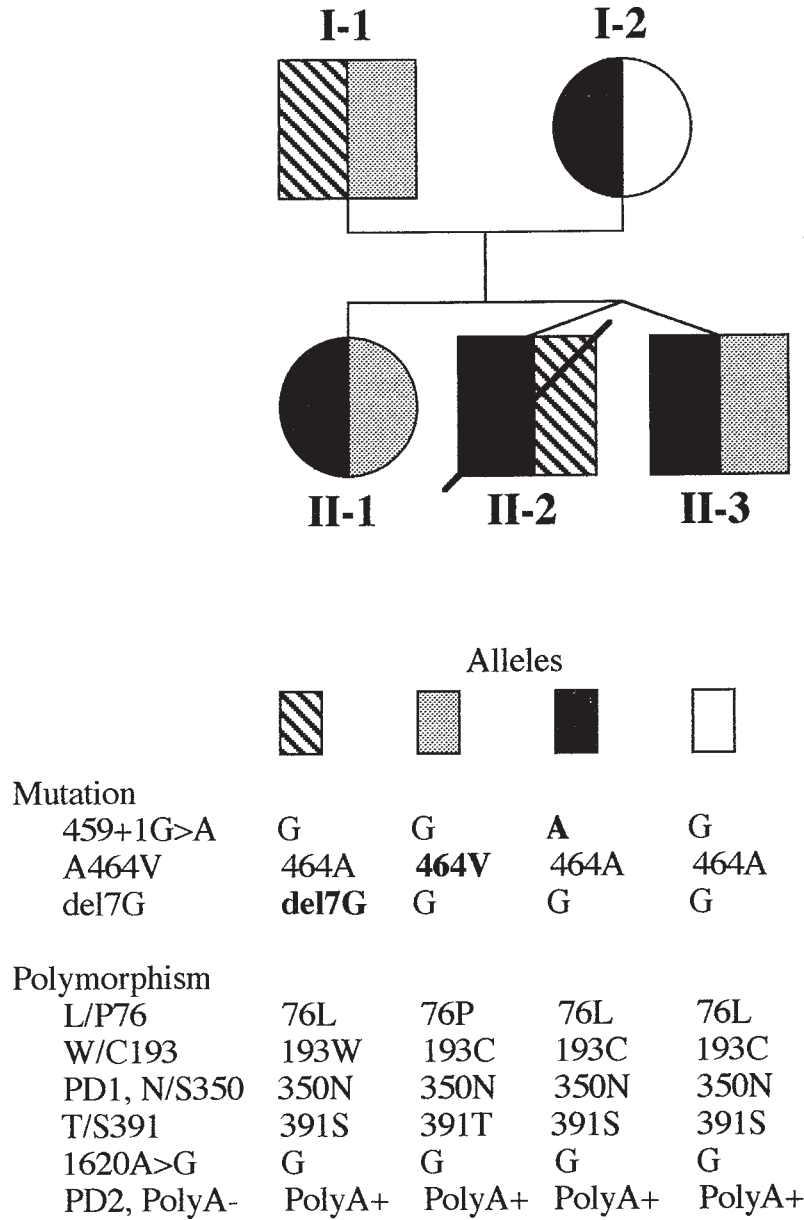


FIGURE 1. Pedigree of the present family, compiling ARSA alleles, ARSA activity (nmoles/mg protein/hour), and GC excretion (nmoles/mg lipid) in the individual probands. The three different mutated arylsulfatase A alleles are indicated. In case of the A464V and del7G ARSA alleles the entire genomic sequence was determined; the other allele characteristics rely on PCR based assays or partial sequencing.

PCR Amplification, ARSA-Allele Differentiation, and DNA Sequencing

Genomic DNA was prepared from peripheral blood leukocytes of I-1, I-2, II-1, II-3 by standard procedures (Sambrook et al., 1989) and in case of patient II-2 from paraffin-embedded, formalin-fixed brain tissue, according to the method described by Kösel and Graeber (1994), with the exception that the extracted DNA, on the

Microcon-30 column (Amicon), was washed three times with 500 µl Tris EDTA (10 mM TRIS-HCl, 1 mM EDTA, pH 7.4) before elution. This slight modification improved the DNA quality considerably. All family members were tested for the presence of the common mutations 459+1G>A and P426L as previously described (Berger et al. 1993).

The entire ARSA gene of patient II-3, presenting with the 459+1G>A mutation on one allele, was

TABLE 1. ARSA Activity in Leukocytes and GS Excretion in Urine

Probands	n	ARSA	GS
		nmoles/mg protein/hr ^a	nmoles/mg lipid ^a
This family^b			
I-1 [39]	—	21	50
I-2 [32]	—	48	4
II-1 [12]	—	22	50
II-2 [3]	—	9	280
II-3 [5]	—	12	137
II-3 [9]	—	29	156
Control groups			
MLD homozygotes	23	9 ± 1 (0–24)	148 ± 20 (27–412)
MLD heterozygotes	21	66 ± 23 (31–107)	8 ± 5 (1–16)
MLD/ARSA-PD	6	42 ± 31 (14–89)	18 ± 11 (5–36)
Compound heterozygotes ^c			
ARSA-PD homozygotes	7	48 ± 19 (25–74)	2 ± 2 (0–6)
ARSA-PD heterozygotes	5	81 ± 26 (57–117)	2 ± 2 (0–4)
Wild type (non MLD) (non ARSA-PD)	19	137 ± 53 (75–288)	2 ± 1 (0–5)

^aFigures in parentheses are ranges.

^bFigures in brackets are age [years] of the proband at investigation.

^cMLD and ARSA-PD mutations may be either on the same or on different alleles.

Abbrev: ARSA = arylsulfatase A; GS = galactosylsulfatide; ARSA-PD = arylsulfatase A pseudodeficiency; MDL, metachromatic leukoolystrophy.

amplified in two overlapping fragments (nt –60 to nt 1090 and nt 1017 to nt 2672) by polymerase chain reaction (PCR), using primer Oli. 85/Oli. 35 and Oli. 86/Oli. 87 (Table 2) to amplify fragment 1 and 2 respectively. The primer sequences were obtained from the genomic ARSA sequence available by GenBank (accession No.: X521151). The fragments were amplified in a DNA thermal cycler (HYBAID) by 32 cycles of PCR (1 min at 94°C, 1 min at 58°C and 4 min at 72°C) using 25 pmoles of each primer, 2.5 units of Taq DNA polymerase (Advanced Biotechnologies, Surrey, U.K.). EcoRI, Xba I, or BamHI sites were constructed into each forward and reverse PCR primer, respectively (indicated

within the primer sequence by underlined letters), to facilitate cloning into the Bluescript vector (Stratagene, La Jolla, USA). To identify specifically the two ARSA alleles of the patient, independent clones of fragment 1 were isolated and tested for the presence of the 459+1G>A mutation as described recently (Berger et al., 1993). Only clones without the 459+1G>A mutation were sequenced. Clones of fragment 2 were tested for the presence of the T391S (BsrI) polymorphism, C to G at position 2161 (Polten et al., 1991; Zlotogora et al., 1994), common in the 459+1G>A allele, using PCR primer Oli. 7 and Oli. 14. Only clones without the polymorphism were sequenced, using an automated fluorescence

TABLE 2. PCR Primers Used

Primer number	nt ^a	Sequence (5'→3') ^b
Oli. 7	2388	GGCAGCCCTGGCTGGGGCCCCACTGCCAAA
Oli. 14	3039	CAGGGTCCTGGACAGGTCATAGAGCTGC
Oli. 20	1391	CCTTGCACGGTGGCTGTGACCAGGGCCTGGTCTGCA
Oli. 21	1630	TGGAGTTAGCACTGGGTAGGGGTCAACGGG
Oli. 24	3090	CTGCAAGCCCTGAAACAGCTTCGGCTGCTC
Oli. 35	1719	CAGGGAGTCTCTAGATGGCCCGCGGGC
Oli. 63	2785	GCGGACTGGAAGTACAAGGCTCAC
Oli. 64	2929	GGGGCCAATTCTGTGCACAG
Oli. 85	570	GAGCCTGCTGGATCCAAGTAGCCCT
Oli. 86	1546	CTTCTCTGAAATTCTGCCTCTCAC
Oli. 87	3301	TCCCAGTGAGGATCCATCACATGCC
Oli. 101	3054	CTGCTGGGGGTGTGGCTGGTGGCCAC
Oli. 102	3146	GGACCACCTGGCTGGAGCCGAAG

^aNucleotide numbering is according to the genomic sequence EMBL data bank accession number X521150.

^bUnderlined sequences indicate mismatches compared to the genomic sequence EMBL data bank accession number X521150.

sequencing device (ABI-373A sequencer) and fluorescent dye deoxy terminator sequencing chemistry (ABI) according to the manufacturer's recommendations. Thereafter the entire ARSA gene of the father (I-1) was PCR amplified using Oli. 85 and Oli. 87. The PCR product was subcloned using the TA-Cloning system (Invitrogen, Leek, NL). The two alleles were subdivided by testing for the A464V mutation. The entire coding sequence of the ARSA allele without the A464V mutation was determined.

Test for ARSA Mutation A464V

A 115 bp PCR fragment, amplified with sense primer Oli. 101 and antisense primer Oli. 102, was digested with HaeIII (Promega, Madison, USA). Normal genomic DNA revealed two fragments of 70 and 45 bp, whereas the mutated DNA was not cleaved. PCR conditions were used as described above. Mismatches have been introduced into the forward and reverse PCR primers (indicated within the primer sequence by underlined letters, Table 2), to destroy additional natural HaeIII restriction sites.

Assay of ARSA Pseudodeficiency Allele

ARSA pseudodeficiency mutations (Gieselmann et al., 1989) PD1 (N-glycosylation sequence) and PD2 (loss of polyadenylation signal) were investigated either together as described by Gieselmann (1991), or in two separate reactions. To detect PD2, PCR primer ON A+ and, within a separate reaction, ON A- (primers described in Gieselmann, 1991) together with Oli. 24 and ON Asn were used. For the detection of PD1, primer ON Ser or ON Asn (Gieselmann, 1991) combined with Oli. 64 was used. As in the original PD assay an internal control fragment was amplified as well in each individual PCR reaction using primer ON 26 and ON 27 as described (Gieselmann, 1991). PD1 and PD2 were detected correctly using genomic DNA with confirmed sequences as template.

Testing for ARSA Polymorphisms

L/P76 polymorphism was tested as described by Berger et al., 1996.

W/C193 (BgII) polymorphism (Zlotogora et al., 1994) was investigated using the PCR primers Oli. 20 and Oli. 21 to amplify a 240 bp fragment digested with BgII into a 168 and a 72 bp fragment.

T/S391 (BsrI) polymorphism was investigated as described before for ARSA allele differentiation.

2193+20C/G (BamHI) polymorphism was tested using the PCR primers Oli. 63 and Oli. 64 amplifying a 144 bp fragment cleaved with BamHI into a 54 and a 90 bp fragment.

All PCR conditions were as described before.

RESULTS

Biochemical Findings and Diagnostic Aspects

Table 1 compiles ARSA activity in leukocytes and GS excretion in urine in the individuals of the family investigated and in various control groups. From the biochemical and clinical data sibling II-2 was diagnosed as late infantile MLD. Sibling II-3 was considered to be an MLD patient due to the biochemical findings; this presumptive diagnosis, however, was questioned by the lack of pathological clinical findings. A similar striking discrepancy between MLD-like biochemical data and apparent clinical health was encountered in sibling II-1. The parents of the siblings, obligate heterozygotes, were apparently healthy as well. Whereas the mother (I-2) presented biochemically (ARSA activity and GS excretion) in the range of MLD heterozygotes, the respective values of the father (I-1) were clearly in the MLD range (Table 1). To obtain insight into the genetic background of these puzzling inconsistencies between biochemical and clinical presentation, genotype studies were performed with the results given below.

ARSA Pseudodeficiency

We investigated PD1 and PD2 together in one reaction as well as in separate reactions for PD1 and PD2, since it was demonstrated recently that, in addition to the common ARSA-PD allele with PD1 and PD2 on one allele, these two mutations can occur on separate alleles (Leistner et al., 1995, Ricketts et al., 1996). All probands of the present family were negative, thus, ARSA-PD was ruled out as a possible cause of reduced ARSA activity levels.

ARSA Allele Analysis

ARSA mutation 459+1G>A.

Mutation analysis using a primer-modulated restriction fragment polymorphism showed that the mother (I-2) was affected by the frequent 0-type mutation 459+1G>A and transmitted 459+1G>A to all three children. (Fig 1).

Characterization of the individual

ARSA alleles.

To characterize the individual ARSA alleles within the family the common ARSA-polymorphisms W/C193 (BgII), T/S391 (BsrI), and 2193+20C/G (BamHI) were investigated. Results show that sibling II-2 inherited a different allele from his father (I-1) than siblings II-1 and II-3 (Fig. 1).

To identify the second, 459+1G>A unrelated ARSA allele of sibling II-3, DNA sequence analysis was performed. In an earlier study a thymidine to cytosine transition at nucleotide 376 in exon 2 had been detected, a missense mutation resulting in a substitution of leucine 76 by proline. This mutation was shown to be a common polymorphism (Berger et al., 1996). Furthermore, a cytosine to thymidine transition at nucleotide 2435 in exon 8 was disclosed. This missense mutation results in a substitution of alanine 464 by valine (A464V). To make sure that A464V was not an amplification artifact and to investigate the distribution of this mutation within the family, a PCR based HaeIII restriction assay was performed (data not shown). This assay clearly demonstrated the presence of A464V in the father (I-1) and siblings II-1 and II-3, but neither in sibling II-2, having suffered from MLD, nor in the mother (I-2). A464V was absent in DNA from 50 unrelated controls. In 18 unrelated MLD patients A464V was not encountered, suggesting a low frequency of this mutation in MLD.

To reveal the ARSA mutation which, together with 459+1G>A, led to the manifestation of late infantile MLD in sibling II-2, DNA sequencing had to be done in the father (I-1), since in sibling II-2 only paraffin-embedded, formalin-fixed brain tissue was available, only limited suitable for sequence analysis of the entire gene. Thus, the entire ARSA gene of I-1 was PCR amplified, cloned, and the alleles were distinguished using the A464V detection assay. The ARSA allele without the A464V mutation was sequenced, and a single basepair microdeletion of a guanine residue immediately after the start codon was found. This deletion caused a frameshift, leading to a stop codon after 24 amino acids (del7G, Fig. 1).

In addition to the described mutations and polymorphisms within the coding region (Fig. 1) we observed in both entirely sequenced ARSA alleles 10 further polymorphisms in the non-coding region as compared to the genomic ARSA sequence (emb. X52150). Eight of these polymorphisms (459+56delC, 459+61delC, 459+63insC, 678+36delT, 1101+69insG, 1101+76insC, 1101+197insC, 1101+218insG, and 1204+32delA) were also present in the sequence of chromosome 22q13 (gb U62317), whereas two (459+71G>T, 1101+223C>T) were different.

DISCUSSION

In a family comprised of two unrelated parents and three children, one sibling (II-2) suffered from

classical late infantile MLD with typical biochemical findings, i.e. severe deficiency of ARSA activity and markedly increased urinary GS excretion. The two other siblings (II-1 and II-3), presently at age 15 and 12½, respectively, were clinical normal (II-3) and apparently healthy (II-1), but displayed ARSA and GS values in the range of MLD patients. Similar pathobiochemical findings were seen in the apparently healthy father (I-1). The healthy mother (I-2) presented ARSA and GS clearly in the MLD heterozygote range. To elucidate these striking discrepancies between health state and biochemical findings, ARSA genotypes were investigated in all five probands.

The finding of three different ARSA mutations in our family offered, on a molecular basis, a plausible explanation for intrafamilial phenotypic heterogeneity. Coincidence of three mutations was rather unexpected, since MLD is a rare disorder with an estimated frequency of 0.6 to 1 in 100,000 (Kolodny and Fluharty 1995; Heim et al., 1997).

Sibling II-2, affected with late infantile MLD, was a compound heterozygote for the 459+1G>A mutation, inherited from his mother (I-2), and a novel mutation, del7G, on his paternal allele. 459+1G>A is known to be a frequent 0-type mutation; del7G, due to its molecular mechanism, must be an 0-type mutation as well with lack of detectable ARSA protein and activity. Thus, the 459+1G>A/del7G genotype is an 0/0 genotype, which, according to the described genotype-phenotype correlation (Polten et al., 1991) will cause late infantile MLD, as seen indeed in sibling II-2.

Siblings II-1 and II-3, so far apparently healthy, also carried the 459+1G>A allele from their mother (I-2). The other ARSA allele, however, carried another novel ARSA mutation, A464V, a substitution of alanine by valine at amino acid 464. Alanine 464 is conserved between ARSA and sea urchin arylsulfatase, but not between human arylsulfatases A, B, and C. A464V seems to be a unique or very rare ARSA mutation, since it was not found in 136 unrelated ARSA alleles (18 MLD patients and 50 controls). On the other hand, substitution of alanine by valine at another site of the ARSA gene was described being a mutation causing MLD (Barth et al., 1993). In the case of A464V it is conceivable that the substitution of alanine by valine, due to the related structures of these two amino acids, would lead to a relative mild impairment of ARSA protein and thus would reduce, but not abolish, the enzyme activity. This view seems to be corroborated by the apparently healthy state of siblings II-1 and II-3 as well as of their fa-

ther (I-1), all carrying A464V in combination with an O-type mutation (459+1G>A and del7G, respectively). A comparison of the biochemical data of probands I-1, II-1, and II-3 with the values of control groups (Table 1) lend evidence to the view that the A464V allele may possess enzymatic activity at a level between the known R-type alleles (e.g. P426L) and the common ARSA-PD allele. Interestingly, in ARSA-PD/MLD compound heterozygotes, GS concentrations were higher and ARSA activities lower than in MLD heterozygotes (Table 1). Fortunately, notwithstanding elevated GS excretion in urine, impairment of ARSA activity in probands I-1, II-1, and II-3 seemed to be not that pronounced to affect central or peripheral myelin seriously.

Though clinical heterogeneity within families is not common in MLD (Kolodny and Fluharty, 1995), the striking discrepancies between biochemical and clinical findings in our family do not seem to be a unique phenomenon. Clarke et al. (1989) described two siblings with biochemical findings characteristic for MLD. Whereas one sibling suffered from typical juvenile MLD, beginning at 9 years of age and leading to death at age 18, his brother presented at age 16 years with acute cholecystitis due to sulfatide accumulation, but was neurologically and psychometrically normal at age 21. We could not detect mutation A464V in a DNA preparation from the latter sibling (data not shown). Obviously, ARSA alleles comprise quite a spectrum of mutations, leading biochemically from the lack of enzyme activity (O-type mutations) to medium or even high residual activities (R-type mutations or enzyme pseudodeficiency) and clinically to a spectrum of disease types ranging from severe MLD to ARSA-PD without clinical findings. The R-type mutation I179S, for example, although relatively frequent in causing juvenile or adult MLD in compound heterozygotes with an O-type mutation (e. g. I179S/459+1G>A), was never found as a homozygous MLD genotype. This might be due to a relatively mild functional ARSA impairment by the I179S allele and functionally sufficient enzyme activity in the homozygote state.

Our results demonstrate very clearly that pathological biochemical data—ARSA activity and GS excretion within the common range of MLD patients—need not necessarily be paralleled by clinical manifestation of MLD. Although the final clinical outcome in probands II-1 and II-3 is not yet clear, the healthy state of their father (proband I-1) at an age of 42 years gives rise to hope for a favorable prognosis. For future biochemical diag-

nosis of MLD it should be kept in mind that, in certain cases, ARSA alleles may be present which, in combination with O-type mutations, exhibit enzyme activity levels and GS excretions in the pathological range, but may not cause clinical outbreak of MLD.

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