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Identification of a Mutation in the Arylsulfatase A Gene of a Patient with Adult-Type Metachromatic Leukodystrophy

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Summary

To analyze the genetic abnormality in a Japanese patient with adult-type metachromatic leukodystrophy (MLD), we first elucidated the genomic organization of the human arylsulfatase A (ASA) gene and then compared the nucleotide sequences of exons and splice junctions of the mutant ASA gene to those of a normal control. We have identified a new mutation, a G-to-A transition in exon 2, which results in amino acid substitution of Asp for ^{99}Gly. In a transient expression study, COS cells transfected with the mutant cDNA carrying ^{99}Gly→Asp did not show an increase of ASA activity, which confirms that the mutation is a cause of adult-type MLD.

Introduction

Metachromatic leukodystrophy (MLD) is a lysosomal storage disease caused by a deficiency of arylsulfatase A (ASA) (E.C.3.1.6.1). It is characterized by accumulation of sulfatide in the white matter of the central nervous system and in the peripheral nerves (Kolodny and Moser 1983).

MLD is known to have three clinical phenotypes, namely, late infantile type, juvenile type, and adult type. The clinical symptoms of the late-infantile-type MLD start between the first and the second years. Children with late infantile type do not usually survive beyond the first decade. Juvenile-type MLD designates those cases with age at onset between 3 and 16 years. Adult-type MLD may begin at almost any age beyond puberty. In each of these variants the earliest signs are gait disturbance, mental regression, and urinary incontinence. In the childhood variants, other common signs are blindness, loss of speech, quadriparesis, and peripheral neuropathy. In the adult type, dementia is the major presenting sign, and the disease may progress slowly over several decades (Kolodny and Moser 1983). In addition to these clinical phenotypes, deficiency of ASA is occasionally found in apparently healthy individuals, and is called ASA pseudodeficiency (Dubois et al. 1975). The molecular basis for the heterogeneity of clinical phenotypes is not fully understood.

Very recently, molecular cloning of a full-length cDNA for human ASA was reported (Stein et al. 1989). With the availability of the ASA cDNA, loss of a polyadenylylation signal and an *N*-glycosylation site were discovered as the cause of ASA pseudodeficiency (Gieselmann et al. 1989), which confirms the previous hypothesis that MLD and ASA pseudodeficiency are caused by allelic mutations (Bach and Neufeld 1983; Chang and Davidson 1983).

To better understand the molecular mechanisms of adult type MLD, we first elucidated ASA gene organizations and identified a mutation in ASA gene of a patient with adult-type MLD by nucleotide sequence analysis of the ASA gene referring to ASA cDNA sequences (Stein et al. 1989).

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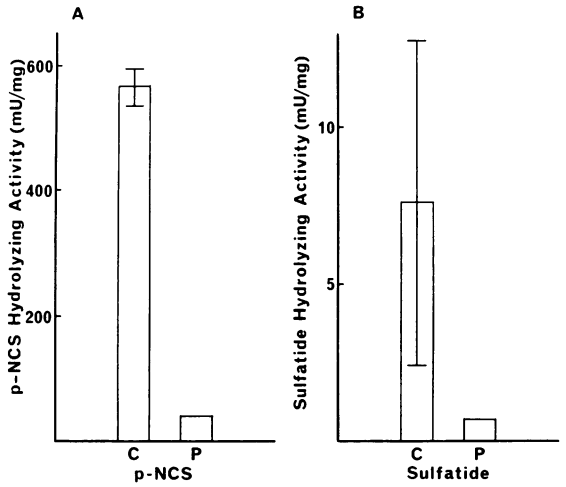


Figure 1 ASA activities toward *p*-nitrocatechol sulfate (*A*) and sulfatide (*B*) in cultured skin fibroblasts. C = control; P = adult-type MLD patient. Vertical bars represent SEM (n = 3). ASA activity of the adult type MLD was markedly reduced to 7% and 9% of normal values toward *p*-nitrocatechol sulfate and sulfatide, respectively.

Material and Methods

Case Report

The patient is a 35-year-old female. There is no consanguinity in her parents. At the age of 24, slowly progressing dementia appeared, and by the age of 30 she became severely demented and required assistance for daily activities. A computed tomography scan revealed brain atrophy and low density of white matter, and nerve conduction velocities were found to be decreased to 22.3 m/s in ulnar nerve (normal range 59.9 ± 5.7 m/s) and 14.1 m/s in tibial nerve (normal range 49.9 ± 5.2 m/s). Pronounced reduction in myelinated fibers, thinning of myelin, and accumulation of metachromatic materials were observed by sural nerve biopsy. ASA activities toward *p*-nitrocatechol sulfate and sulfatide in cultured skin fibroblasts of the patient were found to be markedly reduced, to 7% and 9% of the normal values, respectively (fig. 1) (Baum et al. 1959; Porter et al. 1972; Fluharty and Edmond 1978).

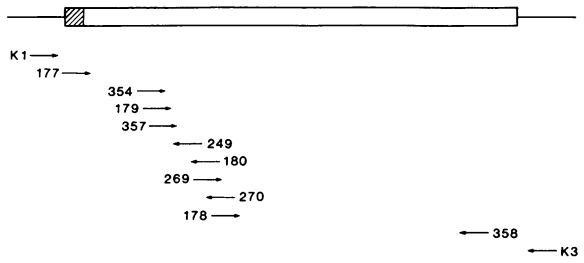


Figure 2 Schematic illustration of ASA cDNA and positions of oligonucleotide primers. The open box represents the region coding for the mature ASA protein and the hatched box represents the signal peptide.

Nucleotide Sequence Analysis of Arylsulfatase A Genes

ASA genes of a normal subject and the patient were amplified in three parts from genomic DNA extracted from leukocytes by PCR (Saiki et al. 1988) using oligonucleotide primer pairs of (1) K1 and 180, (2) 178 and K3, and (3) 269 and 270 (fig. 2; table 1). The conditions for PCR consist of 30 cycles of 1 min dena-

Table 1

List of Oligonucleotide Primers

Oligonucleotide Primer	Nucleotide No. of ASA cDNA[a]	Sequence of Oligonucleotide Primer
K1	− 118 to − 94	5′-GGGAATTCTATTTGGGTCCGGGGTCTCAGGGAA-3′
K3	1648 to 1625	5′-GGGAATTCTCAGATGTGCAAGTCTCCACTGGTG-3′
177	− 8 to 16	5′-CCATGTCCATGGGGGCACCGCGGT-3′
178	494 to 517	5′-CCACTCCTTGCGACGGTGGCTGTG-3′
179	263 to 286	5′-ACCCTGGCGTCCTGGTGCCCAGCT-3′
180	517 to 498	5′-GGGAATTCCACAGCCACCGTCGCAAGGA-3′
249	459 to 440	5′-CTGGTCGTGGGAGTACGGGA-3′
269	434 to 455	5′-TAGGCATCCCGTACTCCCACGA-3′
270	567 to 684	5′-GGGAATTCCGCCTCCACGGACAGGTT-3′
354	238 to 259	5′-CGGCTCCCGGTTCGGATGGGCA-3′
357	282 to 305	5′-CAGCTCCCGGGGGGACCTGCCCCT-3′
358	1424 to 1403	5′-CCTCGCCCCGGGCCACCTGGCT-3′

NOTE.—K1, K3, 180, and 270 contain an *Eco*RI linker sequence at the 5′ end.

[a] From Stein et al. 1989.

turation at 93°C, 1 min annealing at 55°C, and 4 min extension at 72°C. The PCR products were subcloned into a plasmid vector, pUC19 (Yanisch-Perron et al. 1985), and then sequenced by the dideoxynucleotide chain terminator method using double stranded DNA as a template (Sanger et al. 1977; Chen and Seeburg 1985).

Northern Blotting Analysis

Twenty micrograms of total RNA extracted from cultured skin fibroblasts of a normal subject and the patient were electrophoresed through 1.2% denaturing agarose gel containing 0.66 M formaldehyde, transblotted to a nitrocellulose membrane, and hybridized with ^{32}P-labeled full-length human ASA cDNA (pcD2-ASA7), which was isolated in our laboratory during this work. The filter was finally washed in 0.1 × SSC, 0.1% SDS for 30 min at 68°C (Maniatis et al. 1989).

Molecular Cloning of Human ASA cDNA

First, human ASA cDNA was amplified by PCR with oligonucleotide primer pairs of (1) 177 and 180 and (2) 179 and K3 (fig. 2; table 1) using total plasmid DNA prepared from a human fibroblast cDNA library (pcD2 vector cDNA library; Chen and Okayama 1987) as a template. Two fragments of cDNA were subcloned into pUC19 and connected at the *Xma*I site. Using the 5′ fragment of the ASA cDNA as a probe, full-length human ASA cDNAs (pcD2-ASA2, pcD2-ASA7, and pcD2-ASA8) were isolated from pcD2 vector cDNA library (Maniatis et al. 1989). All the cDNAs were 2 kbp in length and showed identical restriction maps.

In Vitro Mutagenesis

A part of the ASA cDNA (pcD2-ASA2) was amplified by PCR using oligonucleotide primer pairs of 357 and 358 (fig. 2; table 1), of which primer 357 carries a G-to-A mutation identified in the exon 2 of the patient's ASA gene. The PCR product was digested with *Xma*I and ligated to *Xma*I-cleaved pcD2-ASA2. The structure of the mutant cDNA (pcD2-ASA2M) was confirmed by restriction mapping as well as nucleotide sequence analysis.

Transient Expression study

COS cells at a density of 5 × 10^5 cells/25 cm^2 were transfected with 20 μg of plasmid DNAs containing the normal ASA cDNA (pcD2-ASA2, pcD2-ASA7, and pcD2-ASA8) and the mutant ASA cDNA (pcD2-ASA2M) by the calcium phosphate coprecipitation method (Maniatis et al. 1989). The COS cells were harvested 50 h after the transfection and the ASA activities were measured using *p*-nitrocatechol sulfate as a substrate. Furthermore, western blotting analysis was performed using 20 μg of COS cell extracts by the avidin-biotin complex method (Inuzuka et al. 1989).

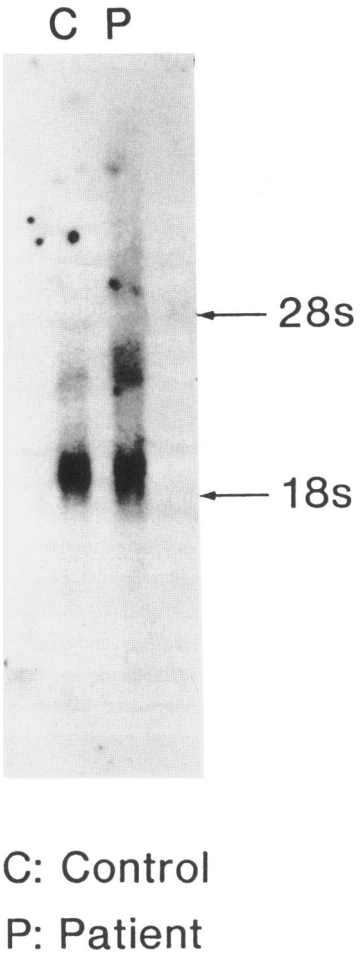


Figure 3 Northern blotting analysis. Twenty micrograms of total RNA extracted from cultured skin fibroblasts of a normal subject and the patient were electrophoresed through 1.2% denaturing agarose gel, transblotted to a nitrocellulose membrane, and hybridized with ^{32}P-labeled full-length human ASA cDNA. The filter was finally washed in 0.1 × SSC, 0.1% SDS at 68°C for 30 min.

HaeIII RFLP

A part of exon 2 of ASA genes including the single-base mutation site was amplified by PCR using the oligonucleotide primers 354 and 249 (fig. 2; table 1). The PCR products were digested by a restriction en-

zyme, *Hae*III, and were electrophoresed through 10% polyacrylamide slab gel followed by staining with ethidium bromide.

Results

On northern blotting analysis, we detected a normal amount of ASA mRNA with the same size in the patient's fibroblasts (fig. 3). Since the result suggests that there is probably no abnormality in the transcription of the patient's ASA gene, we took a strategy of analyzing the exons and splice junctions of the patient's ASA gene.

The exon-intron structures of the ASA gene were determined by nucleotide sequence analysis of the PCR products subcloned into pUC19. The coding region of the human ASA gene was found to be approximately 3 kbp in length and consist of eight exons (fig. 4).

Table 2 shows nucleotide sequences of each splice donor and acceptor site of the human ASA gene. All junctions show consensus sequence. The genomic organization of the ASA gene described here is consistent with the structures described very recently by Kreysing et al. (1990). It was found that the patient's ASA gene had an identical structure.

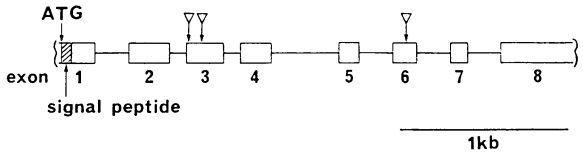


Figure 4 Structure of coding regions of human ASA gene. The coding region of the human ASA gene is approximately 3 kbp in length and consists of eight exons. The three open triangles indicate potential *N*-linked glycosylation sites.

To identify the mutation, we compared nucleotide sequences of each exon and splice junction of the patient's ASA gene with those of a normal subject.

Throughout all exons and splice junctions, we found only one G-to-A transition in exon 2, which results in an amino acid substitution of aspartic acid for ^{99}glycine (fig. 5). The mutation was confirmed by sequencing 2 individual clones.

To confirm the presence of the mutation in the pa-

Table 2

Nucleotide Sequences of Each Splicing Donor and Acceptor Site of Human ASA Gene

Exon	Exon Size (bp)	Sequence of Exon-Intron Junction		Intron Size (kb)
		5′ Border	3′ Border	
1	>226		. . . TCTAG gtaaagaggg	~.2
			218	
			Arg-73	
2	241	tgtcccgcag GGCCG . . .	. . . ACCAG gtaggaacca	~.1
		219	459	
		Arg-73	Gln-153	
3	219	cccgcctcag GGCCC . . .	. . . CTCAC gtaagtatct	~.1
		460	678	
		Gly-154	His-226	
4	170	ttgcccccag CACAC . . .	. . . AATGG gtatgccagc	~.4
		679	848	
		His-227	Gly-283	
5	125	tgtgtcccag ACCTG . . .	. . . TCCCG gtcagtccgc	~.2
		849	973	
		Gly-283	Gly-325	
6	128	ctgcccccag GCGTG . . .	. . . GCAAG gtagggccgg	~.2
		974	1101	
		Gly-325	Lys-367	
7	103	ctgcccccag AGCCC . . .	. . . CCAGG gtaacccctc	~.2
		1102	1204	
		Ser-368	Gly-402	
8	>443	ccctccccag GCTCT . . .		
		1205		
		Gly-402		

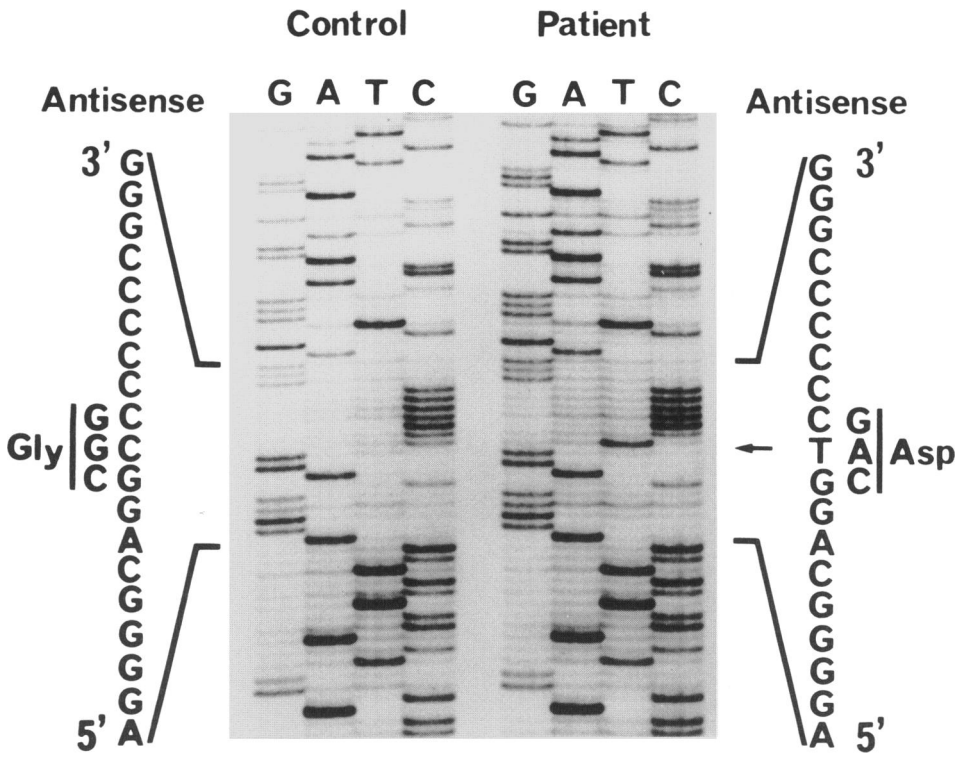
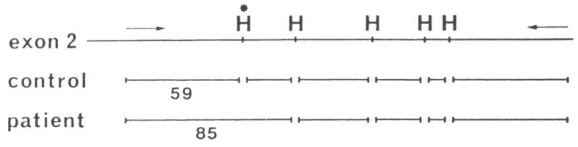
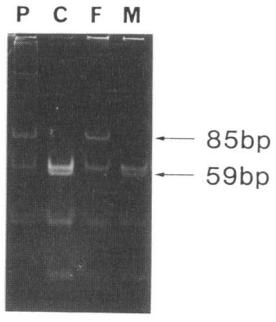


Figure 5 Nucleotide sequence analysis of exon 2 of normal and patient's ASA genes. Only one G-to-A transition was found in exon 2 of the patient's ASA gene, which results in an amino acid substitution of aspartic acid for ^{99}glycine.





P: Patient
C: Control
F: Father
M: Mother

tient's and the parents' genomic DNA, as well as to determine whether the single-base mutation was unique to the patient, we examined the *Hae*III RFLP of the PCR products as described in Material and Methods. The base substitution would result in the loss of the *Hae*III cutting site, which in turn would produce a larger DNA fragment, of 85 bp instead of 59 bp. The patient and the father had both 85-bp and 59-bp DNA fragments, whereas the mother and 12 unrelated controls had only 59-bp fragments (fig. 6). The results were confirmed by extensive digestion by

Figure 6 *Hae*III RFLP analysis of normal, patient's, and parents' ASA genes. A part of exon 2 of the ASA genes including the mutation site was amplified by PCR using primers 354 and 249. The PCR products were digested by *Hae*III and electrophoresed through 10% polyacrylamide slab gel followed by staining with ethidium bromide. Both 85-bp and 59-bp DNA fragments were observed in the patient and the father, whereas only a 59-bp DNA fragment was observed in the mother and normal subjects. A 26-bp fragment generated by *Hae*III digestion of the PCR products derived from the normal allele, not from the ^{99}Gly→Asp mutant allele, comigrate with another 26-bp fragment.

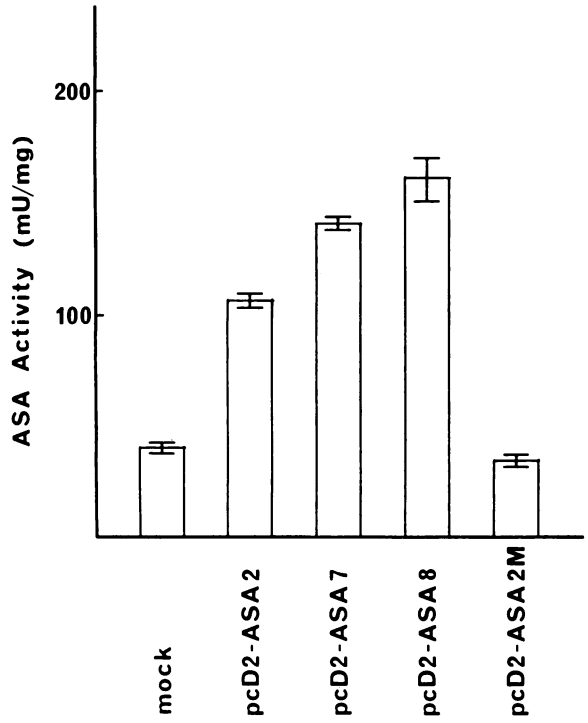


Figure 7 Transient expression study. COS cells at a density of 5×10^5 cells/25 cm^2 were transfected with 20 μg of normal (pcD2-ASA2, pcD2-ASA7, and pcD2-ASA8) and mutant (pcD2-ASA2M) ASA cDNAs. The COS cells were harvested 50 h after the transfection, and the ASA activities were measured using *p*-nitrocatechol sulfate. Vertical bars represent SEM (n = 4).

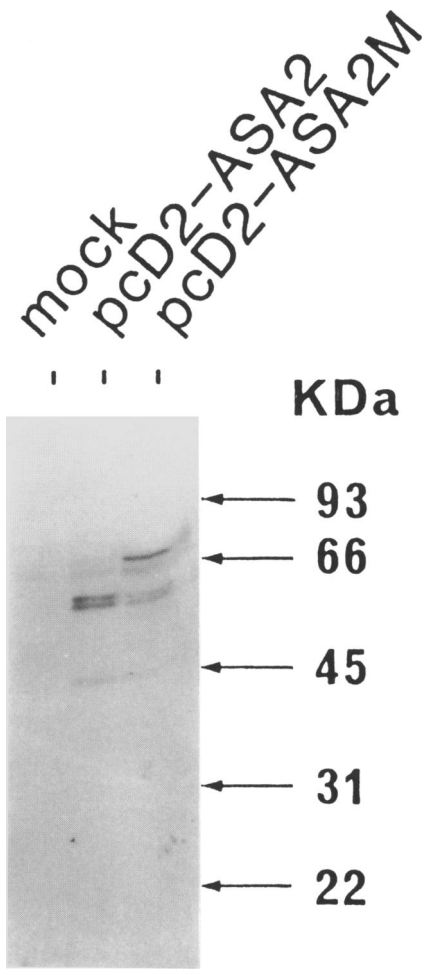


Figure 8 Western blotting analysis. Twenty micrograms of protein extracted from COS cells was electrophoresed through 10% SDS–polyacrylamide slab gel and transblotted to a nitrocellulose membrane. The blot was incubated with anti-ASA rabbit serum and visualized by peroxidase reaction.

*Hae*III, which excludes the possibility of partial digestion.

To confirm that the loss of ASA enzyme activity is caused by the mutation identified in exon 2, we generated a mutant cDNA in the pcD2 vector by in vitro mutagenesis. The mutant cDNA, pcD2-ASA2M, carries the G-to-A mutation but is otherwise identical to pcD2-ASA2.

In a transient expression study, it was clearly demonstrated that COS cells transfected with the mutant cDNA (pcD2-ASA2M) did not show an increase of ASA activity, whereas COS cells transfected with normal cDNA (pcD2-ASA2, pcD2-ASA7, and pcD2-ASA8) showed three to four times as much ASA activity (fig. 7).

In western blotting analysis, the COS cells transfected with the mutant and normal cDNA showed substantial amounts of materials cross-reactive to anti-ASA antibody (fig. 8). In addition to the major bands of cross-reacting materials with apparent molecular weights of 43 kDa and 58 kDa, relatively larger amounts of cross-reacting materials of 66 kDa were observed in COS cells transfected with the mutant cDNA.

Discussion

In the present study, we have described a new point mutation in exon 2 of the ASA gene in a Japanese patient with adult-type MLD. This mutation results in an amino acid substitution of aspartic acid (GAC) for glycine (GGC) at codon 99 of the ASA protein. As shown in *Hae*III RFLP analysis, the single-base mutation was unique to the patient, and the patient had one mutant allele with a ^{99}Gly→Asp mutation de-

rived from the father and another mutant allele with an undetermined mutation derived from the mother.

Since the substitution is from a neutral amino acid to an acidic one, there is a possibility of radically altering the structure of the ASA protein. Actually, in a transient expression study, it was confirmed that the mutation we identified abolished the ASA activity. Furthermore, a slightly different pattern of cross-reacting materials of COS cells transfected with the mutant cDNA suggests a possibility of altered processing of the ASA protein.

Very recently, two mutant alleles were identified by Gieselmann et al. (1990) in late infantile, juvenile, and adult forms of MLD. They proposed a simple two allele–three genotype model which accounts for the phenotype variability of MLD (Gieselmann et al. 1990). One allele carries two point mutations: ^{193}Try →Cys and ^{391}Thr→Ser (allele 193/391). The other allele carries one mutation: ^{426}Pro→Leu (allele 426). A patient with late infantile type was homozygous for allele 193/391. A patient with juvenile type was a compound heterozygote having both alleles, 193/391 and 426. Two adult-type patients were homozygous for allele 426. This model is of interest but unable to explain all MLD cases. As presented in this paper, our adult-type MLD patient does not carry either of the mutations described by Gieselmann et al. (1990) and is a compound heterozygote, having the ^{99}Gly→Asp mutation and another yet undetermined mutation. Therefore, it seems that MLD is genetically more heterogeneous, and there might be additional mutations in the other cases.

Our current knowledge of the molecular basis for the three clinical phenotypes of MLD is still not complete, and detailed analyses of mutations in the ASA gene as well as enzymatic studies of mutant ASA proteins should give us more insight into the molecular mechanisms responsible for each clinical phenotype.

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