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Mutation analysis of the ALD gene in seven Japanese families with X-linked adrenoleukodystrophy

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Abstract The childhood cerebral form of X-linked adrenoleukodystrophy (X-ALD) is a severe congenital metabolic disease without a definite effective therapy except for hematopoietic stem cell transplantation in the appropriate disease stage. Seven Japanese families with X-ALD were analyzed for mutations in the ALD gene (*ALD*). Of the seven families, three were referred to us for prenatal diagnosis, four for carrier detection, and three for confirmation diagnosis of patients. By nucleotide sequencing and/or restriction analysis, all the subjects to be examined were successfully diagnosed. Six different missense mutations in *ALD* were identified. There was a G→A substitution (G512S) in two unrelated families, and a G→A (R617H), a C→T (R660W), a G→C (R163P), a C→T (S606L), or a G→A (G116E) substitution in each of the other five families. Among the six substitutions, five were those reported previously and the other was a novel mutation. In three families, prenatal diagnosis was carried out after genetic counseling.

Key words X-linked adrenoleukodystrophy · ALD gene · Missense mutation · Carrier detection · Prenatal diagnosis · Genetic counseling

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Introduction

X-linked adrenoleukodystrophy (X-ALD) is a congenital peroxisomal disorder caused by mutations in the ALD protein gene (*ALD* or *ABCD1*) on Xq28 (Mosser et al. 1993). Its phenotype is variable, ranging from severe childhood ALD to slowly progressive adrenomyeloneuropathy (AMN) and Addison disease only (Moser et al. 2000). Moreover, there are clinical variations within a family. Childhood ALD is characterized by progressive cerebral dysfunction that is associated with an inflammatory response in the cerebral white matter and by adrenal insufficiency. Biochemically, a saturated very long chain fatty acid (VLCFA), especially hexacosanoic acid (C26:0) and tetracosanoic acid (C24:0), accumulates in nervous system white matter, the adrenal cortex, and in serum. The catabolization of VLCFA to its coenzyme-A (CoA) derivative in the process of beta-oxidation in peroxisome is impaired in X-ALD patients (Lazo et al. 1988); the *ALD* product does not encode VLCFA acyl CoA synthase, but instead encodes peroxisomal membrane ATP-binding cassette (ABC) transporter (Mosser et al. 1993). Several approaches have been tried to treat the neurological manifestations. A dietary therapy called "Lorenzo's oil" does not significantly affect the clinical course (van Geel et al. 1999). Some agents that up-regulate the expression of an *ALD* homologue (*ALDPR*) and some immunosuppressors have been tested (Moser et al. 2000). Shapiro et al. (2000) showed that bone marrow transplantation had a good effect in some patients, although age- and stage-based selection of patients was important.

The diagnosis of X-ALD is based on clinical manifestations, including neurological signs and symptoms, serum VLCFA levels, brain magnetic resonance imaging (MRI) findings, and hormonal data, as well as on ALD gene analysis. However, the serum VLCFA level is sometimes in the normal range in heterozygous women (Moser et al. 1999), and, in some pedigrees, probands may be already dead and thus no materials for DNA analysis are available. Because large genomic rearrangements are infrequent, nucleotide

sequence analysis is necessary for most pedigrees, especially for carrier diagnosis (Boehm et al. 1999).

More than 300 different mutations in *ALD* have been identified and registered in a database (http://www.x-ald.nl/). Among point mutations and large rearrangements reported, base substitutions were most frequent and distributed over the gene. There are no differences in distribution among different populations (Takano et al. 1999) and there is no genotype–phenotype correlation, suggesting the presence of factors that modify the phenotype (Maestri and Beaty 1992).

Here we report the results of mutation analysis of seven Japanese families with ALD.

Subjects and methods

Families studied

The subjects studied included seven Japanese families with ALD or AMN. The family histories and purposes of molecular diagnosis of diseases are summarized in Table 1.

Family 1. This family was described previously (Matsumoto et al. 1994) and included five affected members. The proband with AMN had two carrier daughters according to their serum VLCFA values. The elder daughter was pregnant three times. The younger daughter had one son (grandson) who was diagnosed to be a presymptomatic patient because of a high serum level of VLCFA.

Family 2. The proband was a 14-year-old boy. He had regression of mental development since the age of 8 years, had developed motor dysfunction rapidly, and was bedridden since the age of 12 years. His serum VLCFA level was high, and brain MRI demonstrated a high-intensity area in the anterior lobes. He was receiving gamma-globulin therapy once a month, and his clinical manifestations improved moderately and stabilized thereafter. The grandfather was suspected clinically to have had AMN and died at the age of 43 years.

Family 3. Two brothers with ALD both died around the age of 10 years. A sister was diagnosed as a carrier based on a high serum VLCFA value. She was pregnant and referred to us at 11 weeks of gestation for prenatal diagnosis after receiving genetic counseling.

Family 4. The proband was diagnosed clinically to have had AMN and was deceased. His daughter became pregnant and was referred to us at 11 weeks of gestation for prenatal diagnosis after genetic counseling.

Family 5. The proband was a 20-year-old man. Right hemiplegia occurred at 20 years of age. His serum VLCFA level was high and brain MRI showed a high-intensity area at the left hemisphere, basal ganglia, and the midbrain. There was no other family member with ALD or AMN.

Family 6. A boy complained of visual disturbance at the age of 5 years. Brain computed tomography demonstrated high-intensity areas around the occipital regions. His serum VLCFA and adrenocorticotropic hormone values were high. He seems to be a sporadic case in his family.

Family 7. The proband was a 24-year-old man with Down syndrome. The onset of ALD with a visual disturbance was estimated at around 13 years of age, when he was diagnosed clinically with X-ALD with a high serum VLCFA level and abnormal brain MRI findings. The maternal grandfather was said not to have been able to walk since the age of 50 years and had died 5 years later of uncertain cause. The mother showed a higher level of serum VLCFA, corresponding with that of a carrier. An elder sister of the proband had a normal serum value of VLCFA, but could not be excluded as a carrier. The proband's sister and mother were referred to us for carrier diagnosis.

Mutation analysis of *ALDP*

Genomic DNA and/or RNA were extracted from chorionic villus samples in pregnant women in families 1, 3, and 4. DNA/RNA was also prepared from peripheral blood leukocytes of probands in families 2, 5, 6, and 7, and of their relatives. All these samples were taken after informed

Table 1. *ALD* mutations in seven families analyzed

Family	Family history	Purpose of molecular diagnosis	Base substitution (AA change)	Restriction sites[a]	
				Wild-type	Mutant
1	AMN/ALD	CD/PD	CGC→CAC (R617H)	*Aci*I	*Afl*III
2	AMN/ALD	D	GGC→AGC (G512S)	*Aci*I	*Pst*I
3	ALD	CD/PD	CGG→TGG (R660W)	*Msp*I	—
4	AMN	CD/PD	CGC→CCC (R163P)	*Aci*I	—
5	ALD	D	TCG→TTG (S606L)	—	—
6	ALD	D	GGA→GAA (G116E)	—	—
7	ALD	CD/D	GGC→AGC (G512S)	*Aci*I	*Pst*I

AMN, Adrenomyeloneuropathy; ALD, adrenoleukodystrophy; CD, carrier detection; PD, prenatal diagnosis; D, diagnosis of ALD in a patient
[a] Restriction enzymes cutting wild-type and mutant sequences

Table 2. PCR primers used to amplify *ALDP*

Primer no.	Exon to be amplified	Primer sequences (5′ to 3′)	
		Forward	Reverse
1	1	AGCAACAATCCTTCCAGCCA	AATACCCGGTTCATGCCAGCT
2	1	CTATGGAGCCCACAAAGTCTA	CGGTAGTAGGTCTGCTGGGA
3	1	TACCTGGAGGGCCAACTGGC	AGACTGTCCCCACCGCTCAC
4	2	ATGGCCAGGAAGCCTCTCTG	TGGCCTGGATCCTCTTGGAG
5	3, 4	TGACTTTCCGCTGTCTCTGC	ACCTCAGCACCTGCAGCAGC
6	5	AGACTCCCCAGAATGCAGAG	AGGCTTGCATATGTGCGTGG
7	6	ATTGGGAGCCTCTCAAGGC	GCTGACTCTTCACCACTTCC
8	7	GGAAGTGGTGAAGAGTCAGC	TAGCAGCCTCTGCCCCGCCCCGT
9	8, 9	TGAGCCAAGACCATTGCCCCC	ATGACAGCCGCCTGCTGCTGCCGG
10	10	CGGCTGTCATCAGCAGCCCC	TGCAGGGGCGGGGTGCGTGCATG

PCR, Polymerase chain reaction

consent was obtained; in the case of prenatal/carrier diagnosis, it was obtained after genetic counseling. Primer pairs for polymerase chain reaction (PCR) were synthesized according to the sequences of reported *ALD* compared with the highly homologous *ALDPR* (Holzinger et al. 1999; Table 2). Primers for the sex-determining region Y gene (*SRY*) and the amelogenin gene (*AMELY*) on the Y chromosome were generated for sex diagnosis of the fetuses. PCR and/or reverse transcriptase-PCR was performed with denaturation at 94°C for 30 s, annealing at an appropriate temperature for each primer set for 1 min, and extension at 72°C for 1 min. PCR products were directly sequenced by an ABI 3100 sequencer (PE Applied Biosystems, Foster City, CA, USA) as recommended by the manufacturer.

Results

All subjects studied had a base substitution in *ALD*, leading to a missense mutation (Table 1). The mutations identified were diverse, except in two unrelated families (families 2 and 7).

In family 1, the first fetus (grandson) of the elder daughter was diagnosed as an affected male and was terminated at 20 weeks of gestation. The two fetuses that followed were diagnosed prenatally as normal females by sex determination and PCR-restriction fragment length polymorphism (RFLP) analysis of *ALD*. The younger daughter became pregnant again, and a healthy boy was born after prenatal diagnosis by PCR-RFLP analysis. His umbilical cord blood was stored, and then cord stem cell transplantation was performed in the boy, as strongly desired by the mother, 6 years ago. The boy has no neurological signs or any brain abnormality.

The *ALD* sequencing of a chorionic villus sample for a male fetus of family 3 revealed a C→T mutation at codon 660 (R660W). Because this nucleotide substitution results in a loss of an *Msp*I restriction site, the wild-type and mutant alleles are distinguished by PCR-RFLP analysis. With such an analysis, the pregnant mother and the grandmother in this family were successfully diagnosed as carriers. A few months after termination of the pregnancy, the mother was pregnant again and a second chorionic villus sampling was performed. Sex diagnosis using *SRY* and *AMELY*, and PCR-RFLP analysis, revealed that the fetus in the second pregnancy was a female carrier. The fetus of family 4 was diagnosed as a female by *SRY* and *AMELY* analysis, and then the pregnant mother was analyzed. The results showed that the mother was heterozygous (carrier) for a G-to-C substitution at codon 163 (R163P). In this family, the carrier status of the fetus was not analyzed because of an insufficient amount of specimens. A mutation (GGA→GAA, G116E) in family 6 is a novel one. The proband of family 7 had a G→A substitution at codon 512 (G512S), and an *Aci*I restriction site was lost and a *Pst*I site was newly generated. PCR-RFLP analysis with these enzymes revealed that the mother in this family was a carrier and that the sister of the proband was homozygous for the wild-type allele.

Discussion

Six different missense mutations were detected in seven families in the present series. They included CGC→CAC at codon 617 (R617H) in family 1, GGC→AGC (G512S) in families 2 and 7, CGG → TGG (R660W) in family 3, CGC →CCC (R163P) in family 4, TCG→TTG (S606L) in family 5, and GGA→GAA (G116E) in family 6 (Table 1). Of the six sites where the mutations occurred, four (codons 512, 606, 617, and 660) were those highly conserved in ABC transporter superfamily genes, and mutations at codons 512 and 606 were reported to alter the adenosine triphosphatase activity of the gene product (Roerig et al. 2001). The mutations occurring in our families at codons 606, 617, and 660 are those frequently reported previously (http://www.x-ald.nl/), and that at codon 116 (G116R) has also been reported (Feigenbaum et al. 1996; Lachtermacher et al. 2000). On the other hand, the G116E mutation in family 6 has hitherto been undescribed. The nucleotide substitutions identified in the seven families were distributed along the gene without any mutation hot spots, this finding corresponding to those reported previously (Takano et al. 1999), as shown in Table 3. Also, there was no genotype–

Table 3. Base substitutions in *ALDP* resulting in amino acid change among reported and present Japanese patients with ALD

Codon	Amino acid change	No. of patients		
		Reported	Reference	Present study
98	S→W	1	Ohi et al.	
116	G→E			1
148	N→S	1	Takano et al. 1999	
163	R→P	1	Ohi et al. 2000	1
200	D→N	1	Takano et al. 1999	
214	N→D	1	Takano et al. 1999	
266	G→R	2	Takano et al. 1999	
271	E→K	1	Takano et al. 1999	
296	Y→C	2	Takano et al. 1999	
302	E→K	1	Ueyama et al. 1996	
322	L→P	1	Osaka et al. 1998	
401	R→W	1	Takano et al. 1999	
507	G→V	1	Takano et al. 1999	
512	G→S	1	Yasutake et al. 1995	2
518	R→Q	3	Imamura et al. 1997, Takano et al. 1999	
534	P→L	1	Yasutake et al. 1995	
540	F→S	1	Takano et al. 1999	
544	Q→R	2	Imamura et al. 1997, Takano et al. 1999	
560	P→L	1	Takano et al. 1999	
590	Q→X	1	Uchiyama et al. 1994	
591	R→W	1	Takano et al. 1999	
606	S→L	1	Takano et al. 1999	1
617	R→H			1
660	R→W	2	Yasutake et al. 1995, Takano et al. 1999	1
		28		7

phenotype correlation, as indicated by Maestri and Beaty (1992).

Clinical manifestations of ALD vary not only among patients but also within a family, as also observed in our series of seven families. Patients with a milder form of ALD, such as Addison disease, can be treated all their lives, whereas for individuals with a severe form, especially with the childhood cerebral form, a prenatal diagnosis may be one of the choices for their parents (Moser and Moser 1999). In fact, three of the seven families in the present study were those referred to us for prenatal diagnosis. From this point of view, molecular diagnosis and genetic counseling before and after testing are important. The Ethical Committee for Human Genome and Gene Analysis, Nagasaki University has approved prenatal diagnosis of serious diseases, including X-ALD, after genetic counseling, and genetic counseling in our hospital was done for about 1 h in each case by genetic counselors (Matsumoto et al. 2001).

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