Jpn J Human Genet 39, 345–351, 1994

A POINT MUTATION AT ATP-BINDING REGION OF THE ALD GENE IN A FAMILY WITH X-LINKED ADRENOLEUKODYSTROPHY

Tadashi MATSUMOTO,[1] Tatsuro KONDOH,[1,2] Hideaki MASUZAKI,[3] Naoki HARADA,[4] Tetsuo MATSUSAKA,[1] Ei-ichi KINOSHITA,[1] Goh TAKEO,[5] Mitsuhiro TSUJIHATA,[5] Yasuyuki SUZUKI,[6] and Yoshiro TSUJI[1]

[1]*Department of Pediatrics,* [3]*Department of Gynecology and Obsterics, and* [5]*First Department of Internal Medicine, Nagasaki University School of Medicine, Sakamoto, Nagasaki 852, Japan*
[2]*Department of Pediatrics, Kawatana National Hospital, Kawatana-machi, Higashisonogi-gun, Nagasaki 859–36, Japan*
[4]*Kyushu Medical Science, Hamaguchi-machi, Nagasaki 852, Japan*
[6]*Department of Pediatrics, Gifu University School of Medicine, Tsukasa-machi, Gifu 500, Japan*

Summary A prenatal diagnosis was performed in a family with X-linked adrenoleukodystrophy (ALD). A fetus was at high risk of suffering the disease by segregation analysis and by very long chain fatty acid-CoA synthetase activity assay. A transition (G to A) at codon 617 of the candidate ALD gene was detected by reverse transcription PCR (RT-PCR) based sequencing of the fetal liver RNA. The mutation was located in highly conserved ATP-binding site in this gene and deduced amino acid transversion R617H was thought to be the cause of ALD in this family.
Key Words X-linked adrenoleukodystrophy, prenatal diagnosis, point mutation, ATP-binding site

INTRODUCTION

X-linked adrenoleukodystrophy (ALD) is characterized by progressive neurological deterioration, adrenal insufficiency and the accumulation of very long chain fatty acid (VLCFA) in various tissues and body fluid. The clinical picture varies within a family. There are three major types, progressive adrenoleukodystrophy in childhood, adrenomyeloneuropathy (AMN) in adult, and Addison's disease (Moser and Moser, 1989). The gene for ALD is on the long arm of X chromosome, Xq28 (Human Gene Mapping 11). The cause of ALD has been ascribed

Received February 21, 1994; Revised version accepted June 23, 1994.

to the deficiency of VLCFA-CoA synthetase which is located in peroxisome and functions as a key enzyme for the VLCFA beta-oxidation pathway. Recently, a candidate gene for X-linked ALD has been isolated and some patients have deletion in the gene (Mosser *et al.*, 1993). The isolated "ALD gene" was different from the VLCFA-CoA synthetase gene itself but belonged to the ATP-binding cassette transporter superfamily.

Here, we report a prenatal diagnosis in a family with AMN/ALD and pre-symptomatic ALD by the detection of a point mutation in the ALD gene.

MATERIALS AND METHODS

The proband (II-2) was a 60 years old man with AMN. His two daughters (III-2, 4) were carriers and his grandson (IV-2) was suspected to be a pre-symptomatic ALD (Fig. 1) by serum VLCFA assay (Tsuji *et al.*, 1981). A fetus (IV-1) was consulted to us for prenatal diagnosis. At 15 weeks of gestation amniocentesis was performed and amniocytes were cultured and used for karyotyping, DNA analysis and VLCFA-CoA synthetase assay. DNA was extracted also from peripheral leukocytes of some of the family members. RNA was extracted from the liver of the aborted fetus. For a VLCFA-CoA synthetase assay, lignoceroyl-CoA synthetase activity was measured as described previously (Suzuki *et al.*, 1991).

DNA analyses. Four regions on the Y chromosome (*SRY*, *DYZ3*, *DYS132*, and *DYZ1*) were amplified by PCR (Nagafuchi *et al.*, 1992) for sex determination. Several known polymorphic segments on the long arm of X chromosome were amplified for segregation analysis including coagulation factor VIII (*FVIII*), IX (*FIX*), glocuse-6-phosphate dehydrogenase (*G6PD*), hypoxanthine phosphoribosyl

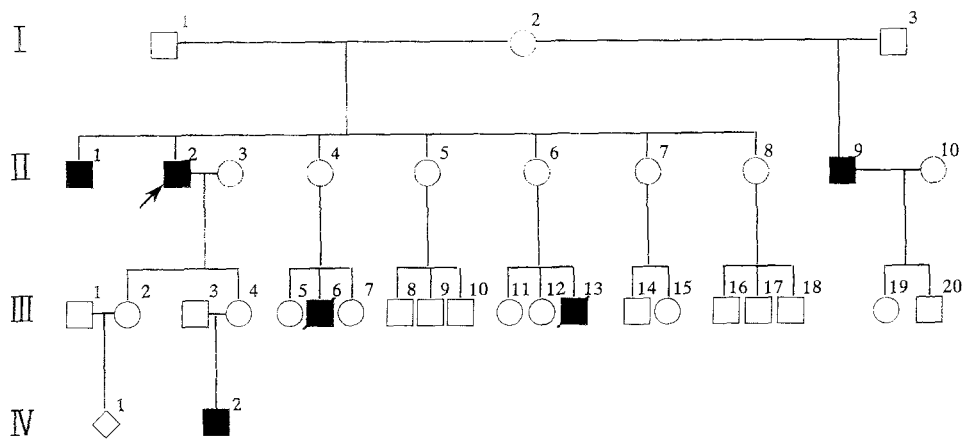

Fig. 1. Pedigree of ALD/AMN family. II-1, 2, 9 are AMN patients. III-6, 13 are ALD patients who were dead and confirmed the diagnosis by autopsy. IV-2 is a pre-symptomatic ALD patients. IV-1 is the fetus consulted for prenatal diagnosis.

transferase (*HPRT*), and GABA receptor alpha (*GABARA*) genes. DNA segments of *DXS424*, *DXS102*, *DXS300*, *DXS425* for dinucleotide repeat polymorphism, and *DXS52* (St14) for variable number tandem repeat (VNTR) polymorphism (Richards *et al.*, 1991). Each primer was synthesized by Gene Assembler Plus (Pharmacia LKB) depending on the reported sequence data (Human Gene Mapping 11, 1991). PCR was done as described previously (Kinoshita *et al.*, 1991) with minor modifications of annealing temperature depending on each primer set. For *FVIII* and *FIX* RFLPs an aliquot of PCR product was digested with *Hin*dIII or *Hha*I.

RT-PCR and sequencing. The ALD cDNA was synthesized by RT-PCR and subcloned in a plasmid vector pSPT18 (Boehringer, Germany). Each primer sequence for RT-PCR were as following; 5 (5′-CTGCGCTACATGCACTCG-3′), 6 (5′-GTGACGATGGGGATGTTCTCG-3′), 7 (5′-GAACATCCCCATCGTCACGCC-3′), and 10 (5′-CCTTGGCCGCCTGGAAGATC-3′). The sequence was analyzed with Silver Sequence TM Kit (Promega, USA) using SP6 or T7 primer as recommended by manufacturer. Electrophoresis was performed on a denaturing gel at 2,400 V for 4 h, then silver stained gel was photographed by a Poraloid camera.

Restriction enzyme digestion of ALD7/10. RNA was extracted from autopsied normal adrenal gland and used for RT-PCR. ALD7/10 PCR products from the fetal liver and the normal adrenal gland was digested with *Aci*I and *Afl*III, then electrophoresis was performed on a 6% polyacrylamide gel.

RESULTS

Karyotyping. The karyotype of the fetus was 46,XY.

DNA analysis. Each DNA fragment was amplified using 4 sets of primers on Y chromosome (data not shown). The FIX gene RFLP was informative: The proband was hemizygous for the 150 bp+80 bp allele. His wife (II-3) was homozygous for the 230 bp allele. The pregnant daughter and her sister had both of the alleles. The pre-symptomatic ALD patient and the fetus were hemizygous for the 150 bp+80 bp allele (Fig. 2). The St14 VNTR polymorphism showed similar result, namely, the proband's allele was transmitted to the pre-symptomatic patient and the fetus (data not shown). Other DNA markers were not informative in this family.

VLCFA-CoA synthetase assay. The lignoceroyl-CoA synthethase activity of the cultured amniocytes was reduced and similar as in ALD fibroblasts (Table 1).

Since the fetus was thought to have the disease from these results, the parent elected termination of pregnancy at 20th week of gestation. The fresh liver specimen of the fetus was used for RT-PCR analysis.

RT-PCR and sequencing. Each amplified cDNA (Fig. 3) was ligated with the vector DNA. At codon 617 a G to A substitution was detected by sequencing

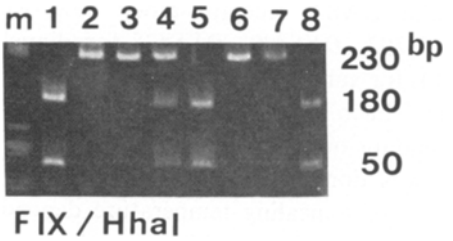

Fig. 2. *Hha*I digested *FIX* PCR product. *FIX* PCR product was digested with *Hha*I and electrophoresed in a 6% polyacrylamide gel and stained with ethidium bromide. It showed two allele polymorphism; 150 bp+80 bp allele (a) and 230 bp allele (b). The proband, fetus and pre-symptomatic male patient were hemizygous for allele (a), two normal men showed hemizygous for allele (b), the proband's wife showed homozygous for allele (b), and two sisters showed heterozygous for allele (a) and (b). m: size marker. Lane 1–8 represents II-2, II-3, III-1, III-2, IV-1, III-3, III-4, and IV-2 in Fig. 2, respectively.

Table 1. Lignoceroyl-CoA synthetase activity in the amniocytes of the fetus and a control were measured in duplicate.

Sample	Activity (pmol/h/mg protein)
Fetal amniocytes	115 (1st), 73 (2nd)
Control amniocytes	202 (1st), 186 (2nd)
ALD fibroblasts (n=7)[a]	75±16
Control fibroblasts (n=5)[a]	283±98

[a] The activity in fibroblasts of ALD patients and normal controls were shown (mean±SD).

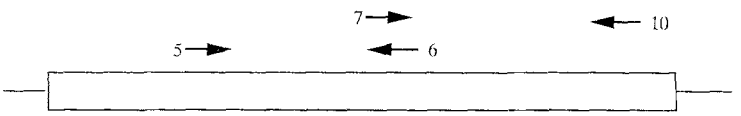

Fig. 3. Two RT-PCR products ALD5/6 and ALD7/10 (with primers 5/6 and 7/10, respectively) were subcloned into vector pSPT18. Coding region is boxed.

of the subclone ALD7/10. RT-PCR product with primer set 7/10 was digested with either *Aci*I or *Afl*III. *Aci*I recognizes normal sequence at codon 617, and *Afl*III does G to A substituted codon 617 (Fig. 4a). From the reported cDNA sequence, *Aci*I cut ALD7/10 into 103, 95, 143, 78, 88, and 8 bp fragments and *Afl*III cut it into 211 and 304 bp fragments. In the fetus, 78+88 bp *Aci*I fragments fused to 166 bp fragment, and 304 bp *Afl*III fragment was cut into 210+94 bp fragments (Fig. 4b).

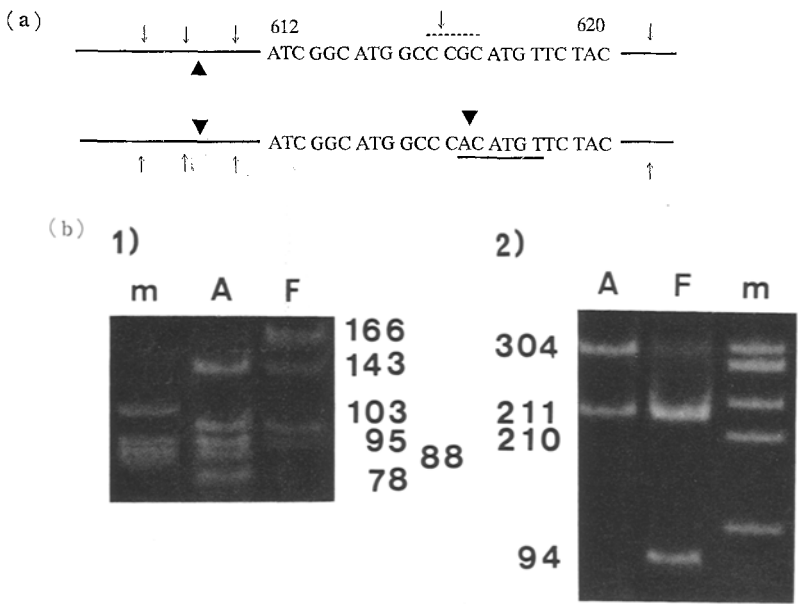

Fig. 4. Restriction enzyme digestion of ALD7/10. a) Nucleotide sequence around codon 617 (codon 613–620) of ALD cDNA and restriction map of ALD7/10. Wild type sequence (upper) has *Aci*I restriction site (dotted line) but not *Afl*III site (underline), and mutant sequence (lower) *vice versa*. Arrows show *Aci*I sites and arrow heads show *Afl*III sites. b) *Aci*I and *Afl*III digested RT-PCR products from normal adrenal gland and the fetal liver. 1) *Aci*I digest showed 88 bp+78 bp fragments in normal adrenal gland-derived cDNA, while undigested 166 bp fragment was seen in the fetal lane. 2) *Afl*III cut the normal 304 bp fragment of adrenal gland to 210 bp+94 bp fragments in the fetus (very weak 304 bp fragment in the fetus was due to partial digestion). m, size marker; A, normal adrenal gland; F, fetal liver. Numbers indicate length of fragments.

DISCUSSION

The high level of serum VLCFA in the AMN/ALD patients (II-1, 2, 9, and III-6, 13) and in the pre-symptomatic male patient (IV-2), and the moderate increase of that in two daughters were compatible with the diagnosis of X-linked ALD/AMN in this family. The male fetus was thought to be abnormal by segregation analysis using polymorphic DNA markers on Xq27 (*FIX*) and Xq28 (*St14*) which are located to centromeric to the ALD locus, although the possibility of a recombination between the ALD and *St14* locus remained. Unfortunately, the telomeric markers such as *G6PD* and *FVIII* were not informative. The biochemical analysis of the VLCFA-CoA synthetase activity with cultured amniocytes added another piece of supporting the conclusion.

The protein coded by ALD gene (ALDP) is a member of the ATP-binding

cassette family of transporters (ABC transporter) (Mosser *et al.*, 1993). ALDP shares high homology with major 70K peroxisome membrane protein (PMP) (Kamijo *et al.*, 1992), which is thought to be the deficit of some types of Zellweger syndrome (Gartner *et al.*, 1992). These two proteins show 56% homology over 210 amino acids at carboxy terminal region. The codon 617 in the carboxy terminal region constitutes the border between the ATP-binding site motif (NBF) and the conserved 12-amino acids segment, and this codon (arginine R) is conserved among the ABC transporters such as PMP 70K, multiple drug resistants 1 (MDR1), cystic fibrosis transmembrane conductance regulator (CFTR) and two peptide transporters (PSF1, 2) (Mosser *et al.*, 1993).

The sequencing of the RT-PCR product in the fetus showed G to A transition at codon 617 of the reported cDNA sequence and the base substitution was confirmed by restriction endonuclease digestion. A substitution from arginine (R) to histidine (H) was deduced by this point mutation. This arginine residue is highly conserved among the ABC transporter superfamily and it seems that this region is necessary for ATP binding and, thus, for transport of the VLCFA-CoA synthetase into the peroxisome (Valle and Gartner, 1993). It is probable that G to A transition in the codon 617 caused AMN/ALD in our family, although it is necessary to elucidate whether this R617H substitution is polymorphism and whether the function of this mutated ALDP is reduced or not. In patients with cystic fibrosis there are also some mutations in NBFs in the CFTR gene (Cutting *et al.*, 1990), thus the ATP-binding site seems very important for these ABC transporters.

The cause of phenotypic variation of AMN/ALD within this family is not known. There may be some unknown factors or some difference in immunological response in each patient (Mosser *et al.*, 1993).

REFERENCES

Cutting GR, Kasch LM, Rosenstein BJ, Zielenski J, Tsui LC, Antonarakis SE, Kazazian HH Jr (1990): A cluster of cystic fibrosis mutations in the first nucleotide-binding fold of the cystic fibrosis conductance regulator protein. Nature **346**: 366–369

Gartner J, Moser H, Valle D (1992): Mutations in the 70K peroxisomal membrane protein gene in Zellweger syndrome. Nature Genet **1**: 16–23

Human Gene Mapping 11. London Conference (1991): Cytogenet Cell Genet **58**: 1663–1696

Kamijo K, Kamijo T, Ueno I, Osumi T, Hashimoto T (1992): Nucleotide sequence of the human 70 kDa peroxisomal membrane protein: a member of ATP-binding cassette transporters. Biochim Biophys Acta **1129**: 323–327

Kinoshita E, Matsumoto T, Kondoh T, Yoshimoto M, Niikawa N, Tsuji Y (1991): Deletion pattern in the 21-hydroxylase gene detected by polymerase chain reaction. Acta Pediatr Jpn **33**: 1–5

Moser HW, Moser AB (1989): Adrenoleukodystrophy. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds). The metabolic basis of inherited disease. McGraw-Hill, New York, pp 1511–1532

Mosser J, Douar AM, Sarde CO, Kioschis P, Feil R, Moser H, Poustka AM, Mandel JL, Aubourg P (1993): Putative X-linked adrenoleukodystrophy gene shares unexpected homology with ABC transporters. Nature **361**: 726–730

Nagafuchi S, Seki S, Nakahori Y, Tamura T, Numabe H, Nakagome Y (1992): PCR detection of structurally abnormal Y chromosome. Jpn J Human Genet **37**: 187–193

Suzuki Y, Shimozawa N, Yajima S, Yamaguchi S, Orii T, Hashimoto T (1991): Effects of sodium 2-[5-(4-chlorophenyl)pentyl]-oxirane-2-carboxylate (POCA) on fatty acid oxidation in fibroblasts from patients with peroxisomal diseases. Biochem Pharmacol **41**: 453–456

Tsuji S, Suzuki M, Ariga T, Sekino M, Kuriyama M, Miyatake T (1981): Abnormality of long-chain fatty acids in erythrocyte membrane sphingomyelin from patients with adrenoleukodystrophy. J Neurochem **36**: 1046–1049

Valle D, Gartner J (1993): Penetrating the peroxisome. Nature **361**: 682–683