Mutational Analysis and Genotype-Phenotype Correlation of 29 Unrelated Japanese Patients With X-linked Adrenoleukodystrophy

Hiroki Takano, MD, PhD; Ryoko Koike, MD, PhD; Osamu Onodera, MD, PhD; Ryogen Sasaki, MD; Shoji Tsuji, MD, PhD

Background: X-linked adrenoleukodystrophy (ALD) is an inherited disease characterized by progressive neurologic dysfunction, occasionally associated with adrenal insufficiency. The classic form of ALD usually has onset in childhood (childhood cerebral ALD), with rapid neurologic deterioration leading to a vegetative state. Adult-onset cerebral ALD also presents with rapidly progressive neurologic dysfunction. Milder phenotypes such as adrenomyeloneuropathy and Addison disease only also have been recognized. Despite discovery of the causative gene, a molecular basis for the diverse clinical presentations remains to be elucidated.

Objectives: To conduct mutational analyses in 29 Japanese patients with ALD from 29 unrelated families, to obtain knowledge of the spectrum of mutations in this gene, and to study genotype-phenotype correlations in Japanese patients.

Methods: The 29 patients comprised 13 patients with childhood cerebral ALD, 11 patients with adult-onset cerebral ALD, and 5 patients with adrenomyeloneuropathy. We conducted detailed mutational analyses of 29 unrelated Japanese patients with ALD by genomic Southern blot analysis and direct nucleotide sequence analysis of reverse transcriptase–polymerase chain reaction products derived from total RNA that was extracted from cultured skin fibroblasts, lymphoblastoid cells, or peripheral blood leukocytes.

Results: Three patients with adult-onset cerebral ALD were identified as having large genomic rearrangements. The remaining 26 patients were identified as having 21 independent mutations, including 12 novel mutations resulting in small nucleotide alterations in the ALD gene. Eighteen (69%) of 26 mutations were missense mutations. Most missense mutations involved amino acids conserved in homologous gene products, including PMP70, mALDRP, and Pxa1p. The AG dinucleotide deletion at position 1081-1082, which has been reported previously to be the most common mutation in white patients (12%-17%), was also identified as the most common mutation in Japanese patients (12%). All phenotypes were associated with mutations resulting in protein truncation or subtle amino acid changes. There were no differences in phenotypic expressions between missense mutations involving conserved amino acids and those involving nonconserved amino acids.

Conclusions: There are no obvious correlations between the phenotypes of patients with ALD and their genotypes, suggesting that other genetic or environmental factors modify the phenotypic expressions of ALD.

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PATIENTS AND METHODS

PATIENTS

A total of 29 Japanese patients with ALD from 29 unrelated families were included in this study (Table 1 and Table 2). Eleven patients had other affected family members or asymptomatic carriers, and 11 patients were apparently sporadic. Family data were not available for 7 patients (Tables 1 and 2). Diagnosis of ALD was made on the basis of clinical findings, and the diagnosis was confirmed by measuring plasma VLCFA levels.30,34 Three patients with ACALD previously were described12 as having large genomic rearrangements; the remaining 26 patients, without such genomic rearrangements, were subjected to mutational analysis based on nucleotide sequence analysis, with informed consent.

MUTATION DETECTION

Genomic Southern blot hybridization analysis for large genomic rearrangements was reported previously.9 Total RNAs were extracted from lymphoblastoid cell lines or cultured skin fibroblasts using the guanidinium isothiocyanate and acidic phenol method30 or from peripheral white blood cells (patients G1986, G2469, and G2022) using an acidic isothiocyanate/phenol/chloroform method (ISOGEN; Nippon Gene Inc, Tokyo, Japan). First-strand complementary DNAs (cDNA) were made from 1 μg of total RNAs using random hexamer primers and Maloney murine leukemia virus–reverse transcriptase (Clontech, Palo Alto, Calif) in a final volume of 20 μL, as described previously.35

The entire coding region of ALDP cDNA was amplified into 4 overlapping DNA fragments (A, B, C, and D) using the 2-round polymerase chain reactions (PCRs) according to previously described methods35 with minor modifications. Two of the 4 fragments (B and D) exactly corresponded to the previously described fragments II (cDNA nucleotide positions 702-1384) and V (cDNA nucleotide positions 1800-2669),26 which were amplified using the same primer sets.35 The remaining 2 fragments (A and C) were generated using the following primers. For amplification of fragment A (cDNA nucleotide positions 323-82126), the primers ALD323F (5′-acctggcctcgcagatcaacc-3′) and ALD821R (5′-acctggcctcgcagatcaacc-3′) were used in the second-round PCR after the first PCR with primers ALD323F (5′-acctggcctcgcagatcaacc-3′) and ALD821R (5′-acctggcctcgcagatcaacc-3′) were used in the second-round PCR after the first PCR with the primers ALD323F and ALD821R.35

The first-round PCR was performed with 2 μL of the first-strand cDNA and 20 pmol of each of the corresponding forward and reverse primers, in a total volume of 25 μL containing Tris hydrochloride, 10 mmol (pH 8.3); potassium chloride, 50 mmol; magnesium chloride, 2.0 mmol; N,N,N-trimethylglycine, 1.7 mol; 300 μmol each of deoxynucleotides, and 2.5 U of Taq DNA polymerase (Takara, Tokyo, Japan) with 32 cycles of 1-minute denaturation (at 96°C), 1½-minute annealing (at 59°C for fragments B and D, 55°C for fragment A, and 57°C for fragment C), and 2-minute extension (at 72°C). The second-round PCR was performed with 2 μL of the first-round PCR products under the same conditions used for the first-round PCR. The second-round PCR products were separated on a 1% agarose gel and purified using a quick gel extraction kit (QIA; Qiagen, Chatsworth, Calif).

The PCR products were directly analyzed for nucleotide sequences by the dideoxynucleotide chain termination method33 using the same primers as the second-round PCR. Additional primers were used for sequencing fragments B (914F and 931R, C (1681F and 1942R), and D (2312F and 2366R).35 Sequence reactions were performed with fluorescein isothiocyanate–labeled primers using a sequencing kit (ThermoSequenase; Amersham, Buckinghamshire, England) or with unlabeled primers using a cycle sequencing kit (Taq FS Dye Terminator; Perkin-Elmer Applied Biosystems, Foster City, Calif) using automated DNA sequencers (ALF II; Pharmacia, Uppsala, Sweden, or ABI 377; Perkin-Elmer Applied Biosystems, Norwalk, Conn). Nucleotides and amino acids were numbered as previously described.20

GENOTYPE-PHENOTYPE CORRELATION

Patients were grouped into 3 clinical phenotypes, including 13 patients with CCALD (age at onset <15 years), 11 patients with ACALD (age at onset >15 years), and 5 patients with AMN. Mutations were divided into 2 categories: mutations expected to cause devastating effects on ALD, such as large genomic rearrangements, frameshift mutations, or nonsense mutations (Table 1), and mutations expected to cause subtle amino acid changes, including missense mutations or small in-frame deletions (Table 2). Amino acids involved in the latter group of mutations were further divided into 4 subgroups based on whether they were in the ABC domain and whether they were conserved in PMP70, mALDRP, and Pxa1p (Table 3). We investigated whether these types of mutations were associated with particular phenotypes. We also reviewed all reported mutations in the ALD gene30,33,35-53 and performed genotype-phenotype correlation analyses for these mutations, as described above.

The clinical presentation of ALD can be highly variable, and various phenotypes can be observed even within the same pedigree.12-14 Adrenoleukodystrophy is biochemically characterized by increased levels of saturated unbranched very long chain fatty acids (VLCFAs) in various tissues, including the white matter of the central nervous system, adrenal gland, testis, erythrocyte membrane, leukocytes, and plasma.15-22 The increased levels of VLCFAs in ALD is generally considered to be the result of impaired peroxisomal β-oxidation of VLCFAs caused by reduced activity of the peroxisomal VLCFA coenzyme A synthetase.23 We also demonstrated that microsomal fatty acid elongation activities are increased in ALD and are strongly suppressed by monounsaturated fatty acids, including erucic acid and oleic acid.23-25 However, the mechanism by which increased levels of VLCFAs in the nervous system lead to demyelination remains to be elucidated. The causative gene for ALD was isolated using positional cloning strategies and was predicted to encode an aden-
osine triphosphate–binding cassette (ABC) transporter consisting of 745 amino acids (ALD protein [ALDP]). The ALDP has a half-transporter structure consisting of an amino-terminal transmembrane domain with 6 transmembrane segments and a carboxy-terminal ABC domain and exhibits a high degree of homology with other half-transporters, including PMP70, mALDRP, and Pxa1p. Although ALDP was found to be localized on the peroxisomal membrane, its role in VLCFA metabolism remains to be clarified.

Large genomic rearrangements in the ALD gene have been identified in 4% to 7% of unrelated patients with ALD. Extensive mutational analyses of the ALD gene have so far revealed 98 individual small nucleotide alterations, which include small nucleotide deletions, insertions, point

### Table 1. Mutations in the ALD Gene That Result in Devastating Effects (Truncation) on ALDP

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Phenotype</th>
<th>Mutation†</th>
<th>Exon</th>
<th>Effect of Mutation‡</th>
<th>Position of Mutation§</th>
<th>Family Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>G4001(s)</td>
<td>ACALD</td>
<td>0.5-kb deletion</td>
<td>1</td>
<td>Disruption of gene structure</td>
<td>Exon 1</td>
<td>No family history</td>
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<tr>
<td>G4002(s)</td>
<td>ACALD</td>
<td>7-kb deletion</td>
<td>7-9</td>
<td>Disruption of gene structure</td>
<td>Exons 7-8</td>
<td>No family history</td>
</tr>
<tr>
<td>G4003(s)</td>
<td>ACALD</td>
<td>10.6-kb deletion</td>
<td>6-10</td>
<td>Disruption of gene structure</td>
<td>Exons 6-10</td>
<td>No family history</td>
</tr>
</tbody>
</table>

### Table 2. Mutations in the ALD Gene That Result in Amino Acid Substitutions or In-frame Deletions

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Phenotype</th>
<th>Mutation†</th>
<th>Exon</th>
<th>Effect of Mutation‡</th>
<th>Position of Mutation§</th>
<th>Amino Acid Identity</th>
<th>PMP70</th>
<th>mALDRP</th>
<th>Pxa1p</th>
<th>Family Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>G4010</td>
<td>ACALD</td>
<td>del.1256-1258</td>
<td>1</td>
<td>del.E291</td>
<td>EAA-like motif</td>
<td>Between Walker A and B</td>
<td>E</td>
<td>E</td>
<td>E</td>
<td>CCALD</td>
</tr>
<tr>
<td>G4011(s)</td>
<td>ACALD</td>
<td>del.2146-2157¶</td>
<td>7</td>
<td>del.HILQ587-590</td>
<td>EAA motif</td>
<td>Between Walker A and B#</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>No family history</td>
</tr>
</tbody>
</table>

**Table 1. Mutations in the ALD Gene That Result in Devastating Effects (Truncation) on ALDP**

**Table 2. Mutations in the ALD Gene That Result in Amino Acid Substitutions or In-frame Deletions**

*ALD indicates adrenoleukodystrophy; ACALD, adult-onset cerebral ALD; CCALD, childhood cerebral ALD; AMN, adrenomyeloneuropathy; (s), apparently sporadic patients; ins., insert; and del., delete.
†Nucleotide numbers in the ALD gene complementary DNA are based on Mosser et al.
‡Amino acids are represented by 1-letter abbreviations, and amino acid residue numbers in ALD protein are based on Mosser et al.
§The domains and motifs in the ALDP were based on Mosser et al.
¶Novel mutations.

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Mutations, and splice-site mutations. The mutation del.1801-1802 has been identified as the most common mutation (approximately 12%-17%), however, most of the other mutations are unique for each pedigree. Furthermore, no definite genotype-phenotype correlations have been identified. These complicated molecular genetic features of ALD could be the result of analyzing patients of diverse ethnic origins. A large-scale analysis of patients from a single population may provide a better understanding of the correlation between genotypes and phenotypes.

There have been only a few reports on mutational analysis in the ALD gene in the Japanese population. We report on mutational analysis in 29 Japanese patients with ALD from 29 unrelated families to obtain knowledge of the spectrum of mutations in this gene and to study genotype-phenotype correlations in Japanese patients.

### RESULTS

### MUTATIONS IN THE ALD GENE

Three patients with ACALD were identified as having large genomic rearrangements on Southern blot hybridization analysis, as described previously (Table 1). In the remaining 26 patients without such genomic rearrangements, we found 21 unrelated mutations, of which 12 were novel mutations (Tables 1 and 2). Most of the mutations were missense mutations, which accounted for 18 (69%) of 26 patients. Frameshift mutations were found in 5 patients, in-frame deletions in 2 patients, and a nonsense mutation in 1 patient. Because none of the deletions or insertions detected in the reverse transcriptase-PCR products involved exonic junctions, it is unlikely that they are caused by mutations involving splice sites. Because these mutations were not observed in 52 normal Japanese chromosomes (data not shown), they are likely to be the causative mutations of ALD.

Although most mutations were unique to each of the unrelated patients, the del.1801-1802 mutation was found in 3 patients (10.0%), and 3 missense mutations—G1182A, G1939A, and G266R—were also associated with CCALD and 2 AMN. Only 4 mutations involved nonconserved amino acids, 3 of which were located outside the ABC domain (1 CCALD and 2 AMN) and 1 was within the ABC domain (1 ACALD). Review of previously published data also generated similar results.

To examine whether specific missense or in-frame deletion mutations are associated with AMN, we focused on mutations associated with AMN. Among the 5 mutations associated with AMN in this study, 2 (del.1801-1802 and G266R) were also associated with CCALD and ACALD. Although each of the remaining 3 mutations (E271K, R401W, and S606L) was identified in only 1 apparently sporadic case of a patient with AMN, a review of the literature indicated that the S606L mutation has been identified in 2 patients with Addison disease only.

### GENOTYPE-PHENOTYPE CORRELATION

We first categorized the mutations into 2 groups. The former group included those expected to cause devastating effects on ALDP, such as large genomic rearrangements, frameshift mutations, or nonsense mutations, and were found in 9 patients (Table 1); all frameshift and nonsense mutations occurred upstream of the region encoding the ABC, which predicted the abolition of ALDP functions as ABC transporter. The latter group comprised missense mutations or small in-frame deletions, which predicted amino acid substitutions or deletions and were found in 20 patients (Table 2). Both groups of mutations were distributed among all the phenotypes of ALD, including CCALD, ACALD, and AMN, suggesting that there is no association of a particular phenotype of ALD with individual mutations.

We considered that analyzing locations of missense mutations or amino acid deletions might be useful for identifying critical regions that affect the function of ALDP and for identifying their association, if any, with particular clinical phenotypes. The amino acids involved in these mutations were divided into 4 groups based on whether they were in the ABC domain and whether they were conserved in PMP70, mALDRP, and Pxa1p (Table 3). Most mutations (16 of 20 mutations), irrespective of their locations, involved conserved amino acids and were distributed among all the phenotypes of ALD (CCALD, ACALD, and AMN). Only 4 mutations involved nonconserved amino acids, 3 of which were located outside the ABC domain (1 CCALD and 2 AMN) and 1 was within the ABC domain (1 ACALD). Review of previously published data also generated similar results.

### COMMENT

### MUTATIONS IN THE ALD GENE

Because of the low frequency (4%-7%) of large genomic rearrangements in the ALD gene, a detailed nucleotide sequence analysis to detect small nucleotide alterations is required for most cases of ALD. Nucleotide sequence analysis of the ALDP cDNA generated from the total RNA enabled us to identify mutations in all 26 patients.
tients analyzed. Because the ALD gene is widely expressed, including in peripheral blood leukocytes, this approach is a simple and highly efficient method for diagnostic nucleotide sequence analysis of the ALD gene, as demonstrated in a previous report.

We identified 21 independent mutations in 26 unrelated Japanese patients, indicating that mutational heterogeneity is prevalent even in the Japanese population, despite its being relatively homogeneous. However, the del.1801-1802 mutation, which has been reported as the most common mutation in previous reports based on studies of white populations (12%-17%), was also the most common mutation among Japanese patients with ALD (12%). Consistent with the previous reports on white patients, most mutations (69%) were missense and unique. These data suggest that the molecular genetic mechanisms leading to mutations in the ALD gene in the Japanese population are as diverse as in white populations. The relatively high incidence of apparently sporadic cases (38%), and the diversity of mutations, may indicate a relatively high incidence of de novo mutations in the ALD gene.

In ALD, 117 (15.7%) of 745 amino acid residues are identical to the corresponding amino acids in homologous half-transporter proteins, including mALDRP, Pxa1p, and PMP70; 54 of 117 conserved amino acids lie outside the ABC domain. Of the 17 mutations, including missense and in-frame deletion mutations identified in the present study, 14 mutations can substitute the amino acids conserved in PMP70, mALDRP, and Pxa1p (Table 2), and 9 are located outside the ABC domain, supporting the assumption that regions other than the ABC domain are also important for the function of ABC proteins. When combined with previously identified mutations, a total of 49 amino acids in the ALD are affected by missense mutations, 35 of which are located outside the ABC domain, including 17 involving conserved amino acids. Taken together, these data indicate that not only the ABC domain but also regions outside the ABC domain are functionally important.

**GENOTYPE-PHENOTYPE CORRELATION**

In our 29 Japanese patients, we did not find any obvious differences in the distributions of clinical phenotypes between mutations leading to protein truncation and those resulting in amino acid substitutions or amino acid deletions. Large genomic rearrangements have already been described in patients with various phenotypes, including CCALD, ACALD, and AMN. Our finding of the del.1081-1082 frameshift mutation being present in patients with CCALD, ACALD, or AMN is in agreement with the finding in a previous study. Note that del.1081-1082—which predicts truncation of a substantial portion of ALD, including the ABC domain—is associated with a mild phenotype of AMN. This finding suggests that the degree of loss of ALD function is not necessarily correlated with the severity of ALD, which is consistent with the results of recent immunocytological studies, indicating that there is no correlation between disease severity and the presence or absence of immunologically detectable ALDP. From analysis of the nature of amino acids affected by missense or in-frame deletion mutations, we again did not identify any correlation between the locations of mutated amino acids and the phenotypic expressions of ALD. Neither were there differences in phenotypic expression between patients with mutations involving conserved amino acids and those with mutations involving nonconserved amino acids. Therefore, similar to the observation in white populations, there do not seem to be simple genotype-phenotype correlations in Japanese patients with ALD.

However, further information on genotype-phenotype associations, especially for genotypes that show little or no phenotypic variation, if any, is important for genetic counseling. In the present study, 1 patient with AMN with no family history was identified to have the C2203T (S606L) mutation, although it had previously been reported to be associated exclusively with the Addison disease only phenotype in other studies. Similar to this mutation, the mutations G1197A (E271K) and C1587T (R401W) were identified in patients with AMN with no family history of ALD in the present study. Review of previous publications indicated that 14 missense mutations are associated exclusively with AMN or Addison disease only, including C696T (R104C), G697A (R104H), C700T (T105D), G832A (S149N), C918G (Q178E), T1045C (L220P), C1137T (T254M), G1266A (A294T), C1551G (R389G), G1552A (R389H), C1638T (R418W), C1930T (S515F), T2084A (M566K), and G2211A (E606K). Analysis of these mutations may provide important insights into the mechanisms involved in variable phenotypic expressions in ALD.

It is well known that more than 1 clinical phenotype can appear within a single pedigree. In 1 kindred, a missense mutation was associated with 5 different phenotypes. In the present study, 1 patient with CCALD with the A829G (N148S) mutation had a brother with AMN. Involvement of immunologic reactions has been implicated in ALD based on the observation that demyelination is associated with perivascular infiltration by lymphocytes and macrophages and immunologic factors may modify the phenotypic expressions of ALD. Taken together, all these data suggest that phenotypic variability in ALD may be affected to a great extent by other genetic or environmental factors.

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