Identification of novel SNPs of \textit{ABCD1}, \textit{ABCD2}, \textit{ABCD3}, and \textit{ABCD4} genes in patients with X-linked adrenoleukodystrophy (ALD) based on comprehensive resequencing and association studies with ALD phenotypes

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Abstract Adrenoleukodystrophy (ALD) is an X-linked disorder affecting primarily the white matter of the central nervous system occasionally accompanied by adrenal insufficiency. Despite the discovery of the causative gene, \textit{ABCD1}, no clear genotype–phenotype correlations have been established. Association studies based on single nucleotide polymorphisms (SNPs) identified by comprehensive resequencing of genes related to \textit{ABCD1} may reveal genes modifying ALD phenotypes. We analyzed 40 Japanese patients with ALD. \textit{ABCD1} and \textit{ABCD2} were analyzed using a newly developed microarray-based resequencing system. \textit{ABCD3} and \textit{ABCD4} were analyzed by direct nucleotide sequence analysis. Replication studies were conducted on an independent French ALD cohort with extreme phenotypes. All the mutations of \textit{ABCD1} were identified, and there was no correlation between the genotypes and phenotypes of ALD. SNPs identified by the comprehensive resequencing of \textit{ABCD2}, \textit{ABCD3}, and \textit{ABCD4} were used for association studies. There were no significant associations between these SNPs and ALD phenotypes, except for the five SNPs of \textit{ABCD4}, which are in complete disequilibrium in the Japanese population. These five SNPs were significantly less frequently represented in patients with adrenomyeloneuropathy (AMN) than in controls in the Japanese population (\(p=0.0468\)), whereas there were no significant differences in patients with childhood cerebral ALD (CCALD). The replication study employing these five SNPs on an independent French ALD cohort, however, showed no significant associations with CCALD or pure AMN. This study showed that \textit{ABCD2}, \textit{ABCD3}, and \textit{ABCD4} are less likely the disease-modifying genes, necessitating further studies to identify genes modifying ALD phenotypes.

Keywords Adrenoleukodystrophy · \textit{ABCD1} · \textit{ABCD2} · \textit{ABCD3} · \textit{ABCD4} · DNA microarray

Introduction

Adrenoleukodystrophy (ALD) is a demyelinating disease caused by mutations of \textit{ABCD1} [1]. This disease affects primarily the white matter of the central nervous system occasionally accompanied by adrenal insufficiency [2–4]. Diagnosis of ALD is usually made by the increased contents of very long chain saturated fatty acids (VLCFAs; >C22:0) in plasma as well as by mutational analysis of \textit{ABCD1} [5–7].
Since 15% of obligate female carriers have normal VLCFA levels [7], mutational analysis is essential for the diagnosis of the carriers. Since the first report of allogenic HSCT for childhood ALD, there has been an increasing number of reports showing efficacies of HSCT for the childhood cerebral form of ALD, if HSCT is performed at early stages of the disease [8–10]. Thus, availability of rapid molecular diagnosis for patients with ALD and carriers is mandatory in the clinical practice for ALD.

ALD is characterized by a broad spectrum of clinical presentations including childhood cerebral form, adrenomyeloneuropathy (AMN), AMN complicated by cerebral demyelination, adulthood cerebral form, and Addison disease. From clinical experience, patients with different clinical phenotypes can be observed even in a single pedigree. In support of this, no clear genotype–phenotype correlations have been observed [11–16], raising the possibility that other genetic or environmental factors are involved in the pleomorphic clinical presentations of ALD.

ABCD1 gene encodes a half-ATP-binding cassette (ABC) transporter, adrenoleukodystrophy protein (ALDP), which is localized to the peroxisomal membrane. ABCD2, ABCD3, and ABCD4 genes are the closest homologues of the ABCD1 gene [17, 18]. It has been shown that the majority of mouse liver ALDP and the 70-kDa peroxisomal membrane protein (PMP70) that is encoded by ABCD3 are homomorphic proteins [19]. Furthermore, it has been shown that ALDP can form homodimers or a heterodimer with the adrenoleukodystrophy-related protein (ALDR) that is encoded by ABCD2 or the PMP70 that is encoded by ABCD3 [16, 20–22], raising the possibility that these ABCD1-related genes function as disease-modifying genes for ALD.

To provide a rapid mutational analysis for ALD, we developed a microarray-based high-throughput resequencing system of ABCD1 (TKYPD01) [23]. Furthermore, to explore the possibility that these ABCD1-related genes function as disease-modifying genes, we established a comprehensive resequencing system for ABCD1-related genes, ABCD2, ABCD3, and ABCD4. On the basis of the comprehensive resequencing of ABCD1, ABCD2, ABCD3, and ABCD4 genes, we identified 11 novel single nucleotide polymorphism (SNPs). Using these novel SNPs as well as previously described SNPs of these genes, we conducted detailed association studies of these SNPs with the clinical phenotypes of ALD.

Materials and methods

Participants

Forty Japanese ALD patients, consisting of 14 patients with childhood cerebral ALD (CCALD), 8 patients with adulthood cerebral ALD (AdultCer), 2 patients with AMN with later development of cerebral ALD (AMN-Cer), 13 patients with AMN, 1 asymptomatic patient, and 2 patients with unknown form, were enrolled in this study. Among the patients, mutations were previously identified in 16 ALD patients by direct nucleotide sequence analysis, while no mutational analyses were conducted for 24 patients.

For replication studies of the results of association studies on Japanese ALD patients showing potential associations of SNPs in ABCD1-related genes with ALD phenotypes, an independent French ALD cohort with well-defined extreme phenotypes consisting of 118 patients with CCALD and 71 patients with pure AMN (AMN with age >45 years as well as with normal brain magnetic resonance imaging) was studied. In addition, 51 ALD patients with AMN-Cer were also analyzed in the French ALD cohort.

Procedures

Primers specific for ABCD1, ABCD2, ABCD3, and ABCD4 were designed using BLAST search and Smith–Waterman method to avoid amplification of the related homologous genes (Fig. 1; ESM Tables 1, 2, 3, and 4). In particular, since there were many segments homologous to exons 8, 9, and 10 of ABCD1, a specific primer pair was designed (Fig. 1). Fifty nanograms of genomic DNA were subjected to polymerase chain reaction (PCR) amplification in a total volume of 50 μL. The PCR conditions were as follows: 94°C for 1 min, followed by five cycles consisting of 94°C for 30 s, 62°C for 30 s, and 68°C for 2 min; five cycles consisting of 94°C for 30 s, 60°C for 30 s, and 68°C for 2 min; and 25 cycles consisting of 94°C for 30 s, 58°C for 30 s, and 68°C for 2 min, followed by a final extension at 68°C for 7 min, using the LA Taq with GC Buffer PCR system (Takara Bio, Otsu, Shiga, Japan).

Resequencing DNA microarrays were used in the analyses of the sequences of ABCD1 (TKYPD01) and ABCD2 (TKYAD01). TKYPD01 and TKYAD01 were designed using the platform of GeneChip CustomSeqTM Resequencing Microarray (Affymetrix, Santa Clara, CA, USA). Since there are substantial homologies between ABCD1 and ABCD2, these genes were placed in independent microarrays (TKYPD01 and TKYAD01). Each PCR product of ABCD1 and ABCD2 was quantified using PicoGreen (Molecular Probes, Eugene, OR, USA) and equimolarly pooled. Poole d PCR products of ABCD1 and ABCD2 were fragmented using DNase I, labeled with biotin, hybridized to DNA microarrays, and subjected to scan and analyses of nucleotide sequences of ABCD1 (TKYPD01) and ABCD2 (TKYAD01) according to the manufacturer's instructions (Affymetrix, Santa Clara, CA, USA). The base calls that were undetermined using the GDAS analysis software (Affymetrix, Santa Clara, CA, USA) were further analyzed.
by manual inspection. Identified mutations and SNPs were confirmed by the direct nucleotide sequence analysis of the PCR products. All the PCR products of ABCD3 and ABCD4 were analyzed by the direct nucleotide sequence analysis. Identified SNPs of ABCD2, ABCD3, and ABCD4 were examined as to whether they were novel SNPs or known SNPs using the J SNP (http://snp.ims.u-tokyo.ac.jp/index_ja.html) and DB SNP (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Snp&cmd=Limits).

Statistical analyses

We compared the allele frequencies of detected SNPs between the subgroups of ALD or between the individual subgroup and the controls by Fisher’s exact test using the JMP 7 software (SAS Institute, Cary, NC, USA). Deviation of the SNP genotypes from the Hardy–Weinberg equilibrium was evaluated using the PEDSTATS program [24]. Linkage disequilibria among the neighboring SNPs were evaluated using Haploview version 4.1 [25].

Results

Resequencing DNA microarray-based mutational analysis of ABCD1 gene in Japanese ALD patients

All the mutations of ABCD1 were clearly identified using the resequencing DNA microarray system including 26 missense, 2 nonsense, and 12 insertion/deletion mutations.
of ABCD1 (Figs. 2 and 3; Tables 1 and 2). Mutations of ABCD1 gene were widely scattered in the entire region of ABCD1 gene. All types of ABCD1 mutations were distributed among all the phenotypes of adrenoleukodystrophy. TM transmembrane domain, EAA-like EAA-like protein motif, Walker A Walker A motif, C sequence nucleotide binding fold conserved sequence, Walker B Walker B motif, fs frameshift

Identification of SNPs of ABCD2, ABCD3, and ABCD4 genes by comprehensive resequencing

Comprehensive resequencing of ABCD2, ABCD3, and ABCD4 genes of the 40 Japanese patients with ALD revealed two novel SNPs, nine SNPs (six known and three novel SNPs), and 13 SNPs (seven known and six novel SNPs), respectively (Fig. 4; Tables 3, 4, 5, and 6; ESM Table 5). Hardy–Weinberg equilibrium was fulfilled for each SNP. The five known SNPs (rs17782508, rs2301345, rs4148077, rs4148078, and rs3742801) were in complete

Table 1: Identified ABCD1 mutations: mutations of ABCD1 that result in devastating effects (frame shifts or nonsense mutations) on adrenoleukodystrophy protein (ALDP)

| Patient number | Phenotype | Mutation of ABCD1 | Effect of mutation of ABCD1 |
|---------------|-----------|-------------------|----------------------------|
| 1             | CCALD     | 488C>AT           | Frameshift at P34           |
| 2             | CCALD     | 2171G>A           | W595X                      |
| 3             | CCALD     | 5'UTR-Ex2 1.4-kb deletion<sup>a</sup> | Disruption of gene structure |
| 4             | AdultCer  | Del. 986C<sup>a</sup> | Frameshift at D200           |
| 5             | AdultCer  | Del. 1801–1802AG<sup>a</sup> | Frameshift at Q472           |
| 6             | AMN-Cer   | Del. 2251 GGTG ins. TGTCT<sup>a</sup> | Frameshift at R622           |
| 7             | AMN       | Ins. 1237T<sup>a</sup> | Frameshift at Y281           |
| 8             | AMN       | Del. 1801–1802AG<sup>a</sup> | Frameshift at Q472           |
| 9             | AMN       | 2171G>A           | W595X                      |
| 10            | AMN       | Del. 2251 GGTG ins. TGTCT<sup>a</sup> | Frameshift at R622           |
| 11            | Unknown   | Del. 1541C<sup>a</sup> | Frameshift at F385           |
| 12            | Unknown   | Ex8-10 0.3-kb deletion<sup>a</sup> | Disruption of gene structure |

Amino acid residue numbers in ALDP are based on Mosser et al. [1]. The domains and motifs in the ALDP are based on Mosser et al. [1]

CCALD childhood cerebral ALD, AdultCer adult with cerebral ALD, AMN-Cer AMN with cerebral ALD, AMN adrenomyeloneuropathy, TM transmembrane domain, Loop 1 loop 1 motif, EAA-like EAA-like protein motif, Walker A Walker A motif, Cons nucleotide binding fold conserved sequence, Walker B Walker B motif

<sup>a</sup>Novel mutation
linkage disequilibrium in the Japanese patients with ALD as well as in the controls, as determined using Haploview version 4.1 (Fig. 4).

Association studies of SNPs of \(ABCD2\), \(ABCD3\), and \(ABCD4\) with the clinical phenotypes of ALD

Using the 11 novel SNPs and 13 previously described SNPs in \(ABCD2\), \(ABCD3\), and \(ABCD4\), we conducted association studies of these SNPs with the clinical phenotypes of ALD (Tables 5 and 6).

For \(ABCD2\), we analyzed two novel SNPs (novel SNP1 and novel SNP2). There were no significant differences in the allele frequencies between patients with cerebral form and those with AMN, or between the patients with individual phenotypes of ALD and the controls. For \(ABCD3\), we analyzed three novel SNPs (novel SNP3, novel SNP4, and novel SNP5) and six previously described SNPs (rs4148058, rs2147794, rs16946, rs681187, rs662813, and rs337592). However, we did not detect any significant associations.

For \(ABCD4\), we analyzed six novel SNPs (novel SNP6, novel SNP7, novel SNP8, novel SNP9, novel SNP10, and novel SNP11) and seven previously described SNPs (rs17782508, rs17182959, rs17158118, rs2301345, rs4148077, rs4148078, and rs3742801). Interestingly, the five previously described SNPs (rs17782508, rs2301345, rs4148077, rs4148078, and rs3742801) that are in complete linkage disequilibrium were significantly less frequently represented in the patients with Japanese AMN than in the controls in the Japanese population (\(p=0.0468\)), whereas

### Table 2  Identified \(ABCD1\) mutations: mutations of \(ABCD1\) that result in amino acid substitutions or in-frame deletions

| Patient number | Phenotype       | Mutation of \(ABCD1\) | Effect of mutation of \(ABCD1\) | Position of mutation |
|----------------|-----------------|-----------------------|-------------------------------|---------------------|
| 13             | CCALD           | 709C>T                | S108L                         | Loop1               |
| 14             | CCALD           | 709C>T                | S108L                         | Loop1               |
| 15             | CCALD           | 829A>G                | N148S                         | TM2                 |
| 16             | CCALD           | 1026A>G               | N214D                         | TM3                 |
| 17             | CCALD           | 1182G>A               | G266R                         | Between TM4 and EAA-like |
| 18             | CCALD           | 1324T>C<sup>a</sup>   | L313P                         | Between EAA-like and TM5 |
| 19             | CCALD           | 1938C>T               | R518W                         | Walker A            |
| 20             | CCALD           | 1939G>A               | R518Q                         | Walker A            |
| 21             | CCALD           | 2017A>G               | Q544R                         | Between Walker A and Cons |
| 22             | CCALD           | 2017A>G               | Q544R                         | Between Walker A and Cons |
| 23             | CCALD           | 2065C>T               | P560L                         | Between Walker A and Cons |
| 24             | CCALD           | 2065C>T               | P560L                         | Between Walker A and Cons |
| 25             | CCALD           | Del. 2145–2156        | Del. HILQ587-590              | Between Walker A and Cons |
| 26             | AdultCer        | Del. 1257–1259        | Del.E291                      | EAA-like            |
| 27             | AdultCer        | 2005T>C               | F540S                         | Between Walker A and Cons |
| 28             | AdultCer        | 2358C>T               | R660W                         | C-terminal to Walker B |
| 29             | AdultCer        | 2385C>A               | H667N                         | C-terminal to Walker B |
| 30             | AMN-Cer         | 1146A>C               | T254P                         | TM4                 |
| 31             | AMN             | 636C>T                | P84S                          | TM1                 |
| 32             | AMN             | 709C>T                | S108L                         | Loop1               |
| 33             | AMN             | 1182G>A               | G266R                         | Between TM4 and EAA-like |
| 34             | AMN             | 1197G>A               | E271K                         | Between TM4 and EAA-like |
| 35             | AMN             | 1215G>A<sup>a</sup>   | G277R                         | Between TM4 and EAA-like |
| 36             | AMN             | 1255C>G               | S290W                         | EAA-like            |
| 37             | AMN             | 1581C>T               | R401W                         | Between TM6 and Walker A |
| 38             | AMN             | 2233C>A               | A616D                         | Cons                |
| 39             | AMN             | 2385C>A               | H667N                         | C-terminal to Walker B |
| 40             | Asymptomatic    | 2211G>A               | E609K                         | Cons                |

Amino acid residue numbers in ALDP are based on Mosser et al. [1]. The domains and motifs in the ALDP are based on Mosser et al. [1] CCALD childhood cerebral ALD, AdultCer adult with cerebral ALD, AMN-Cer AMN with cerebral ALD, AMN adrenomyeloneuropathy, TM transmembrane domain, Loop 1 loop 1 motif, EAA-like EAA-like protein motif, Walker A Walker A motif, Cons nucleotide binding fold conserved sequence, Walker B Walker B motif

<sup>a</sup>Novel mutation
Identified single nucleotide polymorphisms (SNPs) of \textit{ABCD2}, \textit{ABCD3}, and \textit{ABCD4} (upper panel). Comprehensive resequencing of \textit{ABCD2}, \textit{ABCD3}, and \textit{ABCD4} genes of the 40 patients with adrenoleukodystrophy (ALD) revealed two novel SNPs, nine SNPs (six known and three novel SNPs), and 13 SNPs (seven known and six novel SNPs), respectively. Red characters indicate the novel SNPs, blue characters indicate the SNPs identified in the coding region, and black characters indicate the SNPs identified in the noncoding region. Linkage disequilibrium (LD) map of SNPs of \textit{ABCD4} in Japanese patients with ALD and the controls using the Haploview version 4.1 (lower panel). The five known SNPs (rs17782508, rs2301345, rs4148077, rs4148078, and rs3742801) were in complete disequilibrium in Japanese patients with ALD and the controls (LOD=43.97, $r^2=1.0$, $D'=1.0$). Novel SNP7 and the five known SNPs (rs17782508, rs2301345, rs4148077, rs4148078, and rs3742801) were not in strong disequilibrium in Japanese patients with ALD and the controls (LOD=1.15, $r^2=0.037$, $D'=0.706$), although novel SNP7 and the five known SNPs (rs17782508, rs2301345, rs4148077, rs4148078, and rs3742801) were strong disequilibrium only in Japanese patients with ALD (LOD=2.02, $r^2=0.221$, $D'=1.0$). The number in the box indicates the data of $D'$. The color of the box is determined from the LOD score and $D'$. The block was determined using a confidence interval algorithm [33].

Table 3 Summary of identified single nucleotide polymorphism (SNPs) of \textit{ABCD2}, \textit{ABCD3}, and \textit{ABCD4} in 40 adrenoleukodystrophy patients: novel SNPs

| Gene   | Name           | Fragment | Position (UCSC hg18) | Base call | Category             | Amino acid change |
|--------|----------------|----------|----------------------|-----------|----------------------|-------------------|
| \textit{ABCD2} | Novel SNP1 | Exon1 | 38299954             | A/T       | 5' untranslated region |                   |
|       | Novel SNP2 | Exon1 | 38299659             | G/C       | Coding nonsynonymous | A9G               |
| \textit{ABCD3} | Novel SNP3 | Exon4 | 94706096             | A/G       | Coding nonsynonymous | M94V              |
|       | Novel SNP4 | Exon14  | 94727816             | T/G       | Intron                |                   |
|       | Novel SNP5\textsuperscript{a} | Exon15 | 94728352             | G/C       | Intron                |                   |
| \textit{ABCD4} | Novel SNP6 | 5'UTR | 73840784             | T/C       | Upstream at the transcription start site |                   |
|       | Novel SNP7 | 5'UTR | 73839945             | T/C       | Upstream at the transcription start site |                   |
|       | Novel SNP8 | 5'UTR | 73839604             | G/A       | Upstream at the transcription start site |                   |
|       | Novel SNP9\textsuperscript{b} | Exon12 | 73826720             | A/G       | Intron                |                   |
|       | Novel SNP10 | Exon18 | 73823320             | A/G       | Intron                |                   |
|       | Novel SNP11\textsuperscript{b} | Exon18 | 73823116             | T/C       | Intron                |                   |

A total of 24 SNPs of \textit{ABCD2}, \textit{ABCD3}, and \textit{ABCD4} were identified in 40 ALD patients. Among them, 11 SNPs (45.8%) were novel SNPs. The positions of these novel SNPs were based on the UCSC genome browser hg18

\textsuperscript{a} These SNPs were identified only in the cerebral form (childhood cerebral ALD and adult with cerebral ALD)

\textsuperscript{b} These SNPs were identified only in the AMN form
there were no significant differences in the Japanese patients with the cerebral form compared with the controls (Tables 5 and 6).

Given the significant association of the five SNPs (rs17782508, rs2301345, rs4148077, rs4148078, and rs3742801) with the phenotypes of AMN, we then conducted a replication study on an independent French ALD cohort with extreme phenotypes (117 CCALD cases and 71 pure AMN cases). However, we did not find any significant association of these five SNPs with AMN or CCALD. Interestingly, the combination of two intronic SNPs (A (rs17182959) and G (rs7158118)) was significantly more frequently represented in the 51 patients with AMN-Cer than in those with CCALD in the French ALD cohort (p=0.0049). The combination of these two intronic SNPs (A (rs17182959) and G (rs7158118)), however, was not present in any of the Japanese patients with ALD, although one combination of two intronic SNPs (A (rs17182959) and G (rs7158118)) was present in the Japanese controls (Tables 5 and 6; ESM Table 5).

Discussion

Our microarray-based high-throughput mutational analysis system was accurate to detect all the mutations, which were confirmed by direct nucleotide sequence analysis. This system should be highly useful for the mutational analysis of ABCD1 for the diagnosis of patients with ALD, and the diagnosis of the carriers with ALD.

Table 4 Summary of identified single nucleotide polymorphism (SNPs) of ABCD2, ABCD3, and ABCD4 in 40 adrenoleukodystrophy patients: known SNPs

| Gene | Fragment | SNP ID | Category |
|------|----------|--------|----------|
| ABCD3 | Exon1 | rs4148058 | 5′ untranslated region |
| ABCD3 | Exon2 | rs2147794 | Intron |
| ABCD3 | Exon3 | rs16946 | Coding synonymous |
| ABCD3 | Exon7 | rs681187 | Intron |
| ABCD3 | Exon23 | rs662813 | 3′ untranslated region |
| ABCD3 | Exon23 | rs337592 | 3′ untranslated region |
| ABCD4 | 5′ UTR | rs17782508 | Upstream at the transcription start site |
| ABCD4 | Intron1 | rs17182959 | Intron |
| ABCD4 | Intron1 | rs17158118 | Intron |
| ABCD4 | Exon3 | rs2301345 | Coding synonymous |
| ABCD4 | Exon9 | rs4148077 | Coding nonsynonymous |
| ABCD4 | Exon10 | rs4148078 | Coding synonymous |
| ABCD4 | Exon11 | rs3742801 | Coding nonsynonymous |

A total of 24 SNPs of ABCD2, ABCD3, and ABCD4 were identified in 40 ALD patients. Among them, 11 SNPs (45.8%) were novel SNPs. The positions of these novel SNPs were based on the UCSC genome browser hg18.

*These SNPs were in complete disequilibrium in the Japanese population.

Table 5 Association studies of detected single nucleotide polymorphism (SNPs) with the clinical phenotypes of Japanese adrenoleukodystrophy patients: novel SNPs

| Gene | SNP name | Allele frequency (number) | p valuea |
|------|----------|---------------------------|----------|
|      |          | Cerebral form (a total of 44 chromosomes) | AMN (a total of 26 chromosomes) | Control (number of detected SNPs/total number) | Cerebral form vs AMN | Cerebral form vs control | AMN vs control |
| ABCD2 | Novel SNP1 | 3 | 0 | 5/164 | 0.2894 | 0.3700 | 1.0000 |
| ABCD2 | Novel SNP2 | 1 | 0 | 3/164 | 1.0000 | 1.0000 | 1.0000 |
| ABCD3 | Novel SNP3 | 1 | 0 | 0/164 | 1.0000 | 0.2115 | 1.0000 |
| ABCD3 | Novel SNP4 | 0 | 0 | 3/134 | 1.0000 | 1.0000 | 1.0000 |
| ABCD3 | Novel SNP5 | 1 | 0 | 0/160 | 1.0000 | 0.2157 | 1.0000 |
| ABCD4 | Novel SNP6 | 17 | 9 | 67/164 | 0.8019 | 0.8635 | 0.6680 |
| ABCD4 | Novel SNP7 | 2 | 1 | 2/164 | 1.0000 | 0.1974 | 0.3585 |
| ABCD4 | Novel SNP8 | 1 | 0 | 5/164 | 1.0000 | 1.0000 | 1.0000 |
| ABCD4 | Novel SNP9 | 0 | 1 | 0/164 | 0.3714 | 1.0000 | 0.1368 |
| ABCD4 | Novel SNP10 | 4 | 5 | 14/160 | 0.2766 | 1.0000 | 1.0000 |
| ABCD4 | Novel SNP11 | 1 | 0 | 5/160 | 1.0000 | 1.0000 | 1.0000 |

a Results of two-sided Fisher's exact test
Although reverse transcription (RT)-PCR has been preferentially used for the analysis of \(ABCD1\) gene [11, 12, 26] to overcome the difficulty of specifically amplifying the \(ABCD1\) gene owing to the existence of the related highly homologous genes [13, 27], primers allowing the specific amplification of \(ABCD1\) enable the PCR analysis of genomic DNA, which is much easier than RT-PCR analysis. Similar approaches have also been used for SSCP-based [13, 28] and DNA-based diagnostic testing methods [29].

In the Japanese ALD patients, mutations of \(ABCD1\) gene were widely scattered in the entire region of \(ABCD1\) gene. All types of \(ABCD1\) mutation were distributed among all the phenotypes of ALD, including childhood cerebral form, AMN, and adulthood cerebral form, suggesting that there is no association of a particular phenotype of ALD with individual mutations as previously observed in other ALD populations [1, 11–13, 15] (http://www.x-ald.nl/). Even among the frameshift mutations that are clearly expected to cause a complete loss of ALDP functions, such \(ABCD1\) mutations were distributed among all the phenotypes of ALD.

On the basis of the comprehensive resequencing of \(ABCD2\), \(ABCD3\), and \(ABCD4\) genes, we searched for SNPs of these related genes to explore the possibility of these genes as candidate disease-modifying genes for ALD, although it was shown that ALD phenotypes are independent of \(ABCD2\) genotype in two independent association studies of \(ABCD2\) polymorphisms and ALD phenotypes [30]. Although our study did not reveal SNPs significantly associated with the clinical phenotypes irrespective of the ethnic background, the SNPs in \(ABCD4\) with suggestive association in the Japanese patients (rs17782508, rs2301345, rs4148077, rs4848078, and rs3742801) were in complete disequilibrium in the Japanese population.

In this study, we identified as many as 11 novel SNPs in \(ABCD2\), \(ABCD3\), and \(ABCD4\) genes in addition to the 13 previously described SNPs. These findings indicate that there are still numerous novel SNPs with the number comparable to that of previously described SNPs; furthermore, this study places great emphasis on the role of comprehensive resequencing in the discovery of novel SNPs in relevant genes. The novel SNPs as well as previously described ones in \(ABCD2\), \(ABCD3\), and \(ABCD4\) genes should be useful for further association studies on ALD and other peroxisome diseases and on the biological implications associated with these SNPs.

### Table 6 Association studies of detected single nucleotide polymorphism (SNPs) with the clinical phenotypes of Japanese adrenoleukodystrophy patients: known SNPs

| Gene | SNP ID     | Allele frequency (number) | Control (number of detected SNPs/total number) | \(p\) value<sup>a</sup> | Cerebral form vs AMN | Cerebral form vs control | AMN vs control |
|------|------------|---------------------------|-----------------------------------------------|--------------------------|----------------------|------------------------|---------------|
| \(ABCD3\) | Rs4148058  | 11                         | 5                                      | 22/164                   | 0.7697               | 0.1010                  | 0.3820         |
|       | Rs2147794  | 6                          | 2                                      | 34/152                   | 0.7009               | 0.2880                  | 0.0740         |
|       | Rs16946    | 6                          | 3                                      | 35/164                   | 1.0000               | 0.2933                  | 0.3019         |
|       | Rs681187   | 17                         | 9                                      | 75/158                   | 1.0000               | 0.3109                  | 0.2890         |
|       | Rs662813   | 18                         | 10                                     | 42/152                   | 1.0000               | 0.0984                  | 0.1435         |
|       | Rs337592   | 2                          | 4                                      | 19/152                   | 0.1855               | 0.1714                  | 0.7512         |
| \(ABCD4\) | Rs17782508 | 9                          | 2                                      | 42/164                   | 0.1921               | 0.5575                  | 0.0468         |
|       | Rs17182959 | 10                         | 7                                      | 40/128                   | 0.5599               | 0.3386                  | 0.8163         |
|       | Rs17158118 | 10                         | 7                                      | 33/162                   | 0.5599               | 0.8344                  | 0.4454         |
|       | Rs2301345  | 9                          | 2                                      | 42/164                   | 0.1921               | 0.5575                  | 0.0468         |
|       | Rs4148077  | 9                          | 2                                      | 42/164                   | 0.1921               | 0.5575                  | 0.0468         |
|       | Rs4148078  | 9                          | 2                                      | 42/164                   | 0.1921               | 0.5575                  | 0.0468         |
|       | Rs3742801  | 9                          | 2                                      | 42/164                   | 0.1921               | 0.5575                  | 0.0468         |

<sup>a</sup>Five SNPs (rs17782508, rs2301345, rs4148077, rs4848078, and rs3742801) were in complete disequilibrium in the Japanese population

<sup>b</sup>Results of two-sided Fisher’s exact test
The diverse phenotypic variations of ALD still remain enigmatic. Recent studies suggest the role of peroxisomes of oligodendrocytes in axonal loss and neuroinflammation [31] and microglial apoptosis as an early pathogenic change in CCALD [32]. With the advancement in our understanding of the pathophysiology of ALD, we hope that we can further probe into the disease-modifying factors on the basis of the molecular pathogenesis of ALD. Genome-wide association studies may well serve as an alternative approach for the identification of disease-modifying genetic factors.

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References

1. Mosser J, Douar AM, Sarde CO, Kioschis P, Feil R, Moser HW, Poustaik AM, Mandel JL, Aubourg P (1993) Putative X-linked adrenoleukodystrophy gene shares unexpected homology with ABC transporters. Nature 361:726–730
2. Schaumburg HH, Powers JM, Raine CS, Suzuki K, Richardson EP Jr (1975) Adrenoleukodystrophy. A clinical and pathological study of 17 cases. Arch Neurol 32:577–591
3. Moser HW (1997) Adrenoleukodystrophy: phenotype, genetics, pathogenesis and therapy. Brain 120:1458–1508
4. van Geel BM, Assies J, Wanders RJA, Barth PG (1997) X-linked adrenoleukodystrophy: clinical presentation, diagnosis, and therapy. J Neurol Neurosurg Psychiatry 63:4–14
5. Igarashi M, Schaumburg HH, Powers J, Kishimoto Y, Kolodny EH, Suzuki K (1976) Fatty acid abnormality in adrenoleukodystrophy. J Neurochem 26:851–860
6. Moser HW, Moser AB, Kawamura N, Murzyn J, Suzuki K, Schaumburg H, Kishimoto Y (1980) Adrenoleukodystrophy: elevated C26 fatty acid in cultured skin fibroblasts. Ann Neurol 7:542–549
7. Moser AB, Kreitzer N, Bezman L, Lu S, Raymond GV, Naidu S, Moser HW (1999) Plasma very long chain fatty acids in 3, 000 peroxisome disease patients and 29, 000 controls. Ann Neurol 45:100–110
8. Aubourg P, Blanche S, Jambaque I, Rocchiccioli F, Kalifa G, Naud-Saudreau C, Rolland MO, Debre M, Chaussain JL, Griscelli C, Fischer A, Bougnères PF (1990) Reversal of early neurologic and neuroradiologic manifestations of X-linked adrenoleukodystrophy by bone marrow transplantation. N Engl J Med 322:1860–1866
9. Malm G, Ringden O, Anvret M, von Dobeln U, Hagenfeldt L, Isberg B, Knuttula S, Nennesmo I, Winiarski J, Marcus C (1997) Treatment of adrenoleukodystrophy with bone marrow transplantation. Acta Paediatr 86:484–492
10. Shapiro E, Krivit W, Lockman L, Jambaque I, Peters C, Cowan M, Harris R, Blanche S, Bordigoni P, Loes D, Ziegler R, Crittenden M, Ris D, Berg B, Cox M, Moser H, Fischer A, Aubourg P (2000) Long-term effect of bone-marrow transplantation for childhood-onset cerebral X-linked adrenoleukodystrophy. Lancet 356:713–718
11. Krasemann EW, Meier V, Korsken GC, Hunnemann DH, Hanefeld F (1996) Identification of mutations in the ALD-gene of 20 families with adrenoleukodystrophy/adrenomyeloneuropathy. Hum Genet 97:194–197
12. Takano H, Koike R, Onodera O, Sasaki R, Tsuji S (1999) Mutational analysis and genotype-phenotype correlation of 29 unrelated Japanese patients with X-linked adrenoleukodystrophy. Arch Neurol 56:295–300
13. Kok F, Neumann S, Sarde CO, Zheng S, Wu KH, Wei HM, Bergin J, Watkins PA, Gould S, Sack G, Moser HW, Mandel J, Smith KD (1995) Mutational analysis of patients with X-linked adrenoleukodystrophy. Hum Mutat 6:104–115
14. Ligtelen MJ, Kemp S, Sarde CO, van Geel BM, Kleijer WJ, Barth PG, Mandel JL, van Oost BA, Bolhuis PA (1995) Spectrum of mutations in the gene encoding the adrenoleukodystrophy protein. Am J Hum Genet 56:44–50
15. Berger J, Molzer B, Fae I, Bernheimer H (1994) X-linked adrenoleukodystrophy (ALD): a novel mutation of the ALD gene in 6 members of a family presenting with 5 different phenotypes. Biochem Biophys Res Commun 205:1638–1643
16. Smith KD, Kemp S, Braiterman LT, Lu JF, Wei HM, Geraghty M, Stetten G, Bergin JS, Pevensier J, Watkins PA (1999) X-linked adrenoleukodystrophy: genes, mutations, and phenotypes. Neurochem Res 24:521–535
17. Broccardo E, Trofer-Charlier N, Savary S, Mandel JL, Chimini G (1998) Exxon organisation of the mouse gene encoding the adrenoleukodystrophy related protein (ALDRP). Eur J Hum Genet 6:638–641
18. Lombard-Platet G, Savary S, Sarde CO, Mandel JL, Chimini G (1996) A close relative of the adrenoleukodystrophy (ALD) gene codes for a peroxisomal protein with a specific expression pattern. Proc Natl Acad Sci USA 93:1265–1269
19. Guimarães CP, Domingues P, Aubourg P, Fouquet F, Pujol A, Jimenez-Sanchez G, Sá-Miranda C, Azevedo JE (2004) Mouse liver PMP70 and ALDP: homomeric interactions prevail in vivo. Biochim Biophys Acta 1689:235–243
20. Hillebrand M, Verrier SE, Ohlenbusch A, Schäffer A, Stölling HD, Wouters FS, Gärtnert J (2007) Live cell FRET microscopy: homo- and heterodimerization of two human peroxisomal ABC transporters, the adrenoleukodystrophy protein (ALDP, ABCD1) and PMP70 (ABCD3). J Biol Chem 282:26997–27005
21. Liu LX, Janvier K, Berteaux-Lecellier V, Cartier N, Benarous R, Aubourg P (1999) Homo- and heterodimerization of peroxisomal ABC-transporter protein from human and mouse liver. Proc Natl Acad Sci USA 96:7177–7182
22. Takahashi Y, Seki N, Ishiiura H, Mitsuji S, Matsukawa T, Kishino A, Onodera O, Aoki M, Shimozawa N, Murayama S, Itouya Y, Suzuki Y, Sobue G, Nishizawa M, Goto J, Tsuji S (2008) Development of...
high-throughput microarray-based resequencing system for neurological disorders and its application to molecular genetics of atroplastic lateral sclerosis. Arch Neurol 65:1326–1332
24. Wigginton JE, Abecasis GR (2005) PEDSTATS: descriptive statistics, graphics and quality assessment for gene mapping data. Bioinformatics 21:3445–3447
25. Barrett JC, Fry B, Maller J, Daly MJ (2005) Haplovie: analysis and visualization of LD and haplotype maps. Bioinformatics 21:263–265
26. Lachtermacher MB, Seuanez HN, Moser AB, Moser HW, Smith KD (2000) Determination of 30 X-linked adrenoleukodystrophy mutations, including 15 not previously described. Hum Mutat 15:348–353
27. Sarde CO, Mosser J, Kioschis P, Kretz C, Vicaire S, Aubourg P, Poustka A, Mandel JL (1994) Genomic organization of the adrenoleukodystrophy gene. Genomics 22:13–20
28. Feigenbaum V, Lombard-Platet G, Guidoux S, Sarde C, Mandel JL, Aubourg P (1996) Mutational and protein analysis of patients and heterozygous women with X-linked adrenoleukodystrophy. Am J Hum Genet 58:1135–1144
29. Boehm CD, Cutting GR, Lachtermacher MB, Moser HW, Chong SS (1999) Accurate DNA-based diagnostic and carrier testing for X-linked adrenoleukodystrophy. Mol Genet Metab 66:128–136
30. Maier EM, Mayerhofer PU, Asheuer M, Köhler W, Rothe M, Muntau AC, Roscher AA, Holzinger A, Aubourg P, Berger J (2008) X-linked adrenoleukodystrophy phenotype is independent of ABCD2 genotype. Biochem Biophys Res Commun 377:176–180
31. Kassmann CM, Lappe-Siefke C, Baes M, Brügger B, Mildner A, Werner HB, Natt O, Michaelis T, Prinz M, Frahm J, Nave KA (2007) Axonal loss and neuroinflammation caused by peroxisome-deficient oligodendrocytes. Nat Genet 39:969–976
32. Eichler FS, Ren JQ, Cossoy M, Rietsch AM, Naqpal S, Moser AB, Frosch MP, Ransohoff RM (2008) Is microglial apoptosis an early pathogenic change in cerebral X-linked adrenoleukodystrophy? Ann Neurol 63:729–742
33. Grabriel SB, Schaffner SF, Nguyen H, Moore JM, Roy J, Blumenstiel B, Higgins J, DeFelice M, Lochner A, Faggart M, Liu-Cordero SN, Rotimi C, Adenemo A, Cooper R, Ward R, Lander ES, Daly MJ, Altshuler D (2002) The structure of haplotype blocks in the human genome. Science 296:2225–2229