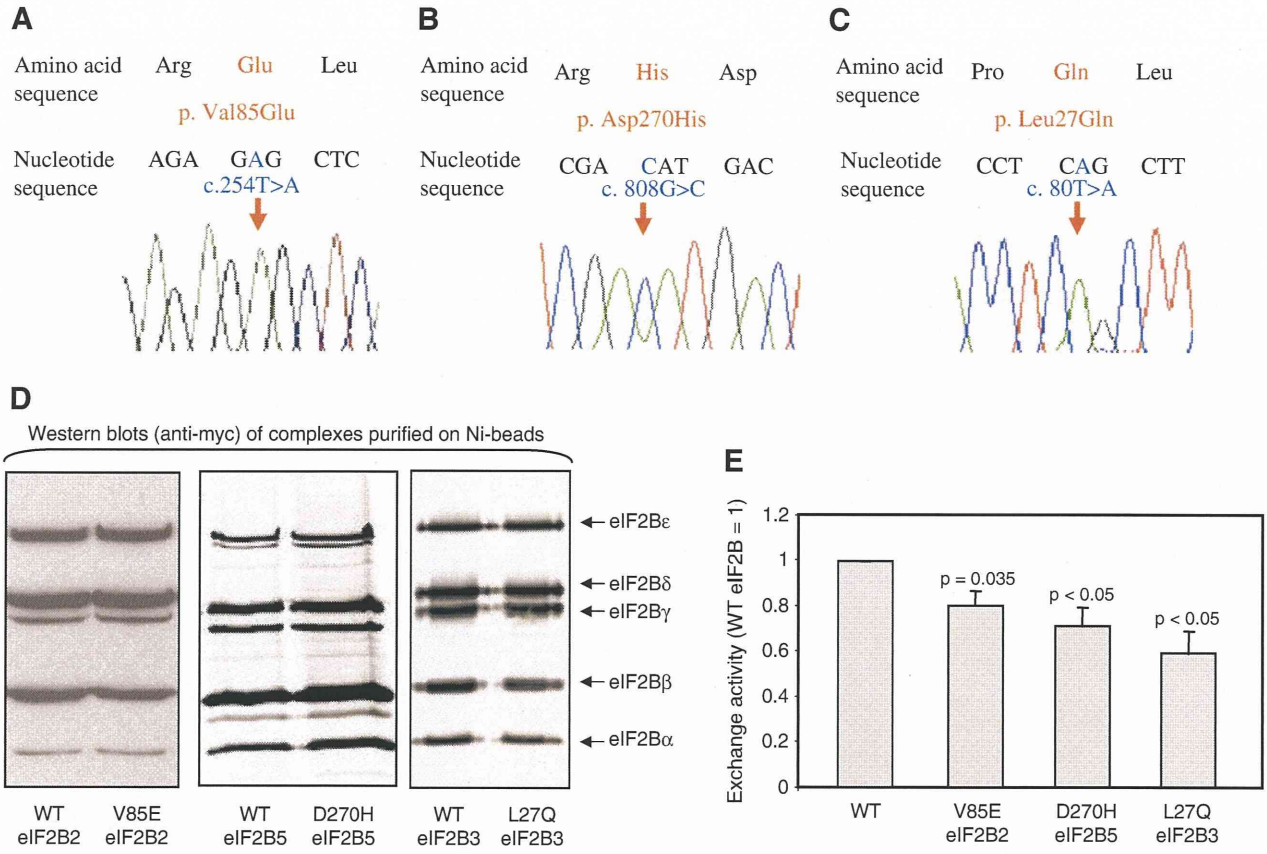

Fig. 1 A homozygous novel (p.Val85Glu) *EIF2B2* was identified (**a**) in case 1. A homozygous novel mutation (p.Asp270His) in *EIF2B5* was identified (**b**) in case 2. A homozygous novel mutation (p.Leu27Gln) in *EIF2B3* was identified (**c**) in case 3. Analyses of formation and activity of eIF2B complexes containing eIF2Bβ[V85E], eIF2Bε[D270H], or eIF2Bγ[L27Q], compared with data on complexes containing the corresponding WT subunits (**d** and **e**). In each case, the other four subunits are also WT. (**d**) Results of Western blotting with anti-myc antibody of eIF2B subunits in complexes isolated by virtue of the His_6-tag on eIF2Bβ, eIF2Bε, or eIF2Bγ, respectively. There are clearly equal amounts of the β, δ, and ε subunits. Although the signals for eIF2Bγ and α are weaker, the signal intensities of the subunits are the same as those of the WT and "mutant" complexes. (**e**) Effects of the mutant eIF2Bβ, eIF2Bε, or eIF2Bγ subunit on activity of eIF2B. There is a decrease of about 20%, 30%, or 40% in the GDP/GTP exchange activity of eIF2B-containing mutant eIF2Bβ (p.Val85Glu), eIF2Bε (p. Asp270His), or eIF2Bγ (p.Leu27Gln), respectively, when compared with eIF2B complexes containing the corresponding WT eIF2B subunits. Each experiment was performed five times, with duplicate or triplicate assays of activity in each case: data are given as % wild-type control±SEM

each of the three cases (case 1; *EIF2B1* and *EIF2B2*, case 2; *EIF2B5*, and case 3; *EIF2B5*) (Supplementary Fig. 2A–C). In accordance with these results, mutational analyses revealed a homozygous mutation (c.375T>A) in *EIF2B2* resulting in the substitution of glutamic acid for valine (p. Val85Glu), a homozygous mutation (c.808 G>C) in *EIF2B5* resulting in the substitution of aspartic acid for histidine (p. Asp270His), and a homozygous mutation (c.80T>A) in *EIF2B3* resulting in the substitution of leucine for glutamine (p.Leu27Gln) in cases 1, 2, and 3, respectively (Fig. 1a–c). No mutations were identified in other subunit genes. These mutations were not described previously and were not present in 96 unrelated Japanese control subjects. Case 3 was the first among cases of adult-onset VWM with the mutation in *EIF2B3*. Case 1 was the second among the adult-onset cases with the mutation in *EIF2B2*.

We then analyzed activities of eIF2B GEF-containing mutant *EIF2B* subunits as previously described [2]. The GDP/GTP exchange activity of eIF2B containing the mutant eIF2Bβ (Val85Glu) subunit decreased by approximately 20% compared with that of eIF2B containing the wild-type (WT) eIF2Bβ subunit (Fig. 1e). Similarly, the GDP/GTP exchange activities of eIF2B containing the mutant eIF2Bγ (Leu27Gln) and eIF2Bε (Asp270His) subunits substantially and significantly decreased compared with that of eIF2B containing WT eIF2Bγ or ε (Fig. 1e). Interestingly, the decrease in the GDP/GTP exchange activity of the eIF2B complexes containing the mutant eIF2Bβ is milder than those previously reported for mutations identified in cases of childhood-onset VWM [2], raising the possibility that mild decreases in eIF2B activity may be associated with later ages at onset. Intriguingly, the residual activity of eIF2B (eIF2B3

[L27Q]) is lower than that of eIF2B (eIF2B2[V85E] or eIF2B5[D270H]), which may be consistent with an earlier age at onset in case 3. Further detailed investigation on a much larger number of cases of VWM will be needed to confirm whether residual activity is related to the age at onset and the variability of the VWM phenotype.

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Disclosure The experiments comply with the current laws of the country in which they were performed. The authors report no conflicts of interest.

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ORIGINAL ARTICLE

Identification of novel SNPs of *ABCD1*, *ABCD2*, *ABCD3*, and *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, *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 *ABCD1* may reveal genes modifying ALD phenotypes. We analyzed 40 Japanese patients with ALD. *ABCD1* and *ABCD2* were analyzed using a newly developed microarray-based resequencing system. *ABCD3* and *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 *ABCD1* were identified, and there was no correlation between the genotypes and phenotypes of ALD. SNPs identified by the comprehensive resequencing of *ABCD2*, *ABCD3*, and *ABCD4* were used for association studies. There were no significant associations between these SNPs and ALD phenotypes, except for the five SNPs of *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 *ABCD2*, *ABCD3*, and *ABCD4* are less likely the disease-modifying genes, necessitating further studies to identify genes modifying ALD phenotypes.

Keywords Adrenoleukodystrophy · *ABCD1* · *ABCD2* · *ABCD3* · *ABCD4* · DNA microarray

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Introduction

Adrenoleukodystrophy (ALD) is a demyelinating disease caused by mutations of *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 *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 pleiomorphic 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 homomeric 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. Pooled 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

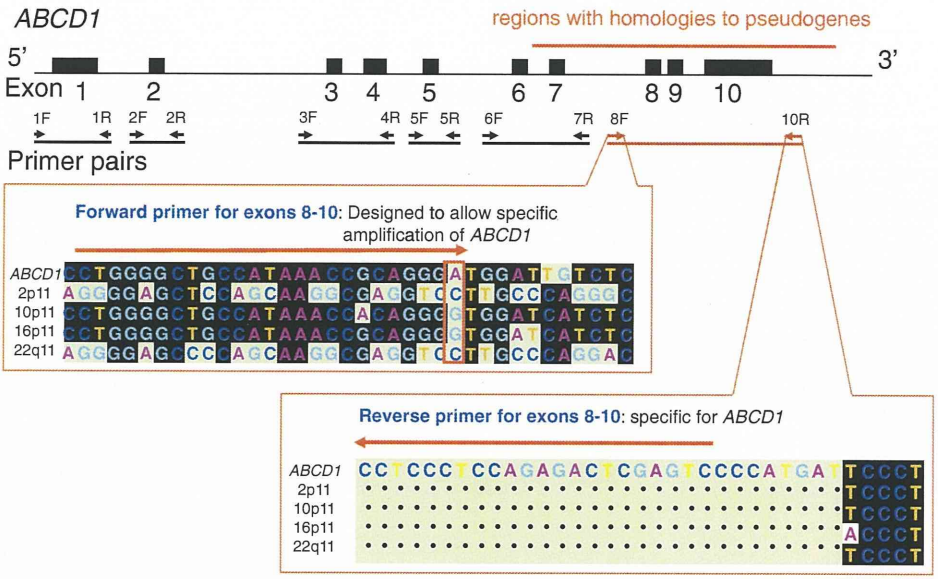

Fig. 1 Primer design for *ABCD1*. All the exons of *ABCD1* were amplified using six primer pairs. There were pseudogenes at 2p11, 10p11, 16p11, and 22q11, which were similar in sequence to exons 7–10 of *ABCD1* gene (92–96%). The forward primer for exons 8–10 was designed to avoid amplification of the related homologous genes. We could design a specific reverse primer for exons 8–10

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

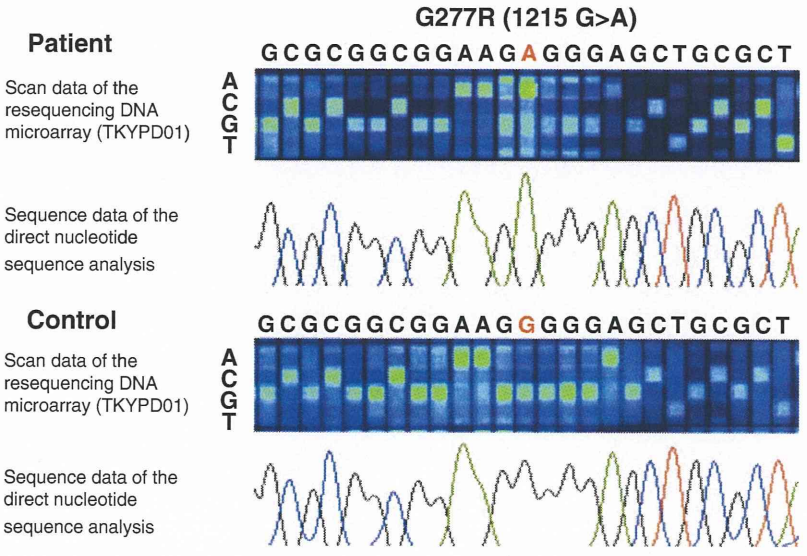

Fig. 2 Scan data of the resequencing DNA microarray and sequence data of the direct nucleotide sequence analysis (*upper panel*: patient, *lower panel*: control). Each *column* shows a base position, and each *row* shows a base call DNA in the scan data of the resequencing DNA microarray. Here, a mutation (G277R) was detected, and the signal intensities around the mutation were reduced because of the mismatch of the mutation site. The sequence data of the direct nucleotide sequence analysis confirmed the scan data of the resequencing DNA microarray

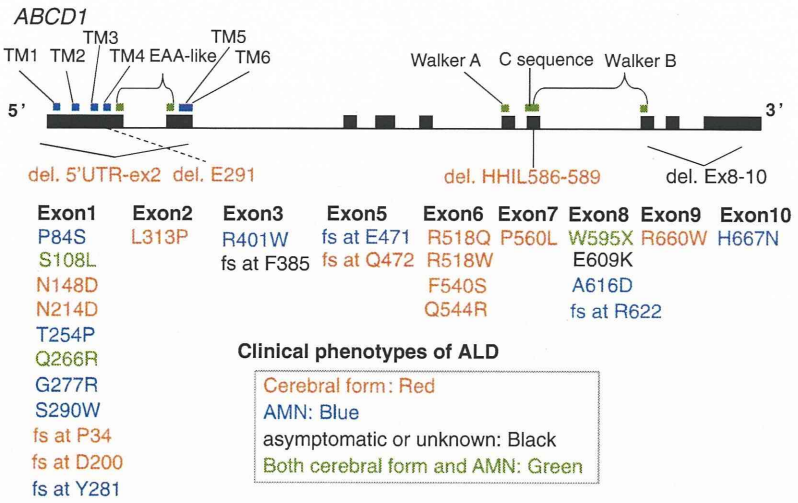

Fig. 3 Identified mutations of *ABCD1*. Mutations of *ABCD1* gene were widely scattered in the entire region of *ABCD1* gene. All types of *ABCD1* mutations were distributed among all the phenotypes of adrenoleukodystrophy. *TM* transmembrane domain, *EAA-like* EAA-like protein motif, *Walker A* Walker A motif, *C sequence* nucleotide binding fold conserved sequence, *Walker B* Walker B motif, *fs* frameshift

of *ABCD1* (Figs. 2 and 3; Tables 1 and 2). Mutations of *ABCD1* gene were widely scattered in the entire region of *ABCD1* gene (Fig. 3; Tables 1 and 2). All types of *ABCD1* mutation were distributed among all the phenotypes of ALD (Fig. 3; Tables 1 and 2). Among the 40 mutations, 11 mutations were novel (Tables 1 and 2). Among the deletion/frameshift mutations that are expected to result in complete loss of ALDP functions, the mutations were distributed among all the phenotypes of ALD (Tables 1 and 2), supporting the previous observations of no genotype–phenotype correlations.

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[a]	Disruption of gene structure
4	AdultCer	Del. 986C[a]	Frameshift at D200
5	AdultCer	Del. 1801–1802AG[a]	Frameshift at Q472
6	AMN-Cer	Del. 2251 GGTG ins. TGTTCT[a]	Frameshift at R622
7	AMN	Ins. 1237T[a]	Frameshift at Y281
8	AMN	Del. 1801–1802AG[a]	Frameshift at Q472
9	AMN	2171G>A	W595X
10	AMN	Del. 2251 GGTG ins. TGTTCT[a]	Frameshift at R622
11	Unknown	Del. 1541C[a]	Frameshift at F385
12	Unknown	Ex8-10 0.3-kb deletion[a]	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

[a] Novel mutation

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[a]	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[a]	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

[a] 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 also 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

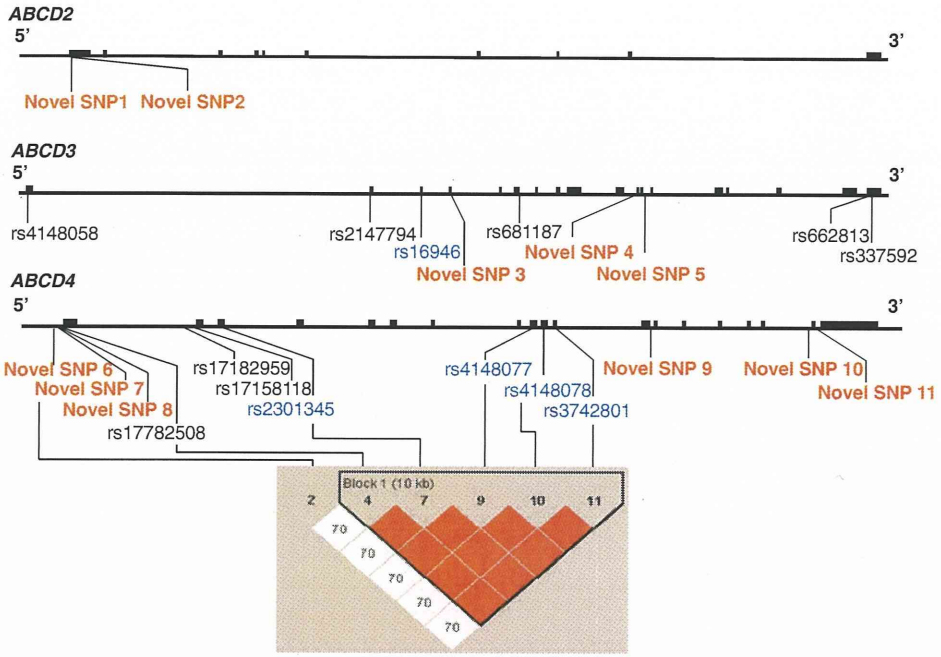

Fig. 4 Identified single nucleotide polymorphisms (SNPs) of *ABCD2*, *ABCD3*, and *ABCD4* (*upper panel*). Comprehensive resequencing of *ABCD2*, *ABCD3*, and *ABCD4* genes of the 40 patients with adrenoleukodystrophy (ALD) revealed two novel SNPs, nine SNPs (six known and three novel SNPs), and 13 SNPs (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 *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 *ABCD2*, *ABCD3*, and *ABCD4* in 40 adrenoleukodystrophy patients: novel SNPs

Gene	Name	Fragment	Position (UCSC hg18)	Base call	Category	Amino acid change
ABCD2	Novel SNP1	Exon1	38299954	A/T	5′ untranslated region	
	Novel SNP2	Exon1	38299659	G/C	Coding nonsynonymous	A9G
ABCD3	Novel SNP3	Exon4	94706096	A/G	Coding nonsynonymous	M94V
	Novel SNP4	Exon14	94727816	T/G	Intron	
	Novel SNP5[a]	Exon15	94728352	G/C	Intron	
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[b]	Exon12	73826720	A/G	Intron	
	Novel SNP10	Exon18	73823320	A/G	Intron	
	Novel SNP11[a]	Exon18	73823116	T/C	Intron	

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

[a] These SNPs were identified only in the cerebral form (childhood cerebral ALD and adult with cerebral ALD)

[b] These SNPs were identified only in the AMN form

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

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

[a] These SNPs were in complete disequilibrium in the Japanese population

Gene	Fragment	SNP ID	Category	Amino acid change
ABCD3	Exon1	rs4148058	5′ untranslated region	
	Exon2	rs2147794	Intron	
	Exon3	rs16946	Coding synonymous	
	Exon7	rs681187	Intron	
	Exon23	rs662813	3′ untranslated region	
	Exon23	rs337592	3′ untranslated region	
ABCD4	5′UTR	rs17782508[a]	Upstream at the transcription start site	
	Intron1	rs17182959	Intron	
	Intron1	rs17158118	Intron	
	Exon3	rs2301345[a]	Coding synonymous	L62L
	Exon9	rs4148077[a]	Coding nonsynonymous	A304T
	Exon10	rs4148078[a]	Coding synonymous	L320L
	Exon11	rs3742801[a]	Coding nonsynonymous	E368K

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 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 value[a]		
		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
	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
	Novel SNP4	0	0	3/134	1.0000	1.0000	1.0000
	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
	Novel SNP7	2	1	2/164	1.0000	0.1974	0.3585
	Novel SNP8	1	0	5/164	1.0000	1.0000	1.0000
	Novel SNP9	0	1	0/164	0.3714	1.0000	0.1368
	Novel SNP10	4	5	14/160	0.2766	1.0000	1.0000
	Novel SNP11	1	0	5/160	1.0000	1.0000	1.0000

[a] Results of two-sided Fisher's exact test

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)			*p* value[b]		
		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
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[a]	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[a]	9	2	42/164	0.1921	0.5575	0.0468
	Rs4148077[a]	9	2	42/164	0.1921	0.5575	0.0468
	Rs4148078[a]	9	2	42/164	0.1921	0.5575	0.0468
	Rs3742801[a]	9	2	42/164	0.1921	0.5575	0.0468

[a] Five SNPs (rs17782508, rs2301345, rs4148077, rs4848078, and rs3742801) were in complete disequilibrium in the Japanese population

[b] 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, rs4148078, and rs3742801) and French patients (rs17182959 and rs7158118) may still deserve further investigation including association studies on other independent cohorts and studies on the biological effects related to these SNPs. Among the SNPs with suggestive association in the Japanese patients (rs17782508, rs2301345, rs4148077, rs4148078, and rs3742801), rs4148077 (A304T) substitutes a hydrophilic amino acid for a hydrophobic amino acid, and rs3742801 (E368K) substitutes a basic amino acid for an acidic amino acid. It would be interesting to investigate if these amino acid substitutions may have relevance to the function of *ABCD4*.

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.

The diverse phenotypic variations of ALD still remain enigmatic. Recent studies suggest the role of perosixomes 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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