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REVIEW ARTICLE

Expanding the concept of peroxisomal diseases and efficient diagnostic system in Japan

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Abstract

The concept of peroxisomal diseases is expanding because of improvements in diagnostic technology based on advanced biochemical analysis and development of next-generation sequencing. For quicker and more accurate diagnosis of as many patients as possible, we developed a new diagnostic system combining the conventional diagnostic system and comprehensive mutational analysis by whole-exome sequencing in Japan. Adrenoleukodystrophy (ALD) is the most common peroxisomal disease. In the cerebral type of ALD, hematopoietic stem cell transplantation is the only treatment in the early stage, and thus prompt diagnosis will improve the prognosis of affected patients. Furthermore, it is also important to identify pre-symptomatic patients by family analysis of probands by providing appropriate disease information and genetic counseling, which will also lead to early intervention. Here, we summarize current information related to peroxisomal diseases and ALD and introduce our efficient diagnostic system for use in Japan, which resulted in the diagnosis of 73 Japanese patients with peroxisome biogenesis disorders, 16 with impaired β-oxidation of fatty acids, three with impaired etherphospholipid biosynthesis, and 191 Japanese families with ALD so far.

Introduction

Peroxisomes are essential organelles in humans and have many metabolic functions such as β-oxidation of saturated very long-chain fatty acids (VLCFA) and unsaturated fatty acids, bile acids synthesis, α-oxidation of phytanic acid, plasmalogen synthesis, hydrogen peroxide degradation, and glyoxylic acid detoxification. Peroxisomal diseases, which are congenital metabolic disorders, are classified into two groups: peroxisome biosynthesis disorders and single enzyme deficiencies (SEDs). Approximately 30 disease-causable genes have been reported, and not only further clinical phenotypes of known disease-causable genes but also newly disease-causable genes have been identified by whole-exome sequencing (WES). Thus, the concept of peroxisomal diseases may still be expanding. In this review, we focus on the expansion of peroxisomal diseases which must be understood to ensure precise diagnosis. We also discuss adrenoleukodystrophy (ALD), which should be diagnosed as early as possible to improve patient prognosis through early intervention. Finally, we introduce a diagnostic system of peroxisomal diseases for use in Japan.

Classification of peroxisomal diseases

Peroxisomal diseases can be classified into two major groups, genetic defects in genes encoding for enzymes that localize in peroxisomes where they exert their functions (SEDs) and genetic defects in genes encoding peroxin (PEX) proteins involved in the import of peroxisomal membranes and matrix proteins (peroxisome biogenesis disorders: PBD) (Table 1).

PBD

Peroxisomal matrix proteins can be imported into peroxisomes through the functions of many PEX proteins via peroxisome-targeting sequences, PTS1 and PTS2; therefore, PBDs, which are caused by mutated *PEX* genes, show similar phenotypes. Indeed, most patients with mutated

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Table 1 Classification of peroxisomal diseases (PD) (disease-causable genes)

A. Peroxisome biogenesis disorders (PBD) (PEX genes)
Zellweger spectrum (PEX 1,2,3,5,6,10,12,13,14,16,19,26)
Zellweger syndrome (ZS)
Neonatal adrenoleukodystrophy (NALD)
Infantile Refsum disease (IRD)
Atypical form (*PEX 1,2,3,6,10,12,16*)
Rhizomelic chondrodysplasia punctata (RCDP) type 1 (*PEX 7*)
type 5 (*PEX 5-long isoform*)
B. Single enzyme deficiencies (SEDs)
Impaired β-oxidation of fatty acids
Adrenoleukodystrophy (ALD) (*ABCD1*)
Acyl-CoA oxidase 1 (ACOX1) deficiency (*ACOX1*)
D-bifunctional protein (DBP) deficiency (*HSD17B4*)
Sterol carrier protein X (SCPx) deficiency (*SCP2*)
2-Methylacyl-CoA racemase (AMACR) deficiency (*AMACR*)
Impaired bile acids synthesis
Acyl-CoA oxidase 2 (ACOX2) deficiency (*ACOX2*)
PMP70 deficiency (*ABCD3*)
Bile acid-CoA: amino acid N-acyltransferase (BAAT) deficiency (*BAAT*)
Impaired α-oxidation of fatty acids
Refsum disease (phytanoyl-CoA hydroxylase deficiency) (*PHYH*)
Impaired etherphospholipid biosynthesis
RCDP type 2 (dihydroxyacetonephosphate acyltransferase deficiency) (*GNPAT*)
RCDP type 3 (alkyldihydroxyacetonephosphate synthase deficiency) (*AGPS*)
RCDP type 4 (fatty acyl-CoA reductase 1 deficiency) (*FAR1*)
Impaired hydrogen peroxide metabolism
Acatalasemia, Hypocatalasemia (catalase deficiency) (*CAT*)
Impaired glyoxylate metabolism
Hyperoxaluria type 1 (alanine: glyoxylate aminotransferase deficiency) (*AGXT*)
Glycolate oxidase (GO) deficiency (*HAO1*)

PEX1, *2*, *5*, *6*, *10*, *12*, *13*, *14*, and *26*, which are involved in both PTS1 and PTS2 protein import, tend to manifest with common clinical features of the Zellweger spectrum including Zellweger syndrome (ZS), neonatal adrenoleukodystrophy (NALD), and infantile Refsum disease (IRD) whose severity is dependent on the importance of the proteins localized in peroxisomes and how the residual function of these proteins exists. ZS is the severest phenotype of PBD and patients present with facial dysmorphism, severe hypotonia from the neonatal period, psychomotor retardation, hepatomegaly with prolonged jaundice and liver dysfunctions, renal cortical microcysts, ventricular enlargement in the brain, and abnormal calcific stippling of multiple joints. These patients typically die in early infancy. NALD patients have less severe clinical phenotypes than ZS and commonly die during the late infantile period, specifically exhibited as mild facial dysmorphism and no chondrodysplasia, whereas developmental regression and intractable seizure occur during the clinical courses, and demyelination and progressive cortical atrophy in the brain are remarkable, which may be because of the longer survival time than that of ZS patients. The phenotype of IRD, the mildest phenotype in the Zellweger spectrum, is very different from that of ZS and is characterized by hearing impairment, retinal degeneration, and mild psychomotor retardation. Patients with IRD typically survive for longer than the second decade of life. In contrast, patients with mutated *PEX3*, *16*, and *19*, which are involved in peroxisomal membrane protein import, tend to manifest with the severe phenotype of the Zellweger spectrum, ZS. Patients in the Zellweger spectrum biochemically show generalized peroxisomal metabolic disturbances, such as accumulation of VLCFA, phytanic and pristanic acids, and intermediate metabolites of bile acids of di-/trihydroxycholestanoic acid (D/THCA) in the plasma as well as decreased plasmalogen (Table 2) [1].

The patients with rhizomelic chondrodysplasia punctata (RCDP) type 1 and type 5 caused by *PEX7* and *PEX5-long isoform* defects, respectively, exhibit limited peroxisomal metabolic abnormalities including plasmalogen synthesis and α-oxidation of phytanic acid, as these gene products are involved in only PTS2 protein imports and result in the common clinical phenotype of RCDP (Table 2). RCDP type 1 has been classified as a skeletal dysplasia characterized by the presence of calcific stippling of multiple joints, disproportionately short stature with symmetric shortening of the proximal extremities, typical craniofacial dysmorphism, and severe mental retardation. Many patients die in the first 2 years of life, but some die in the second decade of life. RCDP type 5 was newly identified by WES and biochemical verification [2].

Furthermore, several other *PEX* defects patients with atypical phenotypes have been reported, as referred to in sub-section 'Peroxisome biogenesis disorders (PBD)'.

SEDs

SEDs tend to exhibit clinical phenotypes based on individual metabolic disturbances due to enzyme deficiencies (Tables 1, 2). Here, we outline the main SEDs as follows. Additionally, atypical phenotypes have been reported, as described in sub-section 'Impaired β-oxidation of fatty acids'.

Table 2 Biochemical abnormalities of peroxisomal diseases in plasma, and Japanese patients diagnosed by Gifu University

	VLCFAs	Phytanic acid[a]	Pristanic acid[a]	Bile acids D/THCA	Plasmalogens	Catalase-positive particles (peroxisomes)	Japanese patients diagnosed by Gifu University[b]
A. Peroxisome biogenesis disorders							
Zellweger syndrome	↑	↑	↑	↑	↓	−	56
Neonatal adrenoleukodystrophy	↑	N ∽↑	N ∽↑	↑	↓	Mosaic or ↓	4
Infantile Refsum disease	↑	N ∽↑	N ∽↑	↑	N ∽↓	Mosaic or ↓	3
Atypical form	N ∽↑	N ∽↑	N ∽↑	N ∽↑	N ∽↓	N ∽↓	6
RCDP type 1, 5	N	↑	N	N	↓	+	Type 1: 4
B. Single enzyme deficiencies							
Adrenoleukodystrophy	↑	N	N	N	N	+	191 families
Acyl-CoA oxidase 1 (ACOX1) deficiency	↑	N	N	N	N	+	5 (atypical: 2)
D-bifunctional protein (DBP) deficiency	↑	N ∽↑	N ∽↑	↑	N	+	11
Sterol carrier protein X (SCPx) deficiency	N	↑	↑	↑	N	+	0
2-Methylacyl-CoA racemase (AMACR) deficiency	N	↑	↑	↑	N	+	0
Acyl-CoA oxidase 2 (ACOX2) deficiency	N	N	N	↑	N	+	0
Refsum disease	N	↑	N	N	N	+	0
RCDP types 2–4	N	N	N	N	↓	+	Type 2: 1, type 3: 2

RCDP rhizomelic chondrodysplasia punctata, *N* normal, *D/THCA* di-/trihydroxycholestanoic acid

[a]Depend on dietary intake

[b]During 1985–July 2018

Impaired β-oxidation of fatty acids

a. ALD:

ABCD1 was identified as the gene responsible for ALD and encodes a peroxisomal membrane protein that transports VLCFA-CoA into the peroxisome. Defects in this protein result in accumulation of VLCFA. However, various phenotypes of ALD may not be correlated with the genotype and VLCFA values, and the onset mechanism of cerebral types remains unknown. ALD will be described in more detail later (Early diagnosis of ALD).

b. Acyl-CoA oxidase 1 (ACOX1) deficiency:

ACOX1 catalyzes the initial steps in peroxisomal fatty acid β-oxidation. Patients with ACOX1 deficiency have decreased muscle tone starting in the neonatal period, convulsions from infancy, and hearing and vision disturbances, but no facial dysmorphism and migration disorder in the brain. These patients die from disease regression during early childhood. Because this enzyme oxidizes only saturated fatty acids as substrates, biochemically the accumulation of VLCFA is the only abnormality. Peroxisomes are larger than usual according to immunocytochemical analysis [3].

c. D-bifunctional protein (DBP) deficiency:

DBP catalyzes the second and third stages of peroxisomal fatty acid β-oxidation. In addition to straight-chain fatty acids, bile acids and branched fatty acids are oxidized by this enzyme as substrates. Patients with DBP deficiency manifest with hypotonia from the neonatal period, poor feeding, facial dysmorphism, hepatomegaly, convulsion from the neonatal period, and mostly die within 2 years of life. Biochemically, in addition to increased VLCFA, DBP deficiency is characterized by the accumulation of D/THCA and phytanic and pristanic acids. Similar to ACOX1 deficiency, peroxisomes present a larger form than usual [3], whereas both clinical and biochemical findings in DBP deficiency are severer than for ACOX1.

d. Sterol carrier protein X (SCPx) deficiency:

SCPx enzyme is involved in the last step of peroxisomal β-oxidation, shows thiolase activity, and

oxidizes branched-chain fatty acids. Patients with SCPx deficiency show biochemically increased pristanic and phytanic acids and D/THCA, whereas VLCFA are not increased and leukoencephalopathy with dystonia and motor neuropathy are observed clinically [4].

e. 2-Methylacyl-CoA racemase (AMACR) deficiency:
AMACR is a peroxisomal enzyme that catalyzes the conversion of 2R-pristanoyl-CoA and 25R-D/THCA to their (*S*)-stereoisomers and the enzymatic defect consequently causes accumulation of plasma pristanic acid and D/THCA in patients who show various clinical findings, such as adult-onset sensorimotor motor neuropathy [5].

Impaired bile acids synthesis

a. Acyl-CoA oxidase 2 (ACOX2) deficiency:
ACOX2 is a peroxisomal branched-chain acyl-CoA oxidase involved in bile acid synthesis. Patients with ACOX2 deficiency have mild developmental delay and mild ataxia, as well as elevated liver enzymes intermittently with liver fibrosis [6]. The patients showed increased D/THCA in the plasma and urine, but no increases in branched-chain fatty acids, phytanic acid, and pristanic acid.

b. Peroxisomal membrane protein 70 (ABCD3) deficiency:
ABCD3 encodes a peroxisomal membrane protein of 70 kDa involved in the transport of branched-chain fatty acids and C27 bile acids into the peroxisomes and increases D/THCA in the plasma of patients. These patients show hepatosplenomegaly with severe liver dysfunction, but with normal developmental milestones [7].

c. Bile acid-CoA: amino acid N-acyltransferase (BAAT) deficiency:
BAAT transfers the bile acid moiety from the acyl-CoA thioester to either glycine or taurine, so the glycine and taurine bile acids conjugates are decreased in the body fluids from the patients. Phenotype of the patients shows familial hypercholanemia [8].

Impaired α-oxidation of fatty acids

Phytanoyl-CoA hydroxylase (PHYH) deficiency (Refsum disease)

Refsum disease is characterized by an increase in phytanic acid due to deficiency of PHYH localized in the peroxisome without pristanic acid accumulation. Many patients with Refsum disease develop symptoms such as retinitis pigmentosa, polyneuropathy (atrophy of lower limb muscles, muscle weakness, sensory paralysis), cerebellar ataxia at approximately 20 years of age, and increased spinal fluid protein. There have been no reports of this disease in Japanese patients.

Impaired plasmalogen biosynthesis

a. Dihydroxyacetonephosphate acyltransferase (GNPAT) deficiency (RCDP type 2)
b. Alkyldihydroxyacetonephosphate synthase (AGPS) deficiency (RCDP type 3)
The first and second steps of plasmalogen biosynthesis are performed in peroxisomes by GNPAT (PTS1 protein) and AGPS (PTS2 protein). Clinical findings of both deficiencies revealed the RCDP phenotype such as rhizomelic shortening of the upper extremities, typical facial appearance, cataract, dwarfism, and severe mental retardation. Biochemically, both types of patients show only decreased plasmalogens in the serum but no increase in phytanic acid.

Expanding peroxisomal disease and efficient diagnostic system in Japan

We have been studying peroxisomal diseases as the only diagnostic center in Japan for more than 30 years and identified the first responsible gene for PBD, *PEX2*, in 1992 [9]. In the conventional diagnostic system, after the screening of peroxisomal metabolites in patients by gas chromatography/mass spectrometry (GCMS) analysis, the disease-causable genes are identified by biochemical, morphological, and molecular analysis [1]. However, in recent years, peroxisomal diseases with new phenotypes and genotypes have been reported because of improvements in analytical techniques of peroxisomal metabolism function and the appearance of next-generation sequencing (NGS). Therefore, it is necessary to develop an efficient diagnostic system combining conventional methods and NGS.

Conventional diagnostic system of peroxisomal disease in Gifu University

When diagnosing peroxisomal disease, we first analyze VLCFA, phytanic acid, docosahexaenoic acid (DHA), and plasmalogens in the serum or plasma from the patients by GCMS analysis [10]. We optionally analyze pristanic acid and other fatty acids by liquid chromatography-mass spectrometry (LCMS) [11]. For definitive diagnosis, gene mutations are evaluated by Sanger sequencing after

identifying the disease-causable gene by biochemical, immunocytochemical, and molecular analysis using fibroblasts from the patients [1].

For example, for the Zellweger spectrum, after immunocytochemical analysis, complementation analysis is conducted among the complementation groups of the Zellweger spectrum by cell fusion, as there are 12 different disease-causable genes in the Zellweger spectrum. The final diagnosis of Zellweger spectrum is determined by mutation analysis of the *PEX* genes. Even in typical cases involving other peroxisomal diseases of RCDP1–5, ACOX1, DBP, SCPx and AMACR deficiencies, Refsum disease, and ALD, the diagnosis can be performed using the conventional system. Many Japanese patients with peroxisomal diseases have been diagnosed at Gifu University since 1985 (Table 2).

Expanding concept of peroxisomal disease

After 2010, new genetic diseases and atypical phenotypes of known diseases were reported for peroxisomal diseases based on advanced peroxisomal biochemical and molecular analysis techniques and WES.

a. Peroxisome biogenesis disorders (PBD):

   In *PEX* defects manifesting as Zellweger spectrum, gene mutations in *PEX10* [12], *PEX16* [13], *PEX2* [14], and *PEX6* [15] have been reported since 2010 and were detected using advanced biochemical and genetic analysis methods to evaluate atypical patients with cerebellar atrophy and ataxia. Furthermore, after WES became widely available, further *PEX* abnormalities were reported in patients with mainly neurodegenerative diseases, such as in *PEX1* [16], *2* [17], *3* [18], *10* [19–21], *12* [22], and *16* [23–25] as well as in those with Heimler syndrome with defects in *PEX1* and *6* [26, 27].

   In recent years, mutations of *DNM1L* [28] and *PEX11β* [29] genes are recognized as peroxisomal fission disorders. Furthermore, muscle–liver–brain–eye (mulibrey) nanism is caused by a defect in TRIM37, which is newly found to be identified as a novel E3 ligase for PEX5-mediated peroxisomal matrix protein import [30]. These diseases may be included in PBD.

b. Impaired β-oxidation of fatty acids:

   Additionally, in β-oxidation enzyme deficiencies, adults with ACOX1 deficiency with cerebellum and brain stem atrophy [31], DBP deficiencies manifesting as Perrault syndrome with ovarian malformation, hearing loss, and cerebellar ataxia [32], and cases with hearing loss, cerebellar ataxia, and retinal pigment degeneration [33] have been reported.

c. Plasmalogen synthesis enzyme deficiency:

   Furthermore, for plasmalogen synthesis in peroxisomes, a patient with severe intellectual disability, epilepsy, and cataracts due to a defect in fatty acyl-CoA reductase 1 that synthesizes the fatty alcohol necessary for the second step of the reaction was reported [34]. This disease was later named as RCDP type 4.

Establishment of efficient diagnostic system for peroxisomal disease in Japan

We have already diagnosed several atypical cases with PEX gene defects and SEDs so far (Table 2); furthermore, to diagnose these various peroxisomal diseases efficiently and accurately, it is necessary to establish a new diagnostic system that combines peroxisomal metabolite screening and genetic analysis by NGS rather than using the conventional system (Fig. 1) .

Particularly, in patients with mild and atypical peroxisomal diseases and those with minor metabolic abnormalities including VLCFA, phytanic acid, or plasmalogens, diagnosis using the conventional system may be difficult. Therefore, we established an efficient diagnostic system in collaboration with Hamamatsu University School of Medicine to search for causal mutations in genes suspected to be involved in peroxisomal diseases by WES. And then, disease-causable genes will be verified biochemically at Gifu University (Fig. 1).

To effectively use this new system in Japan, peroxisomal metabolic screening of undiagnosed patients with neurological diseases such as ataxia, spinocerebellar degeneration, cerebral white matter lesion, retinal pigment degeneration, and hearing loss should be conducted. Although many patients with peroxisomal diseases tend to manifest with severe and serious phenotypes, these subjects may have mild or moderate phenotypes, and thus early intervention of dietary restrictions and replacement therapy after early diagnosis may improve their prognosis.

Early diagnosis of ALD

ALD is a disease of X-linked inheritance. It is the most common peroxisomal disease, and shows relatively higher frequency among hereditary cerebral white matter disorders. ALD impairs β-oxidation of saturated VLCFA, resulting in the accumulation of VLCFA in the tissues and plasma. There are various clinical phenotypes such as the childhood cerebral form, adolescent cerebral form, adult cerebral form, adrenomyeloneuropathy, olivo-ponto-cerebellar form, and Addison's disease. Childhood cerebral form is the most

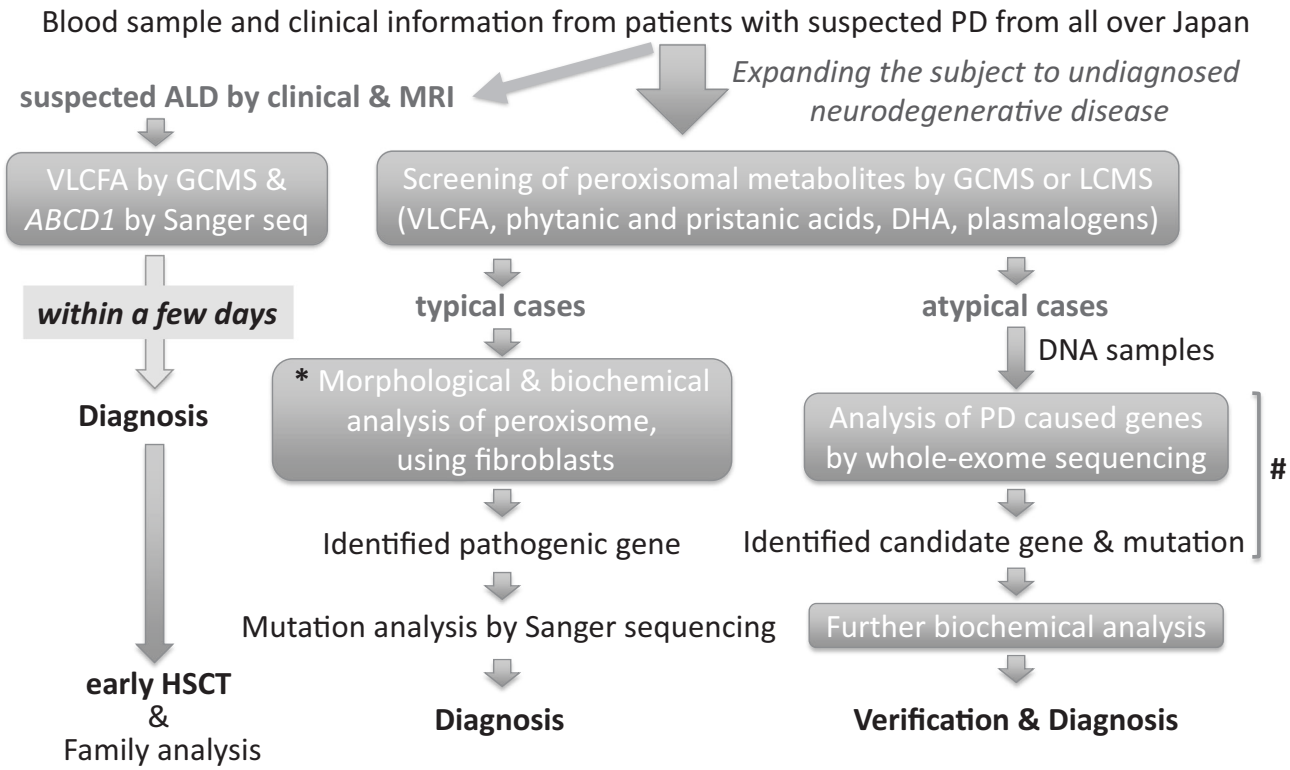

Fig. 1 Efficient diagnostic system of peroxisomal diseases (PD) in Japan. *Skip this step depending on the cases; #To share with Hamamatsu University School of Medicine in this step

common phenotype and is characterized by the progression of intellectual, psychic, visual, and gait disturbances between the ages of 3 and 10 years. The prognosis of cerebral forms is generally very poor and many patients become bedridden within a few years. Hematopoietic stem cell transplantation (HSCT) is currently the only curative approach and can prevent the progression of brain involvement in the cerebral form of ALD; however, HSCT is only effective for patients in the early stages of cerebral symptoms [35].

Additionally, because adrenal insufficiency is deeply involved in the disease state and prognosis, it is necessary to evaluate adrenal function, including ACTH loading test for all male patients with ALD, even in the patients after HSCT. Adrenocortical hormone replacement therapy is important for male ALD patients, but has no effect on their neurologic disturbances. In cases without adrenal dysfunction, adrenal function tests should be conducted yearly.

Efforts for early diagnosis of ALD by Gifu University

We have established a prompt diagnostic system for ALD patients using combined measurements of VLCFA in the serum and mutation analysis of *ABCD1* [36]. Diagnosis can be confirmed *within a few days*, particularly in cases of cerebral-type onset, leading to HSCT as soon as possible (Fig. 1).

Furthermore, pre-symptomatic diagnosis of male ALD patients may be useful for not only early preparing for HSCT but also for proper administration of adrenal hormone to improve their prognosis. Therefore, we provide sufficient disease information of ALD and genetic counseling for male patients at risk of disease in the family of the probands. If consent is obtained from family members at risk, diagnosis is carried out by measuring VLCFA and confirming the mutation in the proband. It should be noted that de novo mutations occur in approximately 4–10% of probands [36]. It is desirable to diagnose the disease until the age of 2 years from the evidences of the earliest onset age of adrenal insufficiency and cerebral type, and thereafter, as soon as possible. It is currently not possible to predict the phenotypes and prognosis before onset. Therefore, it is necessary to present long-term follow-up guidelines to patients and doctors. After diagnosis of pre-symptomatic ALD is given to male patients, follow-up every 6 months to detect subtle neuropsychological signs and abnormalities of brain MRI and electrophysiological examination are necessary until 12 years of age, and after which brain MRI and neurological examination should be performed yearly [37]. When any abnormalities are found, HSCT should be conducted as soon as possible. The adrenal

function test should be also conducted at least every year after diagnosis. In the case of carrier diagnosis for females at risk, genetic counseling should be recommended after adulthood or at the timing of pregnancy planning for babies.

It was reported that three out of five patients, who had their disease diagnosed before clinical onset and received HSCT at the stage of abnormal MRI findings without cerebral symptoms, developed myelopathy in a long-term follow-up study [38]. This means that the effect of inhibiting the progression of inflammatory demyelination in the cerebral type was confirmed in HSCT, while the inhibitory effect of adrenomyeloneuropathy onset may not be observed.

HSC gene therapy

ALD patients at the early stage of cerebral-type disease were administered Lenti-D gene therapy, which involved infusion of autologous $CD34^+$ cells transduced with the elivaldogene tavalentivec (Lenti-D) lentiviral vector as a Phase II/III investigational trial [39]. Based on the results, the Food and Drug Administration in the US has granted the Breakthrough Therapy designation to Lenti-D™ for treating patients with the cerebral type of ALD on 23 May 2018 [http://investor.bluebirdbio.com/news-releases/news-release-details/fda-grants-breakthrough-therapy-designation-lenti-dtm-treatment#].

Newborn mass screening (NBS)

In New York State, neonatal mass screening for ALD was initiated since 30 December 2013. During the first 3 years, New York State screened over 700,000 newborns and identified 45 babies with ALD, 22 boys and 23 girls [https://adrenoleukodystrophy.info/clinical-diagnosis/newborn-screening]; this should also be considered in Japan. However, several limitations must be overcome, including the establishment of a precise diagnostic algorithm even for diagnosing female ALD patients and other peroxisomal diseases, the system of genetic counseling for family members of the patients found by NBS, and a long-term follow-up system for affected male ALD patients.

How to overcome ALD in Japan

Currently, early diagnosis is the most important factor in overcoming ALD; therefore, it is important to disseminate the disease concept of ALD and maintain a prompt diagnostic system to detect VLCFA and *ABCD1* mutations. Furthermore, it is important to detect pre-symptomatic patients by familial analysis from the probands through genetic counseling and ensure their long-term follow-up nationwide. These efforts will enable the introduction of NBS in Japan.

Conclusion

The prognosis of many peroxisomal patients is expected to be improved by the efficient diagnostic system combined with peroxisomal metabolite screening and NGS, as well as by the rapid diagnostic system for postsymptomatic ALD and efforts to detect pre-symptomatic ALD patients.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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