INCIDENCE OF PEROXISOMAL DISORDERS IN JAPAN

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Summary Japanese patients with peroxisomal disorders in the pediatric field were screened. Very long chain fatty acid analysis in the serum sphingomyelin was introduced since 1987 and was useful for the first screening of peroxisomal disorders. Seventy-five patients were diagnosed since 1980: 15 patients with Zellweger syndrome, 2 with neonatal adrenoleukodystrophy (ALD), 1 with rhizomelic chondrodysplasia punctata, 1 with Zellweger-like syndrome, 2 with acyl-CoA oxidase deficiency, 2 with bifunctional enzyme deficiency and 52 with X-linked ALD. The incidence of peroxisome-deficient disorders was estimated to be approximately 1 in 800,000 births which is far less than that in the USA. However, the incidence in Okinawa Islands was 1 in 30,000. Japanese Zellweger patients belonged to 5 complementation groups (A, B, C, E, F) and the patients in Okinawa Islands belonged to groups A and C. The results of this screening were useful for genetic counseling, prenatal diagnosis, carrier detection and early medical care of patients with peroxisomal disorders.

Key Words peroxisomal disorders, screening, very long chain fatty acid(s), complementation group

INTRODUCTION

Inborn errors of peroxisomal metabolism are newly recognized disorders with multiple organ dysfunction and poor prognosis (Lazarow and Moser, 1995). Peroxisomal disorders are categorized into two groups. The first group contains diseases with defective peroxisome assembly and the second group contains diseases with single enzyme deficiency. Zellweger syndrome and neonatal adrenoleukodystrophy (ALD) belong to the first group and are characterized by morphologically absent peroxisomes and generalized loss of metabolic functions of

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peroxisomes (Lazarow and Moser, 1995). Nine complementation groups have been identified among these peroxisome-deficient disorders (Shimozawa *et al.*, 1993a). Rhizomelic type chondrodysplasia punctata (RCDP) and Zellweger-like syndrome (Suzuki *et al.*, 1988) also belong to the first group. The second group includes X-linked ALD (Moser *et al.*, 1995) and isolated deficiencies of peroxisomal β -oxidation enzymes such as acyl-CoA oxidase, bifunctional enzyme and 3-ketoacyl-CoA thiolase (Suzuki *et al.*, 1994).

Screening of these peroxisomal disorders is essential for early medical care, genetic counseling and prenatal diagnosis. Here we report the results of screening for the peroxisomal disorders in Japan with respect to the incidence and biochemical and cytogenetic aspects.

MATERIALS AND METHODS

During the past 8 years (1987–1994), 1,086 serum samples (702 male samples and 384 female samples) from 812 subjects were analyzed. Age of subjects were as follows: 0 year (y) (202 samples), 1 y (76), 2 y (36), 3 y (47), 4 y (22), 5 y (29), 6 y (15), 7 y (29), 8 y (33), 9 y (21), 10–17 y (105), $18 \leq y$ (169). Samples were sent exclusively from Departments of Pediatrics and Pediatric Neurology (910 samples), and Internal Medicine (75). About 150 samples were analyzed every year.

Peroxisomal disorders with defective β -oxidation were screened by means of saturated very long chain fatty acid (VLCFA) analysis in serum sphingomyelin (Tsuji *et al.*, 1981) with slight modification. Shortly, total fatty acids were extracted with chloroform-methanol using 0.5 ml of serum as starting material. Sphingomyelin was separated on thin layer chromatography (TLC) and methylated with 5% methanolic HCl. Fatty acids were extracted with *n*-hexane, purified by TLC, and then analyzed by gas chromatography. A Hewlett Packard 5890A gas chromatograph equipped with a splitless capillary injection system and a fused silica capillary column ultra-1 (25 m, 0.32 mm) was used. The column temperature was raised from 60°C to 180°C at 15°C/min, to 250°C at 4°C/min and then to 280°C at 15°C/min and maintained for 5 min.

Diagnosis was made by means of lignoceric acid oxidation activity (Suzuki *et al.*, 1991), dihydroxyacetone phosphate acyltransferase (DHAP-AT) activity (Schutgens *et al.*, 1984), immunoblot analysis (Suzuki *et al.*, 1986), indirect immunofluorescence staining of peroxisomes (Suzuki *et al.*, 1990) and complementation analyses (Yajima *et al.*, 1992; Suzuki *et al.*, 1994), using cultured skin fibroblasts. These methods were also applied for the prenatal diagnoses, using cultured amniocytes or chorionic villous tissues and cells.

RESULTS

VLCFA analysis

Samples from male subjects were more frequent than those of female subjects, due to the high incidence and repeated analyses of samples from patients with X-linked ALD (Asano *et al.*, 1994). Most of infant samples were screened because of psychomotor retardation, hypotonia, liver dysfunction and dysmorphism. Adult samples were mainly measured for the screening of X-linked ALD and family analysis.

There were no significant sex- or age-related differences in the ratios of C24:0/C22:0 (Table 1) in the control subjects. Ratios of C24:0/C22:0, C25:0/C22:0 and C26:0/C22:0 were increased in all patients with Zellweger syndrome, Zellweger-like syndrome, neonatal ALD, isolated deficiency of peroxisomal β -oxidation enzymes and X-linked ALD (Table 2). There was no false-positive or false-negative result in these patients. Patients with Zellweger syndrome, neonatal ALD and Zellweger-like syndrome showed higher ratios than those in patients with X-linked ALD. Most of the heterozygotes of X-linked ALD showed increased ratios. The ratio in a patient with RCDP was normal as described (Lazarow and Moser, 1995).

Table 1. C24:0/C22:0 ratios in the different age or sex groups of control subjects.

	C24:0/C22:0
0-3 months (n=24)	0.63 $\pm$ 0.14
4-12 months (n=16)	0.60 $\pm$ 0.12
1-6 years (n=41)	0.60 $\pm$ 0.11
7-18 years (n=34)	0.58 $\pm$ 0.09
18 $\leq$ years (n=20)	0.61 $\pm$ 0.12
Male (n=108)	0.61 $\pm$ 0.11
Female (n=64)	0.63 $\pm$ 0.11
Total (n=172)	0.62 $\pm$ 0.11

Table 2. Very long chain fatty acids in serum sphingomyelin.

Disease	C24:0/C22:0	C25:0/C22:0	C26:0/C22:0
Zellweger (n=12)	1.99 $\pm$ 0.43	0.16 $\pm$ 0.06	0.16 $\pm$ 0.07
Neonatal ALD	1.69	0.19	0.10
Zellweger-like	2.17	0.19	0.10
Bifunctional enzyme def.	1.52	0.09	0.08
Acyl-CoA oxidase def.	1.76	0.09	0.04
RCDP	0.53	n.d.	n.d.
ALD patients (n=31)	1.36 $\pm$ 0.24	0.08 $\pm$ 0.03	0.03 $\pm$ 0.02
carriers (n=28)	1.21 $\pm$ 0.16	0.07 $\pm$ 0.03	0.03 $\pm$ 0.02
Control (n=155)	0.62 $\pm$ 0.11	0.02 $\pm$ 0.01	0.006 $\pm$ 0.003

ALD, adrenoleukodystrophy; RCDP, rhizomelic type chondrodysplasia punctata.

Year	ZS	NALD	RCDP	ZLS	β	ALD
80	#					#
81						
82						#
83	#					#
84				#		#
85	#					
86	#				#	
87	####					
88		#				####
89	#					#
90	#					#####
91			#		#	#####
92	#	#				#####
93	#					#####
94	#					###
Total	15	2	1	1	4	52

Fig. 1. Peroxisomal disorders biochemically diagnosed in Gifu University from 1980 to 1994. ZS, Zellweger syndrome; NALD, neonatal adrenoleukodystrophy; RCDP, rhizomelic type chondrodysplasia punctata; ZLS, Zellweger-like syndrome; β , isolated deficiencies of β -oxidation enzymes; ALD, adrenoleukodystrophy; #, patient.

Table 3. Peroxisomal disorders in each prefecture.

Prefecture	PBD	ALD	Others	ALD carriers	Total subjects
Tokyo	2	11	—	10	94
Gifu	3	7	—	6	264
Aichi	—	8	1 (RCDP)	3	52
Okinawa	6	—	1 (Bif)	—	11
Nagasaki	—	5	—	8	29
Shizuoka	1	3	—	3	25
Kanagawa	1	2	—	2	12
Hyogo	1	2	—	—	11
Okayama	1	1	—	3	24
Yamanashi	—	2	—	2	8
Kyoto	—	2	—	2	5
Tottori	—	1	1 (Bif)	—	65
Ishikawa	—	1	1 (ZLS)	—	10
Chiba	—	—	2 (AOX)	—	26
Others (26)	2	7	—	5	176
Total	17	52	6	44	812

PBD, peroxisome biogenesis disorders; ALD, adrenoleukodystrophy; RCDP, rhizomelic type chondrodysplasia punctata; Bif, bifunctional enzyme deficiency; ZLS, Zellweger-like syndrome; AOX, acyl-CoA oxidase deficiency.

Peroxisomal disorders in Japan

During the past 15 years (1980–1994), we diagnosed biochemically 75 Japanese patients with peroxisomal disorders (Fig. 1). The number of patients with

X-linked ALD rapidly increased after the start of VLCFA analysis. We diagnosed 37 boys with childhood ALD, 8 presymptomatic boys, 6 adults with adrenomyeloneuropathy and a boy with Addison disease only. Forty-four female heterozygotes (carriers) of X-linked ALD were also diagnosed. Numbers of patients, carriers and total subjects analyzed in each prefecture are listed in Table 3.

Since 1985 when we established the diagnostic system, 13 Zellweger patients and 2 neonatal ALD patients from 15 different families were diagnosed in Japan. With approximately 1.2 million births a year in Japan, the experience in Gifu University alone would account in an incidence of 1 in 800,000 births in Japan. However, the incidence in Okinawa Islands, southwestern part of Japan, was considerably high, that is, about 1 in 30,000 births (Fig. 2). Incidence in Honshu, the main land of Japan, was estimated to be approximately 1 in 1,500,000 births.

Fig. 2. Peroxisome-deficient disorders biochemically diagnosed in Gifu University from 1980 to 1994. ■, patients from Okinawa Islands; ●, patients from Honshu. Alphabet indicates the complementation groups.

Fig. 3. Lignoceric acid oxidation activities in fibroblasts from patients with peroxisomal disorders diagnosed in Gifu University.

Biochemical and complementation studies of peroxisomal disorders

Lignoceric acid oxidation activities were severely reduced in fibroblasts from patients with Zellweger syndrome, neonatal ALD and β -oxidation enzyme deficiency, whereas those of X-linked ALD patients and carriers were mildly reduced (Fig. 3). DHAP-AT activity was deficient in Zellweger syndrome, neonatal ALD and RCDP, whereas that of β -oxidation deficiency was within the normal range (Fig. 4).

Figure 2 shows the complementation grouping of Japanese patients with Zellweger syndrome and neonatal ALD. Five complementation groups (A, B, C, E and F) were identified. Patients in Okinawa Islands belonged to groups A and C. Three patients in Honshu who belonged to groups A and C were also from Okinawa Islands. Other patients in Honshu mainly belonged to groups E and F (Fig. 2).

Fig. 4. Dihydroxyacetone phosphate acyltransferase activity in fibroblasts from patients with peroxisomal disorders diagnosed in Gifu University.

Table 4. Prenatal diagnosis of peroxisomal disorders in Gifu University.

Year	Proband	Disease	Methods	Result
1988	NK	Bif	C24 ox	normal
1990	NK	Bif	C24 ox	normal
1992	MT	RCDP	DHAP-AT, Immunoblot	normal
	SS	NALD	Immunofl, C24 ox	normal
	MM	ZS(F)	Immunofl, PAF-1 gene	carrier
1993	NS	ALD	C24 ox	affected
1994	MM	ZS(F)	Immunofl, PAF-1 gene	affected
	AM	ZS(A)	Immunofl, C24 ox	affected
1995	MN	ALD	ALDP gene, C24 ox	carrier
	MM	ZS(F)	Immunofl, C24:0 ox	normal
	SS	NALD	Immunofl, C24:0 ox	normal

Bif, bifunctional enzyme deficiency; RCDP, rhizomelic type chondrodysplasia punctata; ALD, adrenoleukodystrophy; NALD, neonatal ALD; ZS(F), group F Zellweger syndrome; ZS(A), group A Zellweger syndrome; C24 ox, lignoceric acid oxidation; DHAP-AT, dihydroxyacetone phosphate acyltransferase; Immunofl, immunofluorescence staining of peroxisomes; PAF-1, peroxisome assembly factor-1; ALDP, ALD protein.

Prenatal diagnosis

Table 4 summarizes the results of prenatal diagnoses performed since 1988. Eleven fetuses from 7 families were tested. Six of them were diagnosed as normal, and the resultant babies were healthy. Two fetuses were determined to be heterozygotes of group F Zellweger syndrome (Shimozawa *et al.*, 1993b) and X-linked ALD by means of gene analysis. Three fetuses were judged as affected and the diagnoses were confirmed by autopsy.

DISCUSSION

Peroxisomal disorders are recently recognized inborn errors of metabolism with severe multiple organ dysfunction and poor prognosis. Individual disorders are relatively rare, but many clinical phenotypes are present (Lazarow and Moser, 1995). Especially, diseases with defective peroxisomal β -oxidation and the accumulation of VLCFA have the majority of peroxisomal disorders. Zellweger syndrome, neonatal ALD, infantile Refsum disease, Zellweger-like syndrome, isolated deficiencies of β -oxidation enzymes and X-linked ALD belong to this category. The screening and the diagnosis of these disorders are essential for the early medical care, genetic counseling and prenatal diagnosis. Hence it was considered to be necessary to establish a diagnostic center for the peroxisomal disorders in Japan.

Previously, diagnosis of peroxisomal disorders was made by means of pathological findings. Although the accumulation of VLCFA in X-linked ALD was first reported by Igarashi *et al.* in 1976, it had not been linked to the defective peroxisomal β -oxidation. In 1982, Brown *et al.* first pointed out the possibility of relationship between the accumulation of VLCFA in Zellweger syndrome and the absence of peroxisomes (Goldfischer *et al.*, 1973). Since then, many patients with peroxisomal disorders have been screened by means of VLCFA analysis. In Japan, X-linked ALD had been screened in several laboratories. However, the screening system for various peroxisomal disorders in the pediatric field had not been established. In our experience, peroxisomal disorders with defective β -oxidation could be screened by means of VLCFA analysis of serum sphingomyelin, without any false-positive or false-negative results. Of the subjects, 9.2% (75/812) were diagnosed as having a peroxisomal disorder. Biochemical, immunocytochemical and complementation analyses are indispensable for the precise diagnosis. Screening of other rare peroxisomal disorders such as RCDP, hyperoxaluria type I and inborn errors of bile acids or phytanic acid should be done separately.

The incidence of Zellweger syndrome and neonatal ALD in Japan was very rare (1 : 800,000 in total, 1 : 1,500,000 in Honshu) as compared to western countries. The incidence in the USA is considered to be at least 1 in 125,000 birth (Lazarow and Moser, 1995). The high incidence in Okinawa Islands may be due to the ancestral inbreeding within a small population. The results of the com-

plementation study suggest the presence of two founders of gene mutation in groups A and C Zellweger syndrome in Okinawa. Analysis of genes for groups A and C, although not identified yet, will clarify the genetic basis of these phenomena. The incidence of X-linked ALD in the USA was estimated to be at least 1 in 100,000 (Moser *et al.*, 1995). The incidence of X-linked ALD in Japan is speculated to be as common as in the USA from our data in recent years, although data from other laboratories in Japan are not available. Other diseases such as RCDP, Zellweger-like syndrome and isolated deficiency of β -oxidation enzyme are extremely rare.

Our screening system was useful for the early diagnosis and medical care of presymptomatic boys with X-linked ALD, genetic counseling of the families and for the prenatal diagnosis. Erucic acid (Lorenzo's oil) has a favorable effect on the prevention of neurologic symptoms of X-linked ALD (Moser *et al.*, 1994). Two presymptomatic boys remained symptom-free for several years after starting erucic acid therapy (Asano *et al.*, 1994). Identification of female heterozygotes of X-linked ALD is also an important purpose of the screening. They can receive genetic counseling. Prenatal diagnosis was precisely performed on all subjects.

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