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ORIGINAL ARTICLE

Long-term outcome and intervention of urea cycle disorders in Japan

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Abstract Urea cycle disorders (UCDs) are one of the most frequently inherited metabolic diseases in Japan, with an estimated prevalence of 1 per 50,000 live births. Here, we investigated the clinical manifestations, treatment, and prognosis of 177 patients with UCDs who were evaluated and treated from January 1999 to March 2009. These included 77 cases of neonatal-onset UCDs and 91 cases of late-onset UCDs. The most common UCD was ornithine transcarbamylase deficiency (OTCD), which accounted for 116 out of 177 patients. This result is similar to a previous study performed between 1978 and 1995 in Japan: OTCD accounted for about two-thirds of the total number of UCD cases. We studied the relationship between prognosis and the peak blood ammonia level at the onset in 151 UCD

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patients. Compared with a previous survey conducted in Japan, we found that a greater number of patients survived without any mental retardation despite their peak blood ammonia levels being greater than 360 μmol/l. The 5-year survival rate of patients with OTCD improved to 86% for those with the neonatal-onset type and to 92% for those with the late-onset type. We hypothesize that the increased survival rate is due to early diagnosis and better treatments that are now available in Japan. It is very important to diagnose and treat UCDs, especially OTCD, when the blood ammonia levels in patients are low. The outcome in patients with low blood ammonia levels was better than that in patients with high blood ammonia levels.

Introduction

Urea cycle disorders (UCDs) are one of the most frequently inherited metabolic diseases in Japan, with an estimated prevalence of 1 per 50,000 live births. The urea cycle is the metabolic pathway that eliminates excess endogenous and exogenous nitrogen from the body by detoxification of ammonia into urea. This cycle comprises six different enzymes. Three of these enzymes are found in the mitochondrial matrix [*N*-acetylglutamate synthase (NAGS) EC 2.3.1.1; carbamoylphosphate synthetase 1 (CPS1) EC 6.3.5.5; and ornithine transcarbamylase (OTC) EC 2.1.3.3], and the other three are found in the cytosol [arginosuccinate synthetase (AS) EC 6.3.4.5; arginosuccinate lyase (AL) EC 4.3.2.1; and arginase-1 (AG) EC 3.5.3.1]. Dysfunction of these enzymes causes hyperammonemia. Nagata et al. (1991a) reported the prevalence rate of all five UCDs in Japan: CPS1 deficiency (CPSD; Mc Kusick no. 237300), 1 per 800,000 births; OTC deficiency (OTCD; Mc Kusick no. 311250), 1 per 80,000 births; classic AS deficiency (ASD; Mc Kusick no. 215700), 1 per 530,000 births; AL deficiency (ALD; Mc Kusick no. 207900), 1 per 800,000 births; and AG deficiency (AGD; Mc Kusick no. 207800), 1 per 2,200,000 births. OTCD is an X-linked disorder, whereas CPSD, ASD, ALD, and AGD are autosomal recessive disorders (Matsuda and Tanase 1997). The traditional treatment for UCDs is a low-protein diet. Sodium benzoate and/or sodium phenylbutyrate are used as an alternative pathway therapy (Brusilow et al. 1979; Brusilow 1991). Because arginine is lacking in all UCDs except AGD, arginine can be administered as therapy (Nagasaka et al. 2006). If necessary, treatment with essential amino acids and L-carnitine is recommended (Leonard 2001). Citrulline treatment is recommended for OTCD and CPSD patients (Feillet and Leonard 1998; Batshaw et al. 2001). Hemodialysis is more effective than peritoneal dialysis to rapidly eliminate blood ammonia (Schaefer et al. 1999). If medical therapy fails to control the general condition of UCD patients, a liver transplant is necessary (Uemoto et al. 1997).

There are many reports about the long-term outcome of UCD patients in Japan and overseas (Bachmann 2003; Maestri et al. 1996; Matsuda et al. 1991; Msall et al. 1984; Nagata et al. 1991b; Nassogne et al. 2005; Nicolaides et al. 2002; Uchino et al. 1998). Fifteen years have passed since the previous study was carried out in Japan. We assume that living-donor liver transplants and hemodialysis are more frequently performed now as compared to 15 years ago, and therefore we studied the long-term outcome and treatment of UCDs in Japan today.

Patients and methods

Study patients

In 2009, we sent a questionnaire to 928 institutions, including the departments of pediatrics, endocrinology and metabolism, neonatology, genetics, and transplant surgery, asking doctors if they had diagnosed or provided medical care to UCD patients. Each institution was the medical center for a local area in Japan and had 300 or more beds. Of the 928 institutions, 668 (72%) responded. Of these 668 institutions, 125 had treated patients with UCDs. A second questionnaire was then sent to these 125 institutions in 2009, of which 87 (70%) responded. Based on the reports of doctors who had diagnosed and treated patients with UCDs from January 1999 to March 2009, 177 cases of UCDs were studied. We excluded patients with secondary UCDs (citrin deficiency and lysinuric protein intolerance) and patients with unexplained hyperammonemia. We regarded a patient who visited several institutions as a single patient. The 177 cases of UCDs (CPSD, OTCD, ASD, ALD, and AGD) were diagnosed on the basis of clinical manifestations, family history, enzyme activity, metabolite analysis (blood amino acids and urinary orotic acid), and/or DNA analysis. Cognitive evaluations to diagnose mental retardation were performed by a pediatrician or child psychiatrist who treated patients with UCDs. The patient's IQ was evaluated using standardized tests such as the Wechsler Intelligence Scale for Children (WISC) or the Wechsler Adult Intelligence Scale (WAIS). We described cognitive evaluation according to assessments by these doctors. The previous study (Uchino et al. 1998) was conducted in 1994 and reported on UCD patients from 1978 to 1995. At least 131 patients who were born after January 1996 or manifested symptoms after January 1996 were newly registered.

This study was approved by the ethics committee of the Faculty of Life Science, Kumamoto University.

Statistical analysis

Blood ammonia levels in the hemodialysis group and the nonhemodialysis group as well as the time when a liver

Table 1 Onset time and confirmed diagnosis of 177 patients with urea cycle disorders (UCDs) in Japan. DNA analysis was performed in 73 patients with OTCD (36 male, 37 female) and in 15 patients with CPSD, 10 patients with ASD, and 1 patient with ALD

	Enzyme deficiency		Identifiable mutation		Onset time			Total (*n*)	Total (%)
					Neonatal onset	Late onset	Unknown		
Male OTCD	16/57	28%	23/57	40%	21 (37%)	30 (53%)	6[a](10%)	57	32%
Female OTCD	15/59	25%	16/59	27%	7 (12%)	50 (85%)	2 (3%)	59	33%
CPSD	5/23	22%	10/23	43%	19 (83%)	3 (13%)	1 (4%)	23	13%
ASD	4/28	14%	7/28	20%	21 (75%)	7 (25%)	0	28	16%
ALD	2/9	22%	1/9	8%	8 (89%)	1 (11%)	0	9	5%
AGD	1/1	100%	0/1	1%	1 (100%)	0	0	1	0.6%
Total	43/177	24%	57/177	32%	77 (44%)	91 (51%)	9[b] (5%)	177	100%

OTCD Ornithine transcarbamylase deficiency, *CPSD* carbamoyl-phosphate synthetase 1 deficiency, *ASD* arginosuccinate synthetase deficiency, *ALD* arginosuccinate lyase deficiency, *AGD* arginase-1 deficiency

Neonatal onset ≦28 days from birth; *late onset* >28 days after birth

[a] Includes one treated case before the onset and five cases where onset timing was unknown

[b] Includes one treated case before the onset and eight cases where onset timing was unknown

transplant was performed after the onset were expressed as median and interquartile range (IQR) and analyzed by Mann-Whitney U test with IBM SPSS version 19. A *P* value of<0.05 was considered statistically significant (Supplemental data 1).

Kaplan-Meier curves of estimated survival rate were generated with the error bars representing 95% confidence interval (CI) for the mean, and comparisons between the groups were performed using a two-sided log-rank test by Graphpad Prism 5.

Results

We investigated the methods used to confirm diagnosis and found that enzyme activity was measured in 24% (43/177) of the patients with UCDs, and genotype analysis was performed in 56% (99/177); genotypes were identified in 32% (57/177) of the patients with UCDs. Eleven patients (6%) were diagnosed by DNA and enzymatic analysis. DNA was analyzed in 73 patients (male: 36, female: 37) with OTCD, 15 patients with CPSD, 10 patients with ASD, and 1 patient with ALD. In the analyzed patients, identifiable mutations were detected at a rate of 64% (23/36) in male-OTCD, 43% (16/37) in female-OTCD, 67% (10/15) in CPSD, 70% (7/10) in ASD, and 100% (1/1) in ALD.

Frequency of neonatal-onset and late-onset UCDs

Table 1 presents the rate of enzyme deficiencies, identifiable mutations, and number of UCD patients by age at disease onset: 77 patients (44%) manifested symptoms in the neonatal period and 91 patients (51%) were late-onset (presented symptoms after day 28 post-partum). OTCD was by far the most common UCD, accounting for 36% (28/77) of all neonatal-onset cases and 88% (80/91) of the late-onset cases. Among the CPSD, ASD, and ALD cases, 83% (19/23), 75% (21/28), and 89% (8/9) of patients, respectively, presented symptoms when they were newborns. Figure 1 shows the onset time for patients with OTCD. Of the male and female late-onset OTCD patients, 64% (51/80) presented symptoms between the ages of 1 and 6 years.

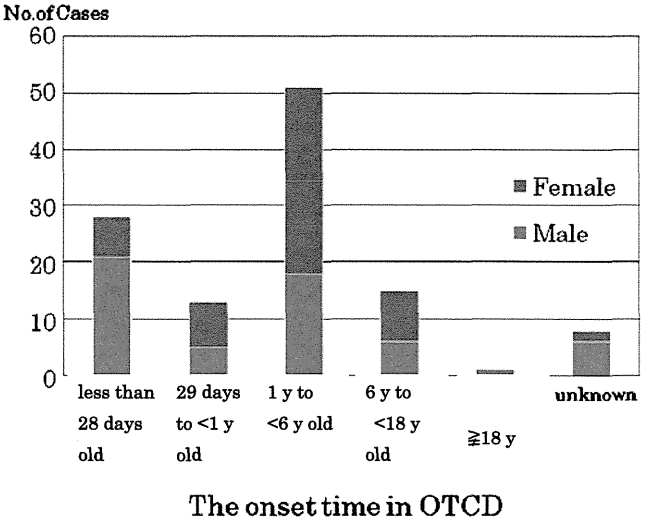

Fig. 1 The onset time in patients with ornithine transcarbamylase deficiency (OTCD). The most frequent onset time for late-onset patients with OTCD was from 1 to 6 years of age. The unknown group include one treated male case before the onset and seven cases where onset timing was unknown (male: *n*=57, female: *n*=59)

Table 2 Survival numbers of urea cycle disorder patients

	Neonatal onset			Late onset			Total
	Nonsurviving patients	Surviving patients	Subtotal	Nonsurviving patients	Surviving patients	Subtotal	
Male-OTCD	4	17	21	3	27	30	57[a]
Female-OTCD	1	6	7	6	44	50	59[b]
CPSD	4	15	19	0	3	3	23[c]
ASD	2	19	21	0	7	7	28
ALD	2	6	8	0	1	1	9
AGD	0	1	1	0	0	0	1
Total	13	64	77	9	82	91	177

OTCD Ornithine transcarbamylase deficiency, *CPSD* carbamoyl-phosphate synthetase 1 deficiency, *ASD* arginosuccinate synthetase deficiency, *ALD* arginosuccinate lyase deficiency, *AGD* arginase-1 deficiency

[a] The onset time of five cases was unknown, and one patient was cured before the onset

[b] The onset time of two cases was unknown

[c] The onset time of one case was unknown

Long-term survival

Table 2 presents survival numbers of patients with UCDs. Seventeen percent (13/77) of neonatal-onset cases and 10% (9/91) of the late-onset cases died.The overall survival rate after the first hyperammonemic attack was 93% (156/168). There was a 90% (69/77) 1-year survival rate for patients with neonatal-onset UCDs and a 95% (86/91) 1-year survival rate for patients with late-onset UCDs (data not shown). Among the deceased patients with neonatal-onset disease, 62% (8/13) died within 1 month after the first hyperammonemic attack. In the late-onset cases, 44% (4/9) of all deaths occurred within 1 month of the first hyperammonemic attack (data not shown).

Figure 2a represents the survival curve of patients with OTCD. The 5-year survival rate of patients with OTCD was 86% for those with the neonatal-onset type and 92% for those with the late-onset type. The 10-year survival rate for the three types of OTCD, except for female neonate-onset OTCD, was more than 80%. One of the seven female patients with neonatal onset of OTCD died 7 years and 4 months after the onset. Figure 2b and c represents the survival age of patients with late-onset OTCD. We compared the Kaplan-Meier survival curves of the previous study (Uchino et al. 1998) with those of the present study. The survival rate of male patients with late-onset OTCD was 89.4% at age 20 years in the present study (n=30) and was 45.0% at age 20 years in the previous study (n=48). The long-term survival rate of male patients with late-onset OTCD was significantly improved in this study (P= 0.004). The survival rate of female patients with late-onset OTCD was 83.8% at age 20 years in the present study (n= 50) and 29.8% at age 20 years in the previous study (n=51). The long-term survival rate of female patients with late-onset OTCD was also improved in this study (P<0.001).

Relationship between peak blood ammonia levels during the first hyperammonemic attack and cognitive development

Figure 3a presents the relationship between peak blood ammonia levels during the first hyperammonemic attack and cognitive outcome. The peak blood ammonia levels at the onset and neurodevelopmental outcomes were known for 151 patients with UCDs. The two patients with a maximum ammonia concentration of less than 60 μmol/l survived, had not developed mental retardation, and had normal brain CT and MRI images and a normal electroencephalogram. When the maximum ammonia concentration was between 60 and 180 μmol/l, 64% (18/28) of patients survived, had not developed mental retardation, and had normal brain CT and MRI images and a normal electroencephalogram. Furthermore, 21% (6/28) of these patients had not developed mental retardation but had abnormal brain CT and MRI images and an abnormal electroencephalogram; 14% (4/28) of these patients survived but had developed mental retardation. Twelve patients died at the onset of the UCD, and the maximum ammonia concentration was recorded for 11 of them, which was greater than 360 μmol/l. Of the 74 patients with a maximum ammonia concentration greater than 360 μmol/l, 15% (11/74) died and 51% (38/74) developed mental retardation. In addition, 15% (11/74) of these patients did not develop mental retardation but had abnormal brain CT and MRI images or an abnormal electroencephalogram; 8% (6/74) of patients did not develop mental retardation and had normal brain CT

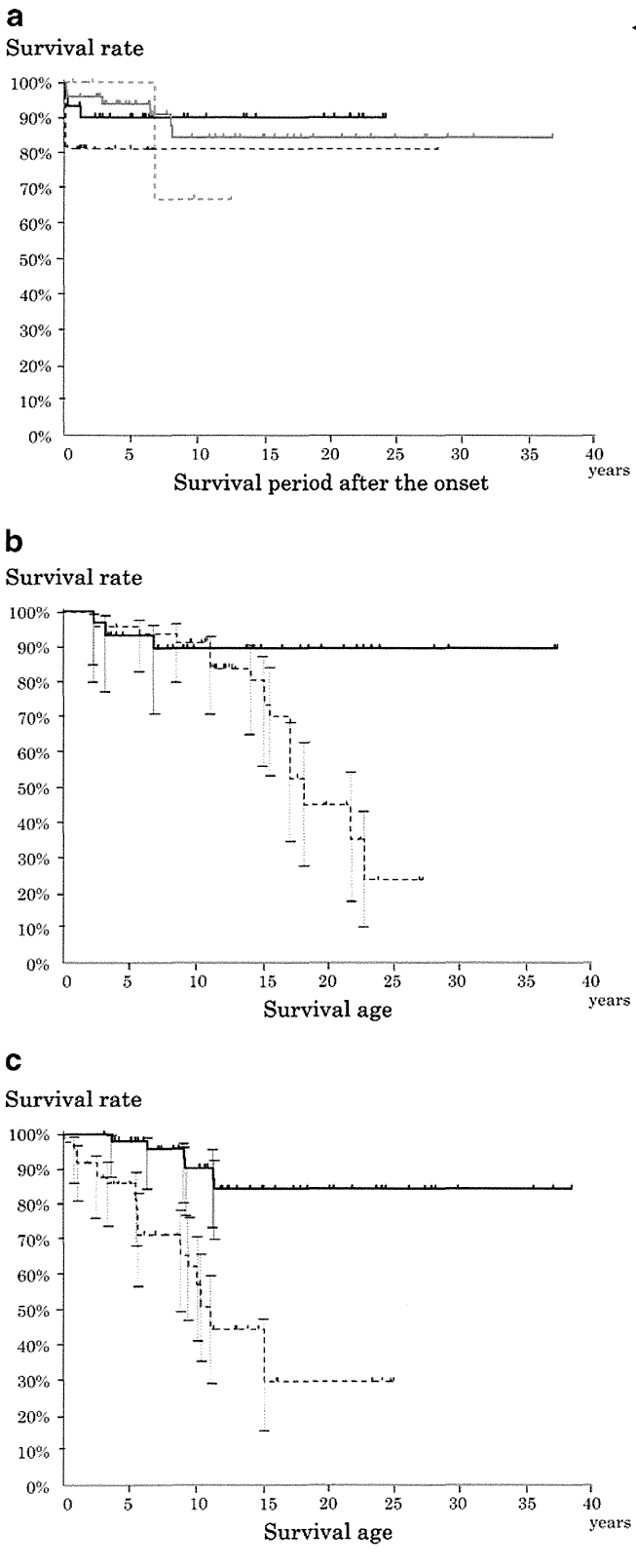

Fig. 2 **a** Survival rate after the onset in patients with ornithine transcarbamylase deficiency (OTCD). Survival plots of OTCD are shown by Kaplan-Meier survival curves. The 10-year survival rate in the three types of OTCD except for female neonate-onset OTCD was more than 80%. One of the seven female neonate-onset OTCD patients died 7 years and 4 months after the onset. [Male late-onset OTCD (*black line*), n=30; female late-onset OTCD (*gray line*), n=50; male neonate-onset OTCD (*black dashed line*), n=21; female neonate-onset OTCD (*gray dashed line*), n=7]. **b** Survival rate at each age in patients with late-onset male OTCD. The survival rates of OTCD patients at each age are shown by Kaplan-Meier survival curves with 95% CI. The survival rate of male OTCD patients in this study was higher than that in the previous study (Uchino et al. 1998). [Male late-onset OTCD from this study (*black bold line*), n=30; male late-onset OTCD from the previous study (*black dashed line*), n=48; P=0.004]. **c** Survival rate at each age in patients with late-onset female OTCD. The survival rates of OTCD patients at each age are shown by Kaplan-Meier survival curves with 95% CI. The survival rate of female OTCD patients in this study was higher than that in the previous study (Uchino et al. 1998). [Female late-onset OTCD (*black bold line*), n=50; female late-onset OTCD from the previous study (*black dashed line*), n=51; P<0.001]

and MRI images and a normal electroencephalogram. Information about mental retardation was unknown for 11% (8/74) of the patients The mental development was unknown for 10 of the 151 patients.

Relationship between peak blood ammonia levels during the first hyperammonemic attack and treatment

Figure 3b and c present the relationship between peak blood ammonia levels during the first hyperammonemic attack and the outcome with and without hemodialysis. Hemodialysis included hemofiltration and hemodiafiltration. Of the 151 patients whose peak ammonia levels were known during the first hyperammonemic attack, 59 patients (39%) received hemodialysis, and the maximum ammonia concentration was greater than 360 μmol/l in 85% (50/59) of these patients. When the maximum ammonia concentration was more than 300 μmol/l and hemodialysis was not performed, 12 patients received peritoneal dialysis. All patients received hemodialysis or peritoneal dialysis when the maximum ammonia concentration was more than 600 μmol/l. Of the patients whose peak ammonia level at the onset was more than 360 μmol/l and who had not developed mental retardation, 66% (12/18) had received hemodialysis. We compared the hemodialysis group with the nonhemodialysis group regarding survival outcome. The median peak ammonia level at the onset in the hemodialysis group was 856 (IQR=966) μmol/l (n=59). The median peak ammonia level at the onset in the nonhemodialysis group was 235 (IQR=220) μmol/l (n=92) (P<0.001) (Supplemental data 1). The long-term survival rate of the hemodialysis group was 84.6% (5 years after the onset), 78.5% (10 years), and 78.5% (15 years) according to the Kaplan-Meier survival curve. The survival rates of the nonhemodialysis group were 96.7% (5 years), 94.8% (10 years), and 94.8% (15 years), which were higher than

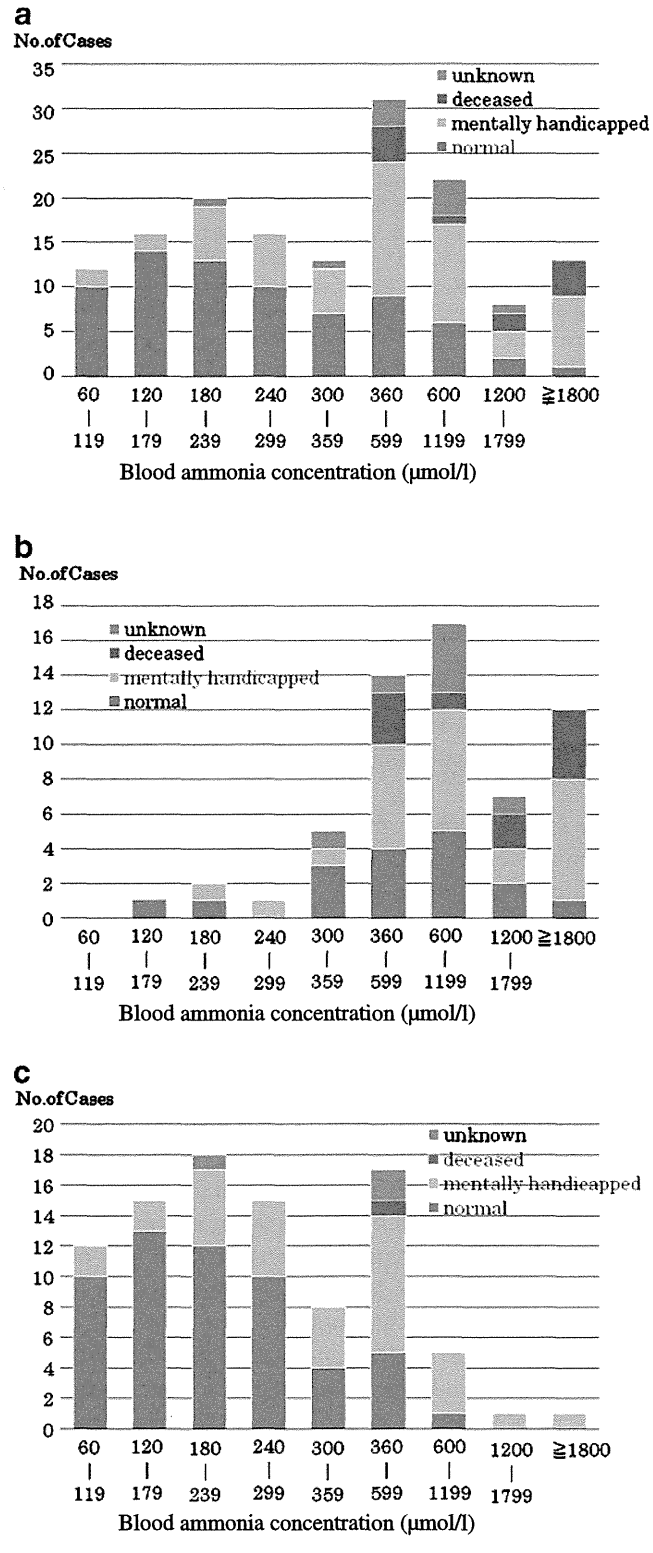

Fig. 3 **a** Relationship between peak blood ammonia levels and neurodevelopmental outcome. Peak plasma ammonia levels were less than 60 μmol/l in 2 cases and greater than 60 μmol/l in 151 cases. The neurodevelopmental outcome was unknown in 10 of the 151 patients. Eighteen urea cycle disorder (UCD) patients with a maximum ammonia concentration greater than 360 μmol/l had normal neurodevelopment. **b** Survival and neurodevelopmental outcome in the hemodialysis group. Fifty-nine (39%) of 151 patients had received hemodialysis, and 85% (50/59) of these patients had a maximum ammonia concentration greater than 360 μmol/l. Twelve patients with ammonia concentrations greater than 360 μmol/l survived and had normal neurodevelopment. **c** Survival and neurodevelopmental outcome in the nonhemodialysis group. Ninety-two (61%) of 151 patients had not received hemodialysis, and 74% (68/92) of these patients had a maximum ammonia concentration of less than 360 μmol/l. Forty-nine patients with ammonia concentrations of less than 360 μmol/l survived and had normal neurodevelopment

those in the hemodialysis group ($P<0.001$) (data not shown).

We assessed the relationship between peak blood ammonia levels and the occurrence of a liver transplant. Forty-two patients received a liver transplant that was independent of their peak blood ammonia level. Only one patient died of complications after the liver transplant. Thirteen of the surviving patients developed mental retardation (data not shown). Table 3 presents the ages at which liver transplants were performed. More than half of the patients received a liver transplant when they were between 1 month and 6 years of age: 26% (11/42) were between 1 month and 1 year of age and 40% (17/42) were between the ages of 1 and 6 years. Liver transplants were performed from 1 month to 18 years and 10 months after the onset (median 0.83, IQR=2.46 years after the onset) (Supplemental data 1). We compared the survival outcome between the liver transplant group ($n=42$) and the non-liver transplant group ($n=127$). The long-term survival rate of the liver transplant group was 100% (5 years after the onset), 94.1% (10 years), and 94.1% (15 years) according to the Kaplan-Meier survival curve. The survival rates of the non-liver transplant group were 89.3% (5 years), 84.3% (10 years), and 81.5% (15 years), which were lower than those of the liver transplant group, although the difference was not statistically significant ($P=0.06$) (Supplemental data 2).

Table 4 presents the treatment for UCDs. For OTCD, arginine was administered in 86% (110/116) of patients, sodium benzoate in 75% (87/116) of patients, and citrulline in 16% (19/116) of patients. A low-protein diet was administered in 64% (74/116) of patients. The combination of arginine and sodium benzoate was used in 72% (83/116) of patients with OTCD, in 61% (14/23) of patients with CPSD, in 79% (22/28) of patients with ASD, and in 100% (9/9) of patients with ALD. After the liver transplants, 91% (40/44) of the patients did not continue with these medications. Two patients with OTCD and two patients with ASD were administered arginine after the liver transplant. After the liver transplants, all patients did not require a low-protein diet, sodium benzoate, or sodium phenylbutyrate.

Table 3 Age at liver transplant for 42 patients with urea cycle disorders

	Less than 1 month old	1 month to <1 year old	1 to <6 years old	6 to <18 years old	≥18 years old	Total
OTCD	0	4	14	7	2	27 (64%)
CPSD	0	6	1	0	0	7 (17%)
ASD	0	1	1	0	5	7 (17%)
AGD	0	0	1	0	0	1 (2%)
Total	0 (0%)	11 (26%)	17 (40%)	7 (17%)	7 (17%)	42 (100%)

OTCD Ornithine transcarbamylase deficiency, *CPSD* carbamoyl-phosphate synthetase 1 deficiency, *ASD* arginosuccinate synthetase deficiency, *AGD* arginase-1 deficiency

Discussion

Thirteen years ago, Uchino et al. (1998) reported that enzyme activity had been measured in almost 70% of patients with UCDs except for those with AGD. At present, however, enzyme activity is measured in only 22% of all patients with UCDs; we hypothesize that doctors may be unwilling to perform the invasive liver biopsy that is necessary for the enzyme assay and this could be a factor for the decline. In a previous survey, DNA mutations were identified in 4% of CPSD patients. Here, we report a higher percentage of identifiable mutations in patients with CPSD than that in the previous report. However, the rate of identifiable mutations in all patients with UCDs was unchanged. At present, many doctors in Japan diagnose UCDs by measuring blood amino acid levels and orotic acid levels in urine and refer to clinical manifestations and family histories of hyperammonemia. DNA analysis is preferred over enzyme assays for a definitive diagnosis. In this report, 24 institutions, including three academic medical centers, did not respond to the second questionnaire despite us sending three requests. Therefore, there may be a bias because some patients are not included in this study.

All types of UCD patients, including female OTCD patients, presented symptoms at any age. As you described, we mean that our ability to compare the results of our study to the previous one are limited, owing to both genetic (private mutations and lyonization in female-OTCD) and external (metabolic crises provoked by nonpredictable common disorders and leading to hyperammonemic crisis) factors that might have affected the outcomes. In Fig. 2b and c, the long-term survival rate was higher than that previously reported, likely due to improved awareness and competence of centers, which were able to intervene efficiently and rapidly. Unlike the previous study, this study reported that 18 patients with ammonia concentrations higher than 360 μmol/l at the onset had normal neurodevelopment. Bachmann (2003) reported that when the ammonia concentration at the onset was higher than 300 μmol/l, none of the patients had a normal neurodevelopment. Determinations of the risk of mental retardation versus normal development can be improved by using the combination of peak ammonia concentration and duration of coma rather than either parameter individually (Bachmann 2005). Furthermore, the age of the patient when exposed to hyperammonemia may also influence the neurological outcome and should be taken into account (Braissant et al. 2002). In general, a peak ammonia concentration of less than 180 μmol/l at the onset was a marker of good prognosis, and a peak ammonia concentration of more than 360 μmol/l was a marker of poor prognosis.

We compared the hemodialysis group with the nonhemodialysis group regarding survival and neurodevelopmental outcome (Fig. 3b and c). A greater number of patients with ammonia concentrations greater than 360 μmol/l had received hemodialysis. Twelve patients with ammonia concentrations greater than 360 μmol/l who had received hemodialysis had normal neurological development. However, patients with ammonia concentrations higher than 360 μmol/l had poor survival and neurodevelopmental

Table 4 Treatment of urea cycle disorders

	Nonprotein formulas	Arginine	Sodium benzoate	Citrulline	Sodium phenylbutyrate	L-Carnitine	Low-protein diet
OTCD	36%	86%	75%	16%	15%	44%	64%
CPSD	52%	78%	74%	30%	17%	39%	74%
ASD	50%	86%	79%	0%	4%	32%	61%
ALD	89%	100%	100%	0%	0%	11%	78%
AGD	0%	0%	100%	0%	0%	0%	0%

OTCD Ornithine transcarbamylase deficiency, *CPSD* carbamoyl-phosphate synthetase 1 deficiency, *ASD* arginosuccinate synthetase deficiency, *ALD* arginosuccinate lyase deficiency, *AGD* arginase-1 deficiency

outcome despite receiving hemodialysis. Many patients without hemodialysis with peak ammonia levels higher than 180 μmol/l died or developed mental retardation. Patients with a peak ammonia level greater than 180 μmol/l at the onset should receive hemodialysis.

In Japan, definitive criteria for liver transplants are controversial. An indication for a liver transplant does not depend on severity at the onset. We considered a liver transplant when hyperammonemia was not improved by medical therapy, if the patient experienced frequent hyperammonemic attacks. We did not evaluate mental development before a liver transplant, therefore we could not describe improved neurodevelopment by a liver transplant. Improved survival outcome might be attributed to a liver transplant. The metabolic derangement was resolved in 41 patients who had a successful liver transplant, and they could consume a normal protein diet without medication. Some patients did not experience hyperammonemia when they were under stress such as common cold. Liver transplants contributed to an improved quality of life in UCD patients. There are some reports about the improved quality of life in UCD patients after liver transplants in Japan and overseas (Whitington et al. 1998; Morioka et al. 2005; Kasahara et al. 2010)

Arginine and sodium benzoate are frequently used as therapy for UCDs in Japan (Table 4). The use of citrulline is recommended in cases of OTCD and CPSD (Feillet and Leonard 1998; Summar 2001). Apart from being expensive, citrulline, sodium benzoate, and sodium phenylbutyrate are not government-approved drugs in Japan, and, therefore, patients have to pay for them. Less than 75% of patients with CPSD and ASD were treated with a low-protein diet. Patients with CPSD and ASD who were not treated with a low-protein diet included patients that received a liver transplant, those who were treated with nonprotein formulas, or those for which information regarding treatment with a low-protein diet was unknown. Reports from other centers about patients who received a liver transplant for a UCD described the need for citrulline or arginine supplements, although these treatments have not been well accepted in Japan. UCD patients were rarely provided with citrulline or arginine supplements after a liver transplant.

In conclusion, we report the long-term outcome and intervention of UCDs in Japan. Compared with a previous study, we found a lower mortality rate at the onset of UCDs and an improved long-term outcome in patients.

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Metabolic autopsy with postmortem cultured fibroblasts in sudden unexpected death in infancy: Diagnosis of mitochondrial respiratory chain disorders

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ABSTRACT

Mitochondrial respiratory chain disorders are the most common disorders among inherited metabolic disorders. However, there are few published reports regarding the relationship between mitochondrial respiratory chain disorders and sudden unexpected death in infancy. In the present study, we performed metabolic autopsy in 13 Japanese cases of sudden unexpected death in infancy. We performed fat staining of liver and postmortem acylcarnitine analysis. In addition, we analyzed mitochondrial respiratory chain enzyme activity in frozen organs as well as in postmortem cultured fibroblasts. In heart, 11 cases of complex I activity met the major criteria and one case of complex I activity met the minor criteria. In liver, three cases of complex I activity met the major criteria and four cases of complex I activity met the minor criteria. However, these specimens are susceptible to postmortem changes and, therefore, correct enzyme analysis is hard to be performed. In cultured fibroblasts, only one case of complex I activity met the major criteria and one case of complex I activity met the minor criteria. Cultured fibroblasts are not affected by postmortem changes and, therefore, reflect premortem information more accurately. These cases might not have been identified without postmortem cultured fibroblasts. In conclusion, we detected one probable case and one possible case of mitochondrial respiratory chain disorders among 13 Japanese cases of sudden unexpected death in infancy. Mitochondrial respiratory chain disorders are one of the important inherited metabolic disorders causing sudden unexpected death in infancy. We advocate metabolic autopsy with postmortem cultured fibroblasts in sudden unexpected death in infancy cases.

1. Introduction

Sudden unexpected death in infancy (SUDI) is defined as sudden unexpected death occurring before 12 months of age. If SUDI remains unexplained after thorough investigations, it is classified as sudden infant death syndrome (SIDS). The more common causes of SUDI are infection, cardiovascular anomaly, child abuse, and metabolic disorders. However, the many potential inherited metabolic disorders are more difficult to diagnose at autopsy as compared to cardiovascular defects and serious infection. Inherited metabolic disorders may, therefore, be underdiagnosed as a cause of SUDI or misdiagnosed as SIDS. Fatty acid oxidation disorders (FAODs) are one type of the inherited metabolic disorders and may cause as much as 5% of SUDI cases after thorough investigations including metabolic autopsy [1–5]. In a review of SUDI cases with respect to potential FAODs, we found a case of carnitine palmitoyltransferase II deficiency [6]. In that study, we performed fat staining of liver, postmortem acylcarnitine analysis, and genetic analysis, advocating the importance of metabolic autopsy in SUDI cases.

Mitochondrial respiratory chain (MRC) disorders were first identified in 1962 [7]. MRC disorders have a frequency of about at least 1:5000 newborns and are the most common disorders among inherited metabolic disorders [8]. However, there are few published reports regarding the relationship between MRC disorders and SUDI. Studies of MRC disorders have not progressed because of technical difficulties or variability in clinical manifestations [9]. In sudden death cases especially, clinical features are unclear and postmortem changes complicate molecular analysis.

In the present study, we performed metabolic autopsy in 13 Japanese cases of SUDI in order to determine whether MRC disorders could be detected or not. We performed fat staining of liver and postmortem

Abbreviations: CS, citrate synthetase; FAODs, fatty acid oxidation disorders; MRC, mitochondrial respiratory chain; OXPHOS, oxidative phosphorylation; SIDS, sudden infant death syndrome; SUDI, sudden unexpected death in infancy.

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acylcarnitine analysis according to the previous methods. In addition, we analyzed MRC enzyme activity in frozen organs as well as in postmortem cultured fibroblasts. With such metabolic autopsy, we were able to detect one probable case and one possible case of MRC disorders. These cases might not have been identified without metabolic autopsy. MRC disorders are important diseases causing SUDI and metabolic autopsy might be helpful for forensic scientists and pediatricians to diagnose MRC disorders that might not otherwise be identified.

2. Materials and methods

2.1. Subjects

Between October 2009 and September 2011, forensic autopsy was performed on 588 cases at our institute, 22 of whom were under 12 months of age. Following macroscopic examination, nine cases could be diagnosed but 13 cases (Table 1) did not have any characteristic appearance and remained undiagnosed. In this study, we reviewed these 13 undiagnosed cases (8 males, 5 females) with age ranging from 1 to 10 months.

2.2. Autopsy

Autopsies were performed within 24 h following death. Blood was obtained from the femoral vein. Heart and liver specimens were immediately cut and frozen at −80 °C. Dermis, which was cut and sterilized, was cultured at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) containing 10% fetal bovine serum, 1% penicillin streptomycin glutamine, and 2.5% amphotericin B (Life Technologies, Indianapolis, IN). Once cultures were established, fibroblasts were frozen at −80 °C.

2.3. Sudan III staining

Liver samples preserved in 4% phosphate-buffered formaldehyde solution were frozen, cut into 10-μm sections, and stained by the Sudan III method for fat staining.

2.4. Postmortem blood acylcarnitine analysis by tandem mass spectrometry

Whole blood samples obtained at autopsy were blotted onto one spot on Guthrie cards. They were subjected to acylcarnitine analysis by tandem mass spectrometry and compared with the previously determined normal range [6].

Table 1
SUDI cases.

Case no.	Age/sex	Height/weight (cm/kg)	Circumstances	Fever	Remarks
1	4 mo/M	68/7.5	Sleeping	−	
2	10 mo/F	70/8.8	Sleeping	−	Sister: undiagnosed encephalitis
3	10 mo/F	71/7.7	Sleeping	+	Cesarean section
4	9 mo/M	67/7.5	Sleeping	−	
5	4 mo/M	60/5.7	Sleeping	−	Hydrocephalia
6	6 mo/M	68/8.0	Sleeping	−	
7	1 mo/F	51/3.6	Sleeping	−	Twins, preterm birth
8	10 mo/M	72/9.9	Sleeping	−	Developmental disease (right side of the body paralysis)
9	6 mo/F	64/8.9	Sleeping	−	Bronchitis
10	4 mo/M	65/7.4	Sleeping	−	Cesarean section
11	1 mo/M	58/4.8	Sleeping	−	
12	5 mo/M	59/4.2	Sleeping	−	Preterm birth
13	2 mo/F	53/3.9	Sleeping	−	Low-birth-weight infant

Abbreviations: F, female; M, male; mo, month; SUDI, sudden unexpected death in infancy.

2.5. Enzyme analysis

The activity of mitochondrial respiratory chain complexes I, II, III, and IV was assayed in the crude post-600-g supernatant of heart and liver, and in isolated mitochondria from skin fibroblasts as described previously [10]. The activity of each complex was presented as a percent ratio relative to the mean value [9]. The activity of complexes I, II, III, and IV was also calculated as the percent relative to citrate synthetase (CS), a mitochondrial enzyme marker or complex II activity [10].

2.6. Ethics

This study was approved by the Ethics Committee of the Osaka University Graduate School of Medicine.

3. Results

3.1. Microscopic examination

One of the common features in diagnosing MRC disorders is hepatic steatosis. We therefore performed Sudan III staining to examine whether vacuoles caused by fatty degeneration were present in hepatocytes. Diffuse microvesicular steatosis was detected in case 5 (Fig. 1A). No Sudan III-positive vacuole was detected in case 13 (Fig. 1B) and the other cases, for example, case 2 (Fig. 1C).

3.2. Postmortem blood acylcarnitine analysis

We performed acylcarnitine analysis by tandem mass spectrometry using whole blood samples. In all samples, data were within the normal range. These data suggested that no case was affected by FAODs (data not shown).

3.3. Enzyme analysis of MRC complexes in heart, liver, and cultured fibroblasts

The enzyme activity of each complex was compared with the CS ratio and complex II ratio. Lower than 20% activity of any complex in a tissue or lower than 30% activity of any complex in a cell line meets the major criteria. Lower than 30% activity of any complex in a tissue or lower than 40% activity of any complex in a cell line meets the minor criteria according to Bernier et al. [11].

In heart, 11 cases of complex I activity met the major criteria of MRC disorders and one case of complex I activity met the minor criteria (Fig. 2A). In liver, three cases of complex I activity met the major criteria of MRC disorders and four cases of complex I activity met the minor criteria (Fig. 2B). In cultured fibroblasts, one case (case 5) of complex I activity met the major criteria of MRC disorders and one case (case 13) of complex I activity met the minor criteria (Fig. 2C, Table 2). The activity of complexes II, III, and IV was maintained in almost all cases.

3.4. Diagnosis

A definite diagnosis is defined as the identification of either two major criteria or one major plus two minor criteria. A probable diagnosis is defined as either one major plus one minor criterion or at least three minor criteria. A possible diagnosis is defined as either a single major criterion or two minor criteria, one of which must be clinical [11].

All the cases had a clinical symptom of sudden death, meeting one minor criterion. In the enzyme activity, eleven cases (cases 2, 4–13) met the major criteria and we could make a probable diagnosis in these 11 cases. The other two cases (cases 1 and 3) met the minor criteria and we could make a possible diagnosis.

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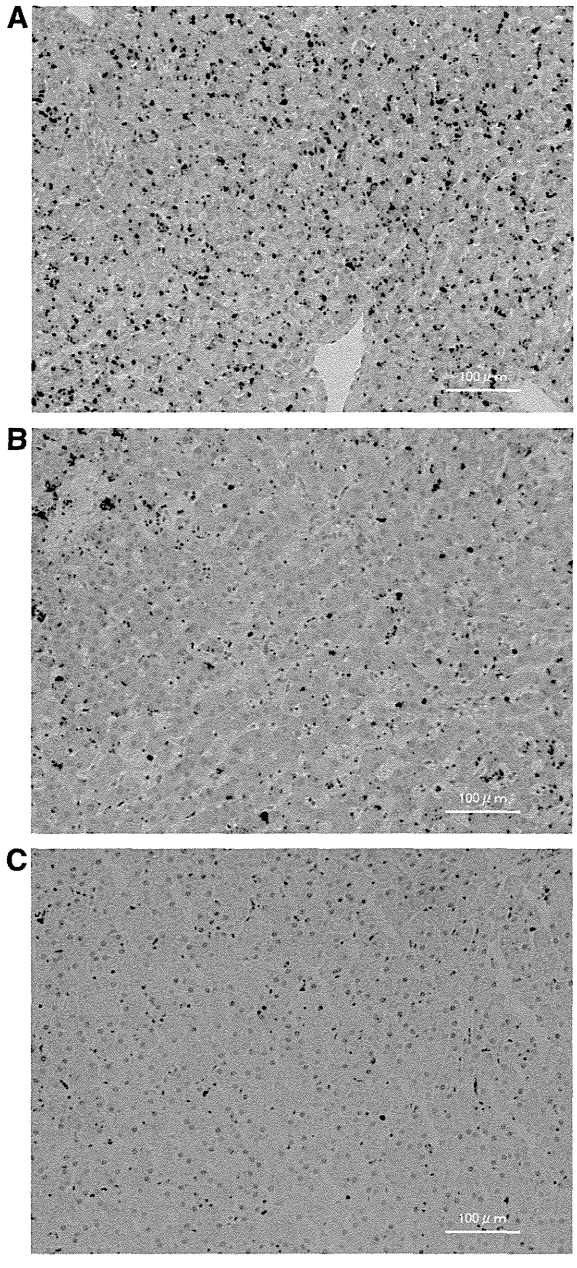

Fig. 1. Microscopic examination of liver (Sudan III staining): (A) case 5, (B) case 13, and (C) case 2. Diffuse microvesicular steatosis was detected in case 5 (A). No Sudan III-positive vacuole was detected in case 13 (B) and the other cases, for example, case 2 (C).

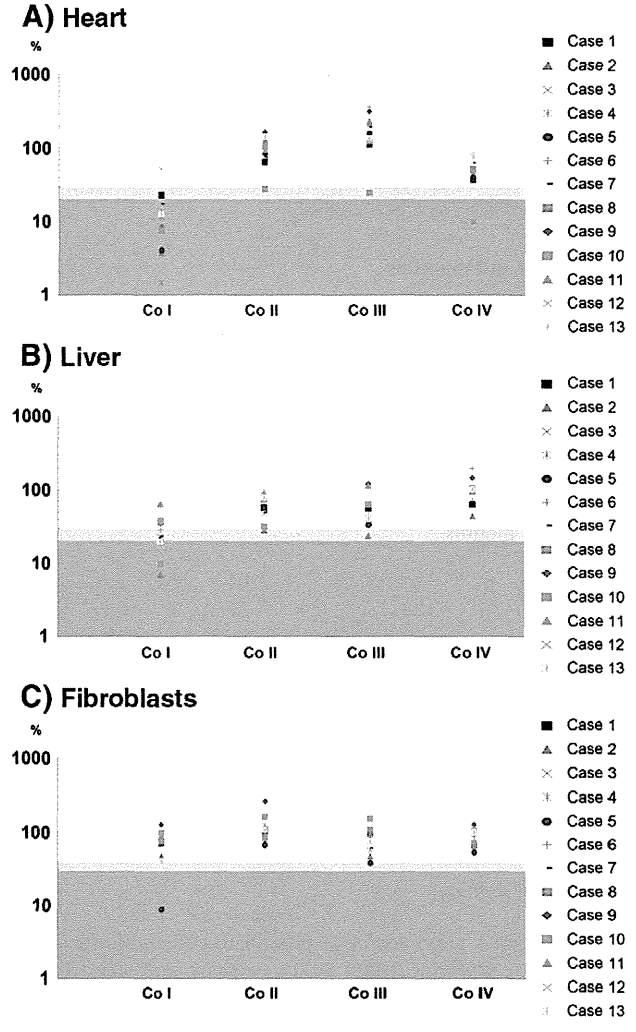

Fig. 2. Enzyme activity of MRC complexes in heart (A), liver (B), and cultured fibroblasts (C). In heart, 11 cases of complex I activity were under 20% of the CS ratio, meeting the major criteria and one case of complex I activity was under 30% of the CS ratio, meeting the minor criteria (A). In liver, three cases of complex I activity were under 20% of the CS ratio, meeting the major criteria and four cases of complex I activity were under 30% of the CS ratio, meeting the minor criteria (B). In cultured fibroblasts, one case (case 5) of complex I activity was under 30% of the CS ratio, meeting the major criteria and one case (case 13) of complex I activity was under 40% of the CS ratio, meeting the minor criteria (C). The activity of complexes II, III, and IV was maintained in almost all cases. The enzyme activity of each complex was compared with the CS ratio. Lower than 20% activity in a tissue or lower than 30% activity in a cell line (dark blue) meets the major criteria. Lower than 30% activity in a tissue or lower than 40% activity in a cell line (light blue) meets the minor criteria. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

Mitochondria are essential organelles that exist in all nucleated mammalian cells. They provide the energy required for normal cell function through oxidative phosphorylation (OXPHOS). OXPHOS includes MRC complexes (complexes I, II, III, and IV) and ATP synthase (complex V) [12], which use reduced coenzymes from the tricarboxylic acid cycle and molecular oxygen, generating cellular energy in the form of ATP [13].

The infantile or early neonatal period demands high energy. Patients with MRC disorders are unable to produce adequate energy, which may thus compromise them in the first days of life or during infancy. MRC disorders affect most organ systems and present variable clinical manifestations from prenatal complications through acute neonatal decompensation and death to adult-onset disorders. Therefore, it is not surprising that MRC disorders are also one of the causes of SUDI. However, there are few reports on a relationship between MRC disorders and SUDI [12,14].

We have previously reviewed SUDI cases with respect to FAODs and found a case of carnitine palmitoyltransferase II deficiency [6]. In that study, we advocated the importance of metabolic autopsy [15], including fat staining of liver, postmortem acylcarnitine analysis, and genetic analysis. Using this protocol, most FAODs, some amino acid oxidation disorders, and some organic acid oxidation disorders could be diagnosed.

However, MRC disorders are difficult to diagnose. First, they present variable clinical manifestations and non-specific features such as failure to thrive or hepatic, cardiac, renal, gastrointestinal, endocrine, hematological, or other symptoms [10,16]. Second, although blood

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Table 2
Enzyme assay of mitochondrial respiratory chain complexes in cultured fibroblasts.

	Enzyme activity (%)[a]			
	Co I	Co II	Co III	Co IV
Case 5				
CS ratio	9	66	38	53
Co II ratio	13	–	71	58
Case 13				
CS ratio	39	106	76	98
Co II ratio	37	–	73	92

Abbreviations: Co I, complex I; Co II, complex II; Co III, complex III; Co IV, complex IV; CS, citrate synthetase.
[a] Relative to mean CS and Co II of the normal controls.

lactate levels and muscle morphology can be used as a screening test, some confirmed patients were normal [10]. Third, genomic mutational analysis is difficult because MRC complexes are composed of 13 subunits encoded by mitochondrial DNA and over 70 subunits encoded by nuclear genes. In addition, nuclear genes are related to many assembly factors, membrane dynamics, nucleotide transport synthesis, and mitochondrial DNA replication and expression. Therefore, enzyme analysis still remains the most significant diagnostic tool. A definite diagnosis thus requires enzyme analysis [8].

In the present study, we performed enzyme analysis in frozen heart, frozen liver, and cultured fibroblasts. Eleven cases were supposed to be a probable diagnosis and two cases were supposed to be a possible diagnosis. However, it seemed unlikely that such a high proportion would have real MRC disorders. Did we have to take the effect of postmortem changes into consideration?

For forensic autopsy, organ specimens are often preserved in formaldehyde solution and sometimes frozen. These specimens are susceptible to postmortem changes and, therefore, correct enzyme analysis is hard to be performed. Based on the previous report that artifactual loss of complex II activity in autopsy samples preceded that of complex I and the data that complex II activity in the present study was maintained, this low complex I activity might be decreased before death. However, postmortem changes cannot be completely ruled out and this low complex I activity may not therefore be consistent with premortem activity.

We therefore analyzed activity in cultured fibroblasts. Cultured fibroblasts are not affected by postmortem changes and, therefore, reflect premortem information more accurately. In cultured fibroblasts, one case (case 5) of complex I activity met the major criteria and one case (case 13) of complex I activity met the minor criteria. In case 5, complex I activity was distinctively decreased. Sudan III staining of the case revealed hepatic steatosis, consistent with Reye-like syndrome. Reye-like syndrome is one of the characteristic features of MRC disorders [9]. We could therefore make a probable diagnosis (case 5) and a possible diagnosis (case 13) from metabolic autopsy with postmortem cultured fibroblasts.

Case 5 had hydrocephalia and case 13 was a low-birth-weight infant. However, neither was severe. Macroscopic examination did not reveal any abnormal appearance and microscopic examination showed no pathological findings except for steatosis. These cases might not have been identified without postmortem cultured fibroblasts. As with such cases, some MRC disorders reveal no clinical manifestation and no pathological characteristic. We believe it is important to perform metabolic autopsy with postmortem cultured fibroblasts when encountering SUDI cases.

We emphasized the advantage of metabolic autopsy with cultured fibroblasts. First, despite lacking obvious preceding symptoms, MRC disorders could be diagnosed. Second, cultured cells are the only method to retrieve premortem information from the deceased. Third, even frozen samples are affected by postmortem changes and may lead to a false positive diagnosis. However, we have to discuss the disadvantage. MRC disorders showed tissue specificity and the activity of cultured fibroblasts represent normal in some cases. Some of the low complex I activity in heart or liver could represent premortem MRC disorders despite normal activity in cultured fibroblasts. Thus, other molecular investigations may well be added to enzyme analysis. Recently, systematic gene analysis using next-generation sequencing has been reported for the diagnosis of patients with MRC disorders [17]. Further investigations are thus needed.

In conclusion, we detected one probable case and one possible case of MRC disorders among 13 Japanese cases of SUDI. MRC disorders are one of the important inherited metabolic disorders causing SUDI. We advocate metabolic autopsy with postmortem cultured fibroblasts in SUDI cases.

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ORIGINAL ARTICLE

Haplotype analysis of *ESR2* in Japanese patients with spermatogenic failure

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The prevalence of spermatogenic failure (SF) has gradually increased during the past few decades at least in several countries. Although multiple factors would be involved in this phenomenon, one important factor would be excessive estrogen effects via estrogen receptors (ERs). Thus, we performed haplotype analysis of *ESR2* encoding ERβ in 125 Japanese SF patients and 119 age-matched control males, using single nucleotide polymorphisms (SNPs) 1–9 that are widely distributed on the ~120-kb genomic sequence of *ESR2*. Consequently, a linkage disequilibrium (LD) block was detected in an ~60-kb region encompassing SNPs 2–7 in both groups, and four major estimated haplotypes were identified within the LD block. Furthermore, the most prevalent 'TGTAGA' haplotype was found to be significantly associated with SF, with the *P*-value obtained by the Cochran–Armitage trend test (0.0029) being lower than that obtained by a 100 000-times permutation test (0.0038) to cope with the problem of multiple comparisons. The results, in conjunction with our previous data indicating lack of a susceptibility factor on *ESR1* encoding ERα, imply that the specific 'TGTAGA' haplotype of *ESR2* raises the susceptibility to the development of SF.
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Keywords: environmental endocrine disruptors; *ESR2*; estrogenic effects; haplotype analysis; spermatogenic failure; susceptibility

INTRODUCTION

Recent studies have indicated a gradual increase in the prevalence of male genial and reproductive abnormalities during the past few decades at least in several countries.[1] Skakkebaek *et al.*[2] have coined a term 'testicular dysgenesis syndrome' for this phenomenon. As such deterioration of male genital and reproductive health is also observed in many wildlife species,[1,3] it is likely that such adverse changes in males are inter-related events shared in common by the human and the wildlife species.[1,3] In this regard, environmental endocrine disruptors (EEDs) appear to constitute the major factor for this phenomenon, because EEDs are widely spread in the world.[1,3] In particular, exposure to estrogenic EEDs are known to affect male genital and reproductive health.[1,3–5]

The effects of EEDs would primarily be determined by the genetic susceptibility, together with the dosage of exposed EEDs, character of exposed EEDs (for example, estrogenic, anti-androgenic and so on), and the developmental stage of the individuals at the time of EED exposure.[1,3] In this regard, it is known that estrogenic EEDs can bind to both estrogen receptor (ER)α encoded by *ESR1* and ERβ encoded by *ESR2* with low but variable degrees of affinities.[3] Thus, it is likely that genetic susceptibility to estrogenic EEDs is primarily constituted by genetic variations in *ESR1* and *ESR2*.[1,3]

To examine this possibility, we have previously performed haplotype analysis of *ESR1* in Japanese male patients with genial and reproductive abnormalities as well as in control males, using 15 single nucleotide polymorphisms (SNPs 1–15) that are widely distributed throughout the >300-kb genomic sequence of *ESR1*.[6,7] Consequently, we identified an ~50-kb linkage disequilibrium (LD) block spanning SNPs 10–14 in the 3′ region of *ESR1*, and found that homozygosity of a specific 'AGATA' haplotype within the LD block was strongly associated with cryptorchidism ($P = 0.0040$; odds ratio (OR) = 7.55) and hypospadias ($P = 0.000057$; OR = 13.75)[6,7] (and our unpublished updated observation). This finding provides strong evidence that homozygosity of the specific *ESR1* haplotype raises the susceptibility to the development of male genital abnormalities. In this context, we speculate that this effect via the specific *ESR1* haplotype is mediated by EEDs, although there is no direct evidence yet. Indeed, as *ESR1* is expressed in Leydig cells producing testosterone and insulin-like 3,[5,8] it is likely that the specific *ESR1* haplotype primarily enhances estrogenic effects in Leydig cells, compromising their hormonal production capacity.

However, no significant association was found between the specific 'AGATA' haplotype of *ESR1* and spermatogenic failure (SF).[7] In this context, as *ESR2* is clearly expressed in various developmental stages of male germ cells,[5] it may be possible that the deleterious effects of estrogenic EEDs on spermatogenesis may primarily be mediated by ERβ. Thus, we carried out haplotype analysis of *ESR2* in Japanese patients with SF.

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MATERIALS AND METHODS

Subjects

We studied 125 SF patients aged 32–52 years (median 41.0 years), including 80 SF patients utilized in the previous *ESR1* haplotype analysis.[7] The selection criteria included: (1) azoospermia or severe oligozoospermia (<5 million sperms per ml) demonstrated by two consecutive analyses of semen obtained after 4–7 days of abstinence; (2) lack of extragenital anomalies such as cryptorchidism and hypospadias; (3) hypergonadotropic hypogonadism indicative of primary testicular dysfunction; (4) no seminal tract obstruction, varicocele, or retrograde ejaculation; (5) a 46,XY karyotype with no demonstrable structural or numerical abnormality after examining ⩾30 lymphocytes; (6) absence of a Y chromosomal microdeletion after examining 36 loci from *SRY* to *DYZ1*, including multiple Yq loci in the azoospermia factor regions (AZFa, b, c) such as *RBMY* and *DAZ*;[9] (7) no significant expansion of CAG repeat length at exon 1 of *AR* that is known to raise the susceptibility to male reproductive abnormalities;[10] and (8) lack of a disease episode that could affect fertility such as mumps orchiditis. For controls, 119 control adult males with proven fertility aged 24–50 years (median 35.5 year) were similarly analyzed with permission. The ages were similar between the SF patients and control males (Mann–Whitney's *U*-test). All the SF patients and control males were Japanese living in the Tokyo urban area; they were free from particular residential environments such as the vicinity of chemical factories or farms, from specific dietary habits deviated to vegetables or animal/fish proteins, and from intake of drugs with hormonal effects.

SNP analysis

This study was approved by the Institutional Review Board Committees of the authors, and informed consent was obtained from each subject. We examined nine SNPs (SNPs 1–9) that were associated with high minor allele frequencies in the Japanese population (20.3–39.5%) (the NCBI Short Genetic Variations Database (dbSNP); http://www.ncbi.nlm.nih.gov/snp/) and were widely distributed on the ~120-kb *ESR2* genomic DNA sequence including an apparent LD block encompassing exons 1–6 identified in various populations (the International HapMap Project Database; http://hapmap.ncbi.nlm.nih.gov/) (Figure 1a). Genotyping was performed by the 5′ nuclease assay on an ABI PRISM 7000 Sequence Detection System (Life Technologies, Carlsbad, CA, USA),[11] using leukocyte genomic DNA of each subject.

Pearson's χ^2-test with one degree of freedom was applied to test whether the genotyping data are in the Hardy–Weinberg equilibrium. Statistical significance of the differences in allele and genotype frequencies was analyzed by Pearson's χ^2-test, using R environment for statistical computing (http://www.r-project.org/).

Haplotype analysis

Although haplotypes are usually not observed, the haplotypes present in a subject and the frequencies of the haplotypes in a population can be inferred using genotype data at separate loci.[12] In this regard, the degree of LD can be expressed as the pairwise $|D'|$ value (the absolute value for the disequilibrium parameter) that ranges from 0 (complete absence of LD) to 1.0 (complete presence of LD),[13] and a chromosomal region associated with high $|D'|$ values between different loci is defined as a haplotype or an LD block.[14] In this study, haplotype inference was performed by the maximum-likelihood method using expectation maximization algorithm implemented in the software LDSUPPORT.[15,16] The pairwise $|D'|$ values were estimated by the method of Terwilliger and Ott, [12] and a haplotype block was determined by the method of Zhu *et al.*[17] using the software developed by Kamatani *et al.*[18]

The difference in the frequencies of haplotypes between the SF patients and the control males was examined using the estimated population haplotype frequencies by Pearson's χ^2-test, and the OR and the 95% confidence interval (CI) were calculated using the R environment. The association between SF phenotype and estimated haplotypes was tested using PENHAPLO software in a dominant mode (comparison of the frequencies of subjects with one risk haplotype between cases and controls) and in a recessive mode (comparison of the frequencies of subjects with two risk haplotypes between cases and controls).[19] Furthermore, the association between SF phenotype and estimated haplotypes was also examined in a dosage-dependent mode (comparison of the frequencies of subjects with zero, one, and two risk haplotypes between cases and controls) by the Cochran–Armitage trend test,[20,21] using the R environment. To cope with the problem of multiple comparisons, the significant level was determined by a 100 000-times permutation test.[22]

RESULTS

SNP analysis

The results of SNP analysis are summarized in Table 1. Minor allele frequencies of the 9 SNPs were 20.4–46.8% in the SF patients and 27.7–37.3% in control males. The genotype frequencies of SNPs 1–9 were in accord with the Hardy–Weinberg equilibrium. Low *P*-values (<0.05) were identified for the differences in the allele and genotype frequencies of SNPs 1, 4, and 5, with stronger association being identified for the

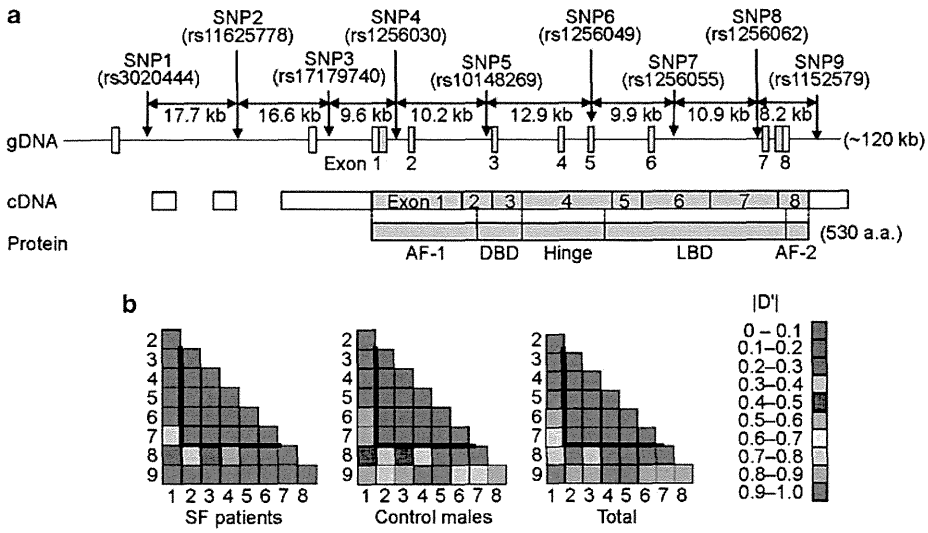

Figure 1 Schematic representation of *ESR2* and its LD maps. (**a**) Physical positions of *ESR2* SNPs 1–9 examined in the present study. The gray and the white boxes represent coding and untranslated regions, respectively. AF-1, activation function 1 (ligand independent); AF-2: activation function 2 (ligand dependent); DBD, DNA-binding domain; LBD, ligand-binding domain. (**b**) Pairwise LD maps. $|D'|$: an absolute value for the disequilibrium parameter.

Table 1 Summary of SNP analysis

Genotyping data				*Statistical data*			
	Genotype	*SF*	*CM*		*P-value*	*OR*	*95% CI*
SNP1	TT	78	58	T vs C	0.028	1.59	1.05–2.42
rs3020444	TC	43	53	TT vs TC+CC	0.032	0.57	0.34–0.95
	CC	4	8	TT+TC vs CC	0.20	2.18	0.64–7.44
SNP2	TT	68	63	T vs C	0.74	1.07	0.72–1.56
rs11625778	TC	48	46	TT vs TC+CC	0.82	0.94	0.57–1.56
	CC	9	10	TT+TC vs CC	0.73	1.18	0.46–3.02
SNP3	GG	77	59	G vs A	0.059	1.49	0.98–2.25
rs17179740	AG	43	52	GG vs AG+AA	0.059	0.61	0.37–1.02
	AA	5	8	GG+AG vs AA	0.34	1.73	0.55–5.45
SNP4	CC	36	55	C vs T	0.0022	1.77	1.23–2.56
rs1256030	CT	61	49	CC vs CT+TT	0.0049	2.13	1.25–3.61
	TT	28	15	CC+CT vs TT	0.045	0.500	0.25–0.99
SNP5	GG	36	55	G vs A	0.0022	1.77	1.23–2.56
rs10148269	AG	61	49	GG vs AG+AA	0.0049	2.13	1.25–3.61
	AA	28	15	GG+AG vs AA	0.045	0.500	0.25–0.99
SNP6	GG	68	64	G vs A	0.74	1.07	0.72–1.60
rs1256049	GA	49	45	GG vs GA+AA	0.92	0.98	0.59–1.61
	AA	8	10	GG+GA vs AA	0.55	1.34	0.51–3.52
SNP7	AA	68	64	A vs G	0.74	1.07	0.72–1.60
rs1256055	AG	49	45	AA vs AG+GG	0.92	0.98	0.59–1.61
	GG	8	10	AA+AG vs GG	0.55	1.34	0.51–3.52
SNP8	AA	59	47	A vs G	0.21	1.27	0.87–1.85
rs1256062	AG	54	57	AA vs AG+GG	0.22	0.73	0.44–1.21
	GG	12	15	AA+AG vs GG	0.45	1.36	0.61–3.04
SNP9	GG	40	45	G vs A	0.12	1.34	0.93–1.92
rs1152579	GA	59	59	GG vs GA+AA	0.34	1.29	0.76–2.19
	AA	26	15	GG+GA vs AA	0.087	0.55	0.28–1.10

Abbreviations: CI, confidence interval; OR, odds ratio; SNP, single nucleotide polymorphism. NCBI rs no. is given for each SNP. SF, 125 patients with spermatogenic failure; CM, 119 control males.

allele rather than the genotype frequencies. In particular, the *P*-values for allele frequencies of SNPs 4 and 5 were markedly low.

Haplotype analysis

The LD map is shown in Figure 1b, and the results of haplotype analysis are summarized in Table 2. An ~60-kb LD block spanning SNPs 2–7 was identified in both the SF patients and control males, with the $|D'|$ value being >0.9 for all the pairs of SNPs 2–7. Within the LD block, four major estimated haplotypes were identified, together with three additional minor haplotypes ('CGTAGA' haplotype in a single control male, and 'TATAGA' and 'CGCGGA' haplotypes in single SF patients). Notably, the frequency of the most prevalent 'TGTAGA' haplotype was significantly higher in the SF patients than in the control males. Furthermore, the 'TGTAGA' haplotype was significantly associated with SF phenotype, with the *P*-value obtained by the Cochran–Armitage trend test (0.0029) being lower than the permutation *P*-value (0.0038). In addition, of the four major haplotypes, the 'TGTAGA' haplotype alone contained the 'T' allele in SNP 4 and the 'A' allele in SNP 5, whereas these two alleles were also identified in two of the three minor haplotypes.

DISCUSSION

The present study revealed the presence of an ~60-kb LD block encompassing SNPs 2–7 of *ESR2* in both the SF patients and control males. In this regard, the allele frequencies obtained in the control males are comparable to those registered in the JSNP Database, and the LD block identified in this study is similar to that reported in the International HapMap Project. These findings argue for the accuracy of our data.

Table 2 Summary of haplotype analysis (SNPs 2–7)

Estimated haplotype	*TGTAGA*	*TACGGA*	*CGCGAG*	*TGCGGA*
SF (n=125)	46.4%	21.2%	26.0%	6.0%
CM (n=119)	32.7%	28.1%	27.3%	11.0%
Comparison of estimated haplotype frequency				
P-value	0.0028	0.096	0.82	0.070
OR	1.77	0.69	0.94	0.52
95% CI	1.21–2.61	0.44–1.06	0.61–1.43	0.25–1.05
Association of estimated haplotype with phenotype				
Dominant mode				
P-value	0.0063	0.078	0.92	0.031
OR	2.08	0.63	0.98	0.46
95% CI	1.23–3.54	0.38–1.05	0.59–1.62	0.22–0.93
Recessive mode				
P-value	0.026	0.34	0.55	0.97
OR	2.16	0.58	0.75	0.95
95% CI	1.09–4.46	0.17–1.79	0.28–1.96	0.037–24.2
Cochran–Armitage's trend test				
P-value	0.0029	0.071	0.75	0.056
For one haplotype				
OR	1.75	0.67	0.94	0.52
95% CI	1.21–2.52	0.44–1.03	0.63–1.39	0.27–1.02
For two haplotypes				
OR	3.06	0.45	0.88	0.27
95% CI	1.46–6.35	0.19–1.06	0.39–1.93	0.07–1.04

Abbreviations: CI, confidence interval; OR, odds ratio; SNP, single nucleotide polymorphism. SF, 125 patients with spermatogenic failure; CM, 119 control males.

Of the four major estimated haplotypes within the LD block, the 'TGTAGA' haplotype was significantly associated with SF. Indeed, the *P*-value obtained by the Cochran–Armitage trend test was below the permutation *P*-value. Furthermore, comparison of the *P*-values obtained from the three types of analyses for the association between SF phenotype and estimated haplotypes implies that the specific 'TGTAGA' haplotype compromises spermatogenesis in a dosage-dependent manner rather than in a simple dominant or recessive manner. In this regard, as the 'T' allele of SNP 4 and the 'A' allele of SNP 5 are almost exclusively present in the 'TGTAGA' haplotype, genotyping of SNPs 4 and 5 can be utilized for the screening of the 'TGTAGA' haplotype.

For *ESR2*, previous studies have suggested an association between SF and an *Rsa*I SNP on exon 5 that does not result in amino acid change (SNP 6 in this study) in Scandinavian and Iranian populations (*P*-value: 0.01 and 0.012, respectively).[23,24] In such studies, as the frequency of AG genotype relative to GG genotype was higher in SF patients than in control males (AA genotype was extremely rare), this would imply that the 'A' allele of SNP 6 is regarded as a marker for a hidden true susceptibility factor(s) that is probably in an LD status with the 'A' allele of SNP 6. By contrast, the present study showed no association of SF with SNP 6 and rather suggests a dosage effect of the specific haplotype harboring the 'G' allele of SNP 6. Thus, the present data are apparently inconsistent with the previous studies. It might be possible, however, that the true susceptibility factor(s) is linked with the specific 'TGTAGA' haplotype in the Japanese population and resides on a different pattern of haplotype carrying the 'A' allele of SNP 6 in Scandinavian and Iranian populations, because of a recombination between the true susceptibility factor(s) and SNP 6 in either of the ethnic groups. In addition, there might be population-

specific susceptibility factors, and false positive results might be obtained in association studies with multiple comparisons. This matter awaits further studies.

One may argue that although the present study indicates an association of the specific *ESR2* haplotype with SF, there is no direct evidence for estrogenic EEDs being involved in the development of SF. Indeed, it may be possible that an interaction between the specific *ESR2* haplotype and endogenous estrogens rather than estrogenic EEDs actually underlie the development of SF. However, estrogenic effects of EEDs are known to be primarily mediated by ER.[1,3] In addition, as all the SF patients and the control males examined in this study were apparently free from high exposure to EEDs, the amount of exposed EEDs would be similar between the two groups of subjects. Thus, although further studies such as the investigation of subjects with a high risk of EEDs exposure (for example, workers at chemical factories) are necessary, our results would suggest that the specific *ESR2* haplotype constitutes a susceptibility factor for the development of SF in response to estrogenic EEDs in males who live in an ordinary condition with no high risk of EEDs exposure.

Several points should be made with respect to the present study. First, the number of subjects analyzed remains rather small. Second, the true susceptibility factor(s) on the specific haplotype remains to be identified, although the specific 'TGTAGA' haplotype would facilitate the development of SF by enhancing the ERβ signaling. Third, it remains possible that another susceptibility factor(s) is present on *ESR2*. In particular, as only a few of SNPs were examined in non-LD block regions, a different susceptibility factor(s) may be present on the non-LD block regions of *ESR2*. Fourth, several patients may have some unidentified pathologic cause(s) for SF such as single gene disorders. Fifth, there may be some unknown minor genetic and environmental differences between the patients and the control males. In this context, as SF becomes discernible in adulthood, such minor differences, if they exist, may exert unfavorable influences on spermatogenic function for a long time, leading to SF. This may explain why the OR obtained in this study remained low, in contrast to the high ORs identified in cryptorchidism (7.55) and hypospadias (13.75)[6,7] (and our unpublished updated observation) which develop during the fetal life. Sixth, although it is known that EEDs also exert anti-androgenic effects and influence aromatization,[25,26] these have not been examined in this study. Lastly, it remains to be determined whether similar results can be reproduced in other case–control studies.

Despite the above caveats, this study provides a useful clue to clarify the genetic susceptibility to estrogenic EEDs. In summary, we propose that the specific *ESR2* haplotype raises the susceptibility to the development of SF in response to estrogenic EEDs. Further studies including similar haplotype analyses in different ethnic groups from both developed and developing countries will serve to clarify the relative importance of the dosage of exposed EEDs and the genetic heterogeneity obtained in the process of natural human selection, in the presumably EEDs-related phenomenon such as SF.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Chronic Mucocutaneous Candidiasis Caused by a Gain-of-Function Mutation in the STAT1 DNA-Binding Domain

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Chronic mucocutaneous candidiasis (CMC) is a heterogeneous group of primary immunodeficiency diseases characterized by chronic and recurrent *Candida* infections of the skin, nails, and oropharynx. Gain-of-function mutations in *STAT1* were very recently shown to be responsible for autosomal-dominant or sporadic cases of CMC. The reported mutations have been exclusively localized in the coiled-coil domain, resulting in impaired dephosphorylation of STAT1. However, recent crystallographic analysis and direct mutagenesis experiments indicate that mutations affecting the DNA-binding domain of STAT1 could also lead to persistent phosphorylation of STAT1. To our knowledge, this study shows for the first time that a DNA-binding domain mutation of c.1153C>T in exon 14 (p.T385M) is the genetic cause of sporadic CMC in two unrelated Japanese patients. The underlying mechanisms involve a gain of STAT1 function due to impaired dephosphorylation as observed in the coiled-coil domain mutations. *The Journal of Immunology*, 2012, 189: 1521–1526.

Chronic mucocutaneous candidiasis (CMC) is a heterogeneous group of primary immunodeficiency diseases characterized by chronic and recurrent *Candida* infections of the skin, nails, and oropharynx (1). It is often associated with a variety of endocrine or autoimmune disorders. Especially, in autoimmune polyendocrinopathy with candidiasis and ectodermal dystrophy, mucocutaneous candidiasis is accompanied by hypoparathyroidism, adrenal failure, insulin-dependent diabetes mellitus, alopecia, and malabsorption syndrome (2). Although autosomal-dominant forms of CMC are also associated with endocrine disorders, such as hypothyroidism (3), the genetic causes of these disorders had remained unknown until very recently.

In 2011, two groups reported that autosomal-dominant CMC and sporadic CMC are caused by mutations in *STAT1* (4–6). The reported mutations have been exclusively localized in the coiled-coil (CC) domain, leading to gain of STAT1 function due to impaired STAT1 dephosphorylation (4). However, crystallographic analysis and direct mutagenesis experiments indicated that mutations in the DNA-binding domain (DBD) could also cause a resistance to dephosphorylation (7, 8). To our knowledge, this is the first study to demonstrate that a mutation affecting the DBD of STAT1 is the genetic cause of sporadic CMC in two unrelated Japanese patients. The mechanisms involve a gain of STAT1 function due to impaired dephosphorylation of STAT1, as also observed in mutations affecting the CC domain.

Materials and Methods

Patients

Patient 1 is a 12-y-old boy born to nonconsanguineous healthy Japanese parents. He developed severe and recurrent oral thrush since the age of 2 y and was diagnosed with CMC. He has also had recurrent pneumonia, bronchitis, and otitis media caused by *Streptococcus pneumoniae* since the age of 3 y. Chest x-ray and computerized tomography scan demonstrated the presence of bronchiectasis at the age of 5 y. He was noticed to have hypothyroidism with positive anti–thyroid-stimulating hormone receptor Abs, and levothyroxine was initiated at the age of 9 y.

Patient 2 is a boy born to nonconsanguineous healthy Japanese parents. He had poor body weight gain soon after birth. He was diagnosed with CMC at the age of 6 y. He also had recurrent bronchitis, pneumonia, and sinusitis caused by *S. pneumoniae*. He was diagnosed with bronchiectasis at the age of 7 y. At the age of 13 y, he developed hemophagocytic lymphohistiocytosis (HLH). He subsequently presented with autoimmune hemolytic anemia with positive direct and indirect Coombs' tests and thrombocytopenia and was diagnosed as having Evans syndrome. He died suddenly at the age of 14 y and 5 mo from disseminated intravascular coagulation and pulmonary insufficiency of unknown etiology. These two patients were not related (case reports in preparation).

Patient 3 is a 15-y-old girl with CMC. Her father had also been diagnosed with CMC and died of cerebral vasculitis (9). She was demonstrated to have the heterozygous R274Q mutation affecting the CC domain of STAT1. Because this mutation was recently reported as a gain-of-function mutation due to impaired dephosphorylation of STAT1 (4), we studied Patient 3 as a control for investigating the mechanisms of the development of CMC in Patients 1 and 2. Informed consent for genetic analysis was obtained from the patients, their family members, and normal controls under a protocol approved by the Institutional Review Board of Hokkaido University Hospital.

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Abbreviations used in this article: CC, coiled-coil; CMC, chronic mucocutaneous candidiasis; DBD, DNA-binding domain; HLH, hemophagocytic lymphohistiocytosis; STAT1p, phosphorylated STAT1; Wt, wild-type.

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