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Experimental evidence that phenylalanine is strongly associated to oxidative stress in adolescents and adults with phenylketonuria

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ABSTRACT

Few studies have looked at optimal or acceptable serum phenylalanine levels in later life in patients with phenylketonuria (PKU). This study examined the oxidative stress status of adolescents and adults with PKU. Forty PKU patients aged over fifteen years were enrolled, and were compared with thirty age-matched controls. Oxidative stress markers, anti-oxidant enzyme activities in erythrocytes, and blood anti-oxidant levels were examined. Nitric oxide (NO) production was also examined as a measure of oxidative stress. Plasma thiobarbituric acid reactive species and serum malondialdehyde-modified LDL levels were significantly higher in PKU patients than control subjects, and correlated significantly with serum phenylalanine level ($P<0.01$). Plasma total anti-oxidant reactivity levels were significantly lower in the patient group, and correlated negatively with phenylalanine level ($P<0.001$). Erythrocyte superoxide dismutase and catalase activities were higher and correlated significantly with phenylalanine level ($P<0.01$). Glutathione peroxidase activity was lower and correlated negatively with phenylalanine level ($P<0.001$). The oxidative stress score calculated from these six parameters was significantly higher in patients with serum phenylalanine of 700–800 μmol/l. Plasma anti-oxidant substances, beta-carotene, and coenzyme Q_{10} were also lower ($P<0.001$), although the decreases did not correlate significantly with the phenylalanine level. Serum nitrite/nitrate levels, as stable NO products, were higher together with low serum asymmetric dimethylarginine, as an endogenous NO inhibitor. Oxidative stress status is closely linked with serum phenylalanine levels. Phenylalanine level in should be maintained PKU below 700–800 μmol/l even in adult patients.

1. Introduction

Phenylketonuria (PKU) is an autosomal recessive disorder caused by a deficiency in hepatic phenylalanine hydroxylase (PAH; EC 1.14.16.1) and is usually diagnosed early in life. Unless the affected child is maintained on a strict low-phenylalanine diet, PKU leads to mental retardation, seizures, behavioral difficulties, and other neurological symptoms [1]. After the introduction of newborn mass screening for this disorder in the 1960s–70s, affected infants generally achieved normal development through early-intervention dietary treatment. Initially, the low-phenylalanine diet prescribed for classical

Abbreviations: ADMA, asymmetric dimethylarginine; CoQ_{10}, coenzyme Q_{10}; GPx, glutathione peroxidase; MDA-LDL, malondialdehyde-modified LDL; NO, nitric oxide; NOx, nitrite and nitrate; PKU, phenylketonuria; SOD, superoxide dismutase; TAR, total anti-oxidant reactivity; TBARS, thiobarbituric acid reactive species.

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PKU was discontinued by the second decade of life. However, growing evidence suggests that a high serum phenylalanine level can cause neuropsychological and psychosocial problems in diet-off adult patients [2], and that those symptoms improved after resumption of a phenylalanine-restricted diet [3]. Current recommendations are therefore that PKU patients continue the phenylalanine-restricted diet therapy for life, although the optimal serum phenylalanine levels in later life have yet to be established.

High phenylalanine concentrations in the brain correlate with the neurological signs and brain dysfunction characteristic of PKU, although the exact mechanisms and pathophysiology underlying these symptoms remain unclear. The possible culprits include changes in protein synthesis, transport of large neutral amino acids across the blood-brain barrier, synthesis of monoamine neurotransmitters, activity of glutamate receptors, and energy metabolism in the brain [4,5].

Oxidative stress influences many biological functions and long-term prolongation of oxidative stress contributes to the development and progression of various diseases, including neurological and cardiovascular disease. Brain has a high content of lipids that are vulnerable to oxidation, such as unsaturated fatty acids, yet the neural antioxidant defense system is relatively weak [6–8].

Recent studies demonstrated that oxidative stress status is pronounced in PKU patients [9–13]. In addition, it has been shown that, at least in animal models, the administration of anti-oxidants can improve the evolution of PKU [14,15]. However, the available data is limited, and more information is required before any clinically relevant conclusions are derived. The present study examined surrogate markers for oxidative stress and erythrocyte anti-oxidant enzymes in a group of PKU patients aged over fifteen years that showed widely differing dietary patterns and serum phenylalanine levels among individuals. We also investigated nitric oxide (NO) production because it is sensitive to changes in oxidative stress status and is associated with vascular tone, neurological function, apoptosis, and anti-inflammatory responses [16–18].

The purpose of this study was to evaluate oxidative stress status in PKU patients with respect to their serum phenylalanine levels, with the view to establish an optimal or acceptable serum phenylalanine level in later life.

2. Materials and methods

2.1. Subjects

We enrolled 40 PKU patients (28 females and 12 males) ranging in age from 15 to 50 years (mean ± SD, 28.4 ± 11.3 years). Phenylalanine hydroxylase (PAH) deficiency was diagnosed at the participating institutions, based on the absence of neurological deterioration on a low phenylalanine diet, analysis of dihydropteridine reductase activity in erythrocytes, biopterin loading test, and/or pteridine analysis in urine. Patients younger than 33 years were found to have hyperphenylalaninemia by mass screening at around five days of age. The remaining patients (>34 years) presented at hospital at the ages of 1–6 years for precise assessment of mental retardation and delayed motor development, and were found then to have hyperphenylalaninemia. After diagnoses, all patients were placed on a phenylalanine-restricted diet. As adults, these patients received a various range of dietary restrictions, from the maintenance of strict phenylalanine restriction to mildly restricted diets. Consequently, the plasma phenylalanine levels among the patients enrolled in this study ranged from 180 to 1800 μmol/l.

We also enrolled 30 healthy subjects as the control subjects (20 females and 10 males; range, 17–49 years; mean, 29.5 ± 7.5 years), who were matched to the patients in age, body mass index, and biological data.

2.2. Study design

The following blood markers were measured as indicators of oxidative stress: plasma levels of thiobarbituric acid-reactive species (TBARS), representing fatty acid peroxidation; total antioxidant reactivity (TAR), reflecting the ability to attenuate reactive species in the early phase; and, serum levels of malondialdehyde-modified low-density lipoprotein (MDA-LDL), representing oxidized LDL (Ox LDL) [19]. Urinary markers of oxidative stress also measured were acrolein–lysine to reflect the level of lipid peroxidation products in plasma and urine, and urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG) to reflect oxidative DNA damage [19].

Beta-carotene and alpha-tocopherol in plasma were examined as anti-oxidant substances [19]. Plasma coenzyme Q_{10} (CoQ_{10}), an electron carrier from mitochondrial respiratory chain complexes I and II to complex III, was also examined because CoQ_{10} serves as an anti-oxidant and its production is closely linked with phenylalanine metabolism [12,20,21]. Catalase, superoxide-dismutase (SOD), and glutathione peroxidase (GPx) were measured as anti-oxidative enzymes in erythrocytes.

To estimate NO metabolism, serum levels of nitrite/nitrate (NOx) were assayed as stable metabolites of NO, along with asymmetric dimethylarginine (ADMA), a competitor of arginine for NO synthase (NOS; EC 1. 14. 13. 39), and plasma arginine and citrulline, constituting the NO-citrulline cycle.

Blood was drawn from a peripheral vein in the morning after overnight fasting. Serum and plasma were obtained for the determinations of TBARS, TAR, MDA-LDL, alpha-tocopherol, beta-carotene, CoQ10, NOx, ADMA, and amino acids. Erythrocytes were washed in cold 0.9% NaCl solution. Lysates for the determinations of anti-oxidative enzyme activities were prepared by adding 100 μl of washed erythrocytes to 1 ml of distilled water, and then freezing at −80 °C until further analysis. Urine samples (5–15 ml) for oxidative stress markers were collected 0.5–2 h before blood sample collection.

This study protocol was approved by the relevant institutional review boards. All patients or their parents provided written informed consent before the start of the study.

2.3. Assays for blood and urinary oxidative stress markers

Plasma TBARS levels were determined using a fluorometric assay as described previously [22]. Plasma TAR levels, which represent the capacity to attenuate oxidants, was determined based on the luminol chemiluminescence intensity induced by 2, 2′-azo-bis-2-amidinopropane, according to Lissi et al. [23]. Serum MDA-LDL levels were determined using a sensitive enzyme-linked immunosorbent assay (ELISA) with a monoclonal antibody reactive to MDA-apo B as described previously [24]. Plasma beta-carotene, alpha-tocopherol, and CoQ_{10} (ubiquinol-10 plus ubiquinone-10) levels were measured using high-performance liquid chromatography [25].

Urinary acrolein–lysine and 8-OHdG levels were determined using competitive ELISA kits: ACR-Lysine Adduct ELISA (NOF Corp, Tokyo, Japan) and 8-OHdG Check (Institute for the Control of Aging, Shizuoka, Japan), respectively. These values were presented as acrolein–lysine and 8-OHdG to creatinine ratios.

The intra- and inter-assay coefficients of variation were less than 10% for all measurements.

2.4. Assays for anti-oxidative enzyme activities in erythrocytes

The SOD activity was determined by spectrophotometry at 505 nm (RANSOD kit; Randox Laboratories Ltd; Antrim, UK). Catalase activity was determined as described previously [24]. In brief, we monitored the decrease in absorbance at 240 nm in a reaction medium containing 20 mM H_2O_2, 10 M potassium phosphate buffer, pH 7.0, and 0.1–0.3 mg protein/ml. The GPx activity was determined by the method of Wendel

[26]; this involved monitoring the disappearance of NADPH at 340 nm in a medium containing 2 mM glutathione, 0.15 U/ml glutathione reductase, 0.4 mM azide, 0.5 mM tert-butyl-hydroperoxidase, and 0.1 mM NADPH. The intra- and inter-assay coefficients of variation were less than 10% for the respective enzyme assays.

2.5. Assays for NOx and ADMA

Serum NOx levels were measured using the Griess method, with a nitrate/nitrite colorimetric assay kit (Cayman Chemical, Ann Arbor, MI). Serum ADMA levels were determined using an ELISA kit (DLD Diagnostika GmbH, Hamburg, Germany).

2.6. Statistical analyses

Differences between patients and controls were analyzed using Student's *t*-test. The relation between each pair of parameters was estimated using Pearson's correlation test. *P* values ≤ 0.05 were considered statistically significant.

3. Results

3.1. Urinary and blood oxidative stress markers in PKU patients

The data in Table 1 clearly indicate enhanced oxidative stress in PKU patients. Three oxidative stress markers, TBARS, MDA-LDL, and TAR, were significantly different in PKU patients than the control. The mean level of urinary oxidative marker, acrolein–lysine, was significantly higher than in controls, while the 8-OHdG level was similar in the two groups. These data provided strong evidence for increased lipid peroxidation and enhanced oxidative stress in the affected patients.

Erythrocyte SOD and catalase activities were significantly higher in PKU patients, indicating heightened responsiveness to the enhancement of oxidative stress, supporting the above notion. On contrast, GPx activities were significantly lower. Among anti-oxidative substances, beta-carotene and CoQ_{10} levels were significantly lower in PKU patients than those in the controls, while alpha-tocopherol levels were similar in the two groups.

Table 1
Blood and urinary oxidative stress markers and anti-oxidant enzyme activities in erythrocytes.

	PKU patients (n=42)	Healthy controls (n=30)
Blood TBARS (nmol/mg protein)	5.05±1.16¶	3.79±0.46
Blood TAR (nmol/mg protein)	1.36±0.40§	2.15±0.38
Blood MDA-LDL (IU/ml)	61±18§	39±11
Blood alpha-tocopherol (mg/dl)	0.74±0.13	0.85±0.14
Blood beta-carotene (mg/dl)	28.2±15.0§	46.5±13.5
Coenzyme Q_{10} (ng/ml)	482±102§	970±237
Urinary 8-OHdG/Cr (ng/mg Cr)	7.68±1.65	8.23±1.49
Urinary acrolein–lysine/Cr (mmol/mg Cr)	279±142¶	199±99
SOD activity in erythrocytes (unit/mg protein)	1.54±0.25¶	1.12±0.21
Cat activity in erythrocytes (pmol/mg protein)	3.49±0.47¶	2.79±0.34
GPx activity in erythrocytes (mU/mg protein)	0.511±0.168§	0.756±0.122
Blood NOx (μmol/l)	47.2±17.2¶	30.9±10.7
Blood ADMA (μmol/l)	0.44±0.10*	0.61±0.10
ADMA/NOx	0.014±0.008¶	0.020±0.007
Blood arginine (μmol/l)	66±22¶	95±22
Blood citrulline (μmol/l)	32±9*	26±6

* $p<0.05$, compared with the control (Student's *t*-test).
¶ $p<0.01$, compared with the control (Student's *t*-test).
§ $p<0.001$, compared with the control (Student's *t*-test).

3.2. Correlations between serum phenylalanine and parameters of oxidative stress in PKU patients

In PKU patients, blood TBARS, TAR, and MDA-LDL levels correlated significantly with serum phenylalanine levels: TBARS, $r=0.709$; TAR, $r=-0.871$; MDA-LDL, $r=0.663$ (Fig. 1), whereas urinary acrolein levels did not correlate with those of 8-OHdG (acrolein, $r=0.159$; 8-OHdG, $r=0.012$). With respect to anti-oxidative substances that were significantly different between PKU patients and controls, beta-carotene but not CoQ_{10} showed a significant negative correlation with serum phenylalanine levels in PKU patients ($r=-0.421$; Fig. 2). Erythrocyte SOD and catalase in PKU patients correlated significantly with serum phenylalanine levels (catalase, $r=0.672$; SOD, $r=0.647$). In contrast, GPx activity correlated negatively with phenylalanine level ($r=-0.877$; Fig. 3).

3.3. NO metabolism

Serum NOx levels were significantly higher in PKU patients than in the controls, whereas serum ADMA levels and the ADMA: NOx ratios (ADMA/NOx) were significantly lower than the respective control levels (Table 1). Serum arginine levels were also significantly lower in the patient group compared to the control, while serum citrulline

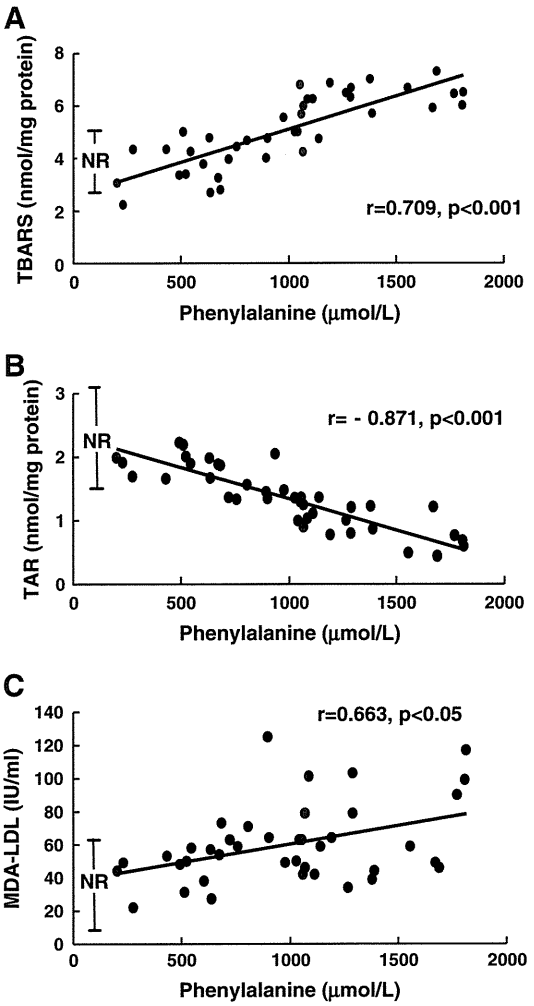

Fig. 1. Scatter graphs of thiobarbituric acid reactive substance (A), total anti-oxidant reactivity level (B), and malondialdehyde-modified LDL level (C) against plasma phenylalanine level. NR, normal range (mean ± 2 SD) obtained from 30 age-matched healthy controls.

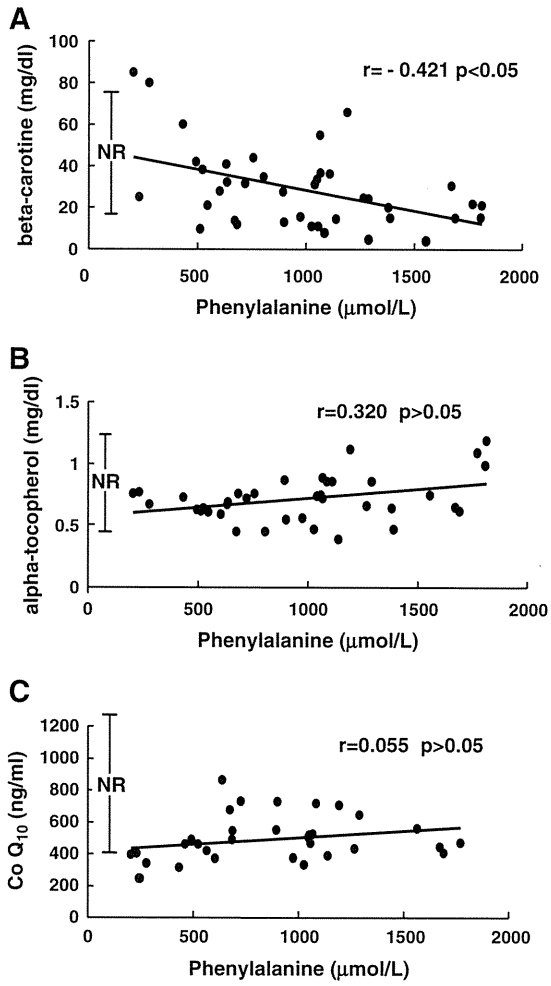

Fig. 2. Scatter graphs of beta-carotene (A), alpha-tocopherol (B), and coenzyme Q_{10} (C) against plasma phenylalanine level. NR, normal range (mean ± 2 SD) obtained from 30 age-matched healthy controls.

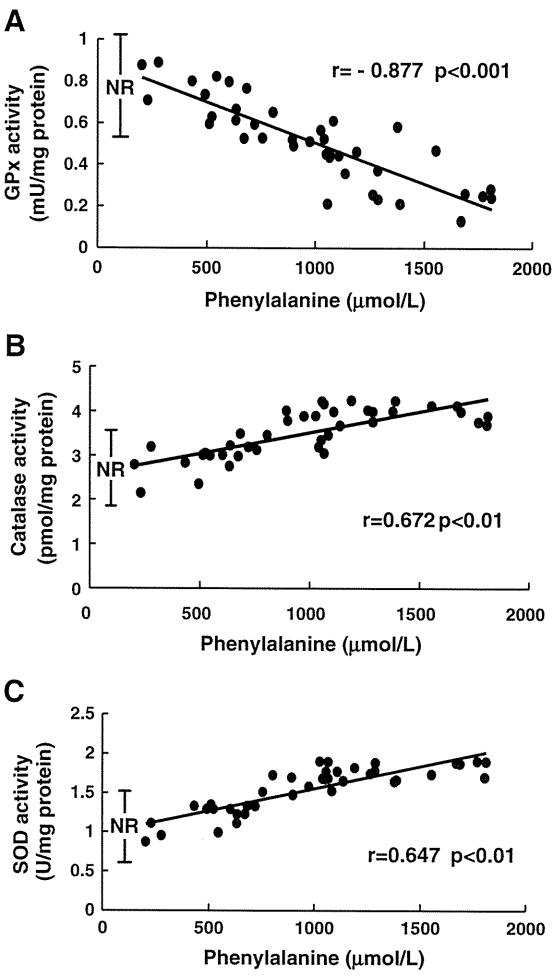

Fig. 3. Scatter graphs of GPx (A), catalase (B), and SOD (C) activities in erythrocytes against plasma phenylalanine level. NR, normal range (mean ± 2 SD) obtained from 30 age-matched healthy controls.

levels were significantly higher in the patient group. (Table 1). In PKU patients, the NOx, ADMA, and ADMA/NOx values did not correlate significantly with serum phenylalanine levels, although NOx tended to be lower in patients with phenylalanine levels of >900 μmol/l (Fig. 4).

3.4. Oxidative stress score

Of note, the values of several parameters of oxidative stress were beyond the range of the respective control values (mean ± 2 SD) in patients with serum phenylalanine levels of >600–800 μmol/l (Figs. 1–3). To evaluate the relationship between serum phenylalanine level and oxidative stress more comprehensively, we scored (0, 1, or 2 points) three blood oxidative stress markers (TBARS, TAR, and MDA-LDL) and three erythrocyte anti-oxidative enzymes (SOD, catalase, and GPx), which exhibited significant correlations with serum phenylalanine levels. For TBARS, MDA-LDL, catalase, and SOD, values ranging mean + 1 SD of healthy controls were scored as 0 point, between mean + 1 SD and + 2 SD were scored as 1 point, and more than mean + 2 SD were scored as 2 points; for TAR and GPx, values between mean − 1 SD of normal control were scored as 0 point, between mean − 1 SD and − 2 SD were scored as 1 point, and those less than mean − 2 SD were scored as 2 points. We calculated the total score as the oxidative stress score (0–12 points). We were able to divide PKU patients into two groups at six points of oxidative stress score as shown in Fig. 5. All patients with less than six points showed less than 800 μmol/l phenylalanine, and all patients with more than six points showed more than 800 μmol/l. In other words, the oxidative stress in the PKU patients changed greatly at the border of 700–800 μmol/l.

4. Discussion

Oxidative stress and its implications have not been studied adequately in PKU, particularly in adult patients. According to earlier reports, irrespective of the blood phenylalanine level, plasma TBARS and TAR levels were increased and decreased, respectively, in PKU children [9], while GPx but not SOD and catalase activities were decreased [9,11]. On the other hand, Artuch et al. [10] reported that anti-oxidant enzyme activities were not changed in PKU patient groups consisting of children, adolescents, and young adults.

We often find it difficult to evaluate the oxidative stress status in children because of the changing anti-oxidative system during growth and development [19]. The present study thus targeted subjects aged over fifteen years, whose oxidative stress status might not be influenced by such factors [19]. Our findings confirmed that oxidative stress was enhanced in the PKU patient group compared to age-matched healthy controls. In addition, there was a significant correlation between serum phenylalanine levels in PKU patients and the magnitude of oxidative stress. In other words, high oxidative stress was noted in patients with

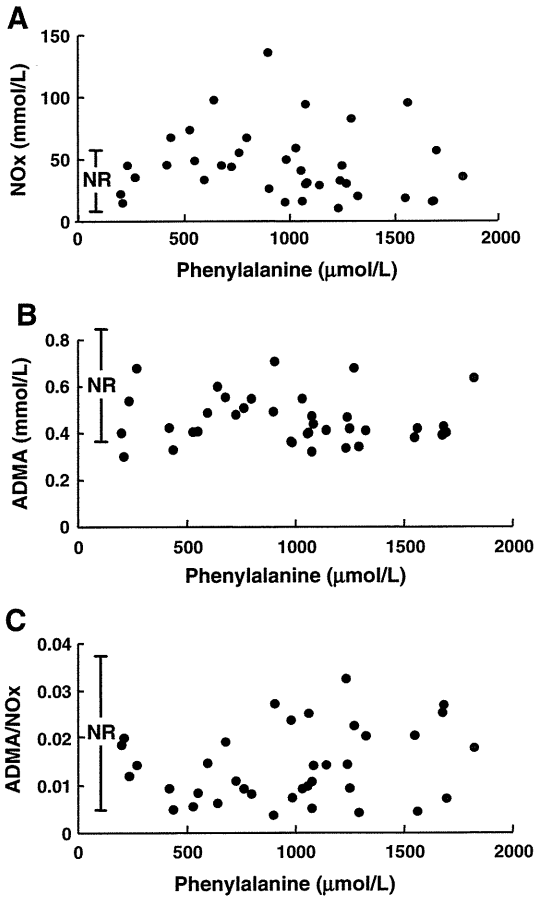

Fig. 4. Scatter graphs of NOx (A), ADMA (B), and NOx/ADMA (C) in erythrocytes against plasma phenylalanine level. NR, normal range (mean ± 2 SD) obtained from 30 age-matched healthy controls.

high serum phenylalanine levels based on the measurements of selected stress status markers (TBARS, TAR, and MDL-LDL), anti-oxidative stress enzymes (SOD and catalase), and anti-oxidants (beta-carotene). These results were supportive of a similar study in animals in which oxidative stress was closely linked with serum phenylalanine level [27].

Of note, CoQ_{10} was considerably reduced in the plasma of PKU patients. CoQ_{10} plays pivotal roles in the mitochondrial respiratory chain, cell signaling, and gene expression, and the pathogenic effects of CoQ_{10} depletion in PKU warrants further investigation. High phenylalanine inhibits mevalonic acid production via the suppression of

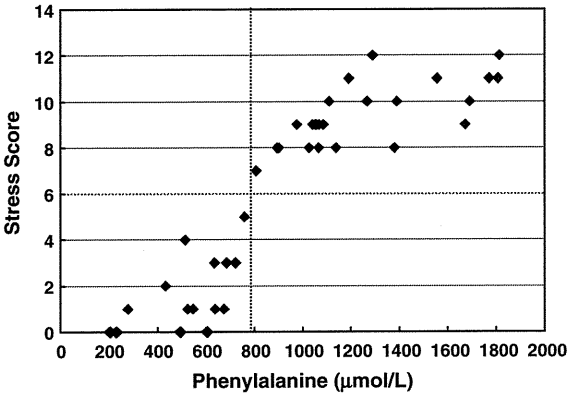

Fig. 5. Scoring of oxidative stress status according to serum phenylalanine level.

3-hydroxy-3-methylglutaryl-CoA reductase, leading to lower plasma CoQ_{10} levels [10,12,21]. In addition, strictly limited dietary intake including phenylalanine has resulted in CoQ_{10} deficiency in some cases, because phenylalanine and mevalonic acid are substrates for CoQ_{10} production [12]. We postulate that these factors also contributed to the low plasma CoQ_{10} level in our patients.

The low GPx activity could be also related to neuropsychiatric disturbances in PKU patients because GPx is highly expressed in brain and plays a pivotal role in the anti-oxidant defense system of the brain [8,28,29]. The mechanisms underlying the inhibition of GPx activity in PKU patients remain to be clarified. It is known that GPx activity is decreased with deficiencies in selenium, an essential cofactor for this enzyme [30]. Selenium levels were measured in approximately 50% of our patient group (19/40), but were normal. Hagen et al. [14] showed that phenylalanine directly suppresses GPx production or promotes GPx degradation in a rat model of hyperphenylalaninemia. In our patients, the GPx activity level exhibited a strong negative correlation with the serum phenylalanine level, suggesting that phenylalanine *per se* inhibits GPx activity.

PKU patients examined in our study also showed changes in parameters of NO metabolism. Considering that NO has a regulatory effect on neurological function, it is plausible that such abnormalities in the NO metabolism could mediate, at least in part, the neurological signs and symptoms in PKU patients [16–18]. In this study, serum NOx levels were increased, but serum ADMA levels and ADMA to NOx ratios were decreased compared to controls, and plasma arginine, an amino acid substrate for NO production, was decreased. A scatter graph of NOx against serum phenylalanine levels displayed a tendency for the NOx level to increase until a serum phenylalanine level of 900 μmol/l and subsequently decrease (Fig. 4). In contrast, ADMA/NOx tended to decrease until 900 μmol/l phenylalanine, and thereafter increase (Fig. 4). Irrespective of NOx level, ADMA levels remained fairly constant. Together, these findings suggested strongly that the regulation of NO production, in particular the inhibition by ADMA, is disturbed in PKU patients. We inferred the increase of NOx in the first phase as a consequence of the pronounced oxidative stress. As a possible mechanism for the decrease in NOx trend in the second phase at higher phenylalanine levels, we speculate that transcriptional suppression of the NOS gene or structural changes in NOS are induced with markedly enhanced oxidative stress [16,18].

Even if the adult brain in PKU develops resistance to high phenylalanine concentrations, such increased levels could still contribute to the development of neuropsychological and psychosocial problems in diet-off adult patients. Therefore, PKU patients are recommended to continue diet therapy for life. On the other hand, the appropriate or ideal serum phenylalanine levels in later life remain to be established. Diet therapy is difficult and therefore it is particularly important to patient quality of life to recommend an optimal blood phenylalanine level. Current recommended levels for adults on dietary treatment vary greatly from 600 to 1300 μmol/l in Europe and the USA. The present study now raises the possibility that increased oxidative stress in PKU patients >15 years of age could be avoided by keeping their serum phenylalanine level below 700–800 μmol/l. This relatively low phenylalanine levels to be maintained have been supported by the results of MR imaging [31–33]. Kono et al. [33] reported that PKU patients with serum phenylalanine levels beyond 550 μmol/l exhibited abnormal findings in posterior cerebral deep white matter on magnetic resonance imaging, irrespective of age.

5. Conclusion

Oxidative stress status is closely linked with serum phenylalanine levels. Based on our findings, we propose that serum phenylalanine level should be controlled below 700–800 μmol/l by dietary treatment even in later life.

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Clinical Transplantation

Progressive familial intrahepatic cholestasis: a single-center experience of living-donor liver transplantation during two decades in Japan

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Abstract: Background: Progressive familial intrahepatic cholestasis (PFIC) results in liver cirrhosis. Therefore, some PFIC patients require liver transplantation (LT). Although three types of PFIC have been identified, their etiologies include unknown mechanisms.
Patients: A total of 717 recipients who underwent living-donor LT (LDLT) at < 20 yr old were enrolled in this study. Among these recipients, 14 PFIC recipients comprising 11 PFIC type 1 (PFIC1) and three PFIC type 2 (PFIC2) were evaluated.
Results: Three of 11 PFIC1 recipients died, while all three PFIC2 recipients survived. Eight of 11 PFIC1 recipients showed steatosis after LDLT. Among the eight steatosis-positive PFIC1 recipients, seven showed severe steatosis and seven were complicated with steatohepatitis. Nine of 11 PFIC1 recipients showed fibrosis after LDLT, and eight of the nine fibrosis-positive PFIC1 recipients showed severe fibrosis. In contrast to the PFIC1 recipients, the PFIC2 recipients did not show any steatosis or fibrosis after LDLT.
Conclusions: The clinical courses and outcomes of PFIC1 recipients after LDLT are still not sufficient owing to steatosis/fibrosis, unlike the case for PFIC2 recipients. As PFIC1 patients will require LT during the long-term progression of the disease, further strategy improvements are required for PFIC1 patients.

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Key words: Byler's disease – liver transplantation – living donor – progressive familial intrahepatic cholestasis – steatosis

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Progressive familial intrahepatic cholestasis (PFIC) refers to a heterogeneous group of autosomal recessive disorders of childhood that disrupt bile formation and present with cholestasis of hepatocellular origin. The actual prevalence remains unknown, but the estimated incidence is one per 50 000–100 000 births. The natural course of PFIC causes portal hypertension, liver failure, cirrhosis, hepatocellular carcinoma, and extrahepatic manifestations. Three types of PFIC have been

identified as follows: (i) deficiency of familial intrahepatic cholestasis 1 (FIC1), so-called Byler's disease, as PFIC type 1 (PFIC1); (ii) deficiency of bile salt export pump (BSEP), so-called Byler's syndrome, as PFIC type 2 (PFIC2); and (iii) deficiency of multidrug resistant 3 (MDR3) as PFIC type 3 (PFIC3). Each mutation is related to hepatocellular transport system genes involved in bile formation (1). Although the etiologies of the three PFIC types have previously been demonstrated, they include unknown mechanisms. Cholestasis is a major clinical sign in all three PFIC types. PFIC should be suspected in children with a clinical history of cholestasis of unknown origin after exclusion of the other main causes of cholestasis (2). A high level of serum bile acid (BA) excludes primary disorders of BA synthesis (3). The phenotypic findings in PFIC1 and PFIC2 are similar, although some slight phenotypic differences have been identified (4–9). Extrahepatic features that have been documented in PFIC1 patients, such as persistent short stature, sensorineural deafness, watery diarrhea, pancreatitis, elevated sweat electrolyte concentration, and liver steatosis (10), have not been reported in PFIC2. Therapy with ursodeoxycholic acid (UDCA) is considered during the initial therapeutic management of children with all types of PFIC (11), although PFIC patients are theoretically associated with a risk of further development of biliary stones, drug-induced cholestasis and intrahepatic cholestasis of pregnancy (ICP) during the disease course. Female PFIC patients under UDCA therapy who reach adulthood with their native liver must not stop UDCA during pregnancy because of the risk of developing severe ICP as observed in a previously reported patient who became pregnant (1, 12). More advanced strategies such as cell transplantation, gene therapy or specific targeted pharmacotherapy may represent alternative therapies for all types of PFIC in the future (13–16). Although UDCA therapy has advantages, especially for PFIC3, the resultant liver cirrhosis in PFIC patients requires liver transplantation (LT) including living-donor LT (LDLT) as a definitive therapy.

Here, we present the data for our PFIC patients after LDLT during two decades (1999–2009) and review the current literature about PFIC.

Patients and methods

Patients

Since 1990, 735 adult and 702 pediatric recipients underwent LT at Kyoto University Hospital. A total of 717 LDLT recipients whose ages at LDLT were < 20 yr were enrolled in this study. Among these LDLT recipients, 11 PFIC1 and three PFIC2 recipients were evaluated. We had no experience of LDLT for PFIC3 patients during these two decades. One PFIC1 patient had a history of paroxysmal atrial fibrillation.

The 14 PFIC recipients comprised five males and nine females, and their age range at LDLT was 0.6–18.2 yr. The time from the diagnosis of PFIC to LDLT was 1417.9 ± 2055.4 d (range, 75–5935 d) in the PFIC1 recipients and 288.7 ± 273.3 d (range, 43–583 d) in the PFIC2 recipients. The values of standard deviation (SD) in height and body weight at LDLT were −4.5 ± 1.8 (range from −7.5 to −1.1) and 2.1 ± 1.0 (range from −3.5 to 0.3), respectively. Thus, the growth retardation was confirmed in all patients. The serum level of total BA was elevated to 439.1 ± 109.8 μmol/mL (range, 299–600 μmol/mL), while the serum level of gamma-glutamyltransferase (γ-GT) was normal at 16.6 ± 4.0 IU/L (range, 12–26 IU/L). The mean Child-Pugh score was 7.9 ± 0.8 points (range, 7–9 points). The mean score of the Model for End-stage Liver Disease (MELD) or Pediatric End-stage Liver Disease (PELD) was 12.3 ± 4.1 points (range, 5–19 points). Pre-operative statuses were 11 cases at home and three were hospitalized cases. The United Network for Organ Sharing (UNOS) statuses were estimated to be 12 cases of status III and two cases of status IIB. The ABO blood groups were characterized as 11 identical cases, two compatible cases and one incompatible case. The donor relationships were 10 fathers, three mothers and one grandmother. The mean donor age was 36.9 ± 7.1 yr (range, 28–47 yr). One donor was HBsAb-positive donor. These data are summarized in Table 1.

The protocol of the study was approved by the Ethics Review Committee for Clinical Studies of Kyoto University Graduate School of Medicine.

Zero-hour liver biopsy in living donor

Basically, the routine checks including computed tomography (CT) number assessments for liver dysfunction, steatosis and non-alcoholic steatohepatitis (NASH) were performed in living donor beforehand.

To confirm the condition of graft, our institution employed zero-h liver biopsy during living-donor operation at the time point before starting the procedures of hepatectomy.

Operation

There were lateral-segment grafts and one case each of extended lateral-segment and left-lobe

Table 1. Profiles of the PFIC patients

Case number	PFIC	Age at LDLT (yr)	Growth retardation		MELD/PELD score (point)	UNOS status	ABO compatibility	GRWR	Histopathological findings	
			Height (SD)	Weight (SD)					Recipients[a] (F)	Living donors[b]
1	Type 1	3.7	−7.3	−3.5	18	III	Compatible	2.80	4	−
2	Type 1	4.1	−3.5	−1.2	5	III	Identical	1.68	4	−
3	Type 1	14.0	−7.5	−2.9	19	III	Identical	1.57	4	−
4	Type 1	14.2	−4.6	−2.2	18	III	Compatible	1.20	4	−
5	Type 1	5.3	−5.5	−2.5	14	IIB	Identical	1.43	4	−
6	Type 1	6.3	−6.4	−2.8	9	III	Identical	1.54	4	−
7	Type 1	18.2	−5.0	−3.5	17	III	Identical	1.29	4	<5
8	Type 1	3.7	−3.3	−1.0	10	III	Identical	2.08	4	0
9	Type 1	4.5	−4.3	−1.8	11	III	Identical	1.57	4	0
10	Type 1	1.8	−3.3	−2.5	14	III	Identical	2.55	4	0
11	Type 1	1.2	−4.0	−2.3	14	III	Identical	4.02	4	<5
12	Type 2	1.4	−3.4	−2.4	19	III	Identical	3.77	4	0
13	Type 2	0.6	−1.1	0.3	9	IIB	Incompatible	2.24	4	0
14	Type 2	4.6	−3.1	−1.3	12	III	Identical	1.39	4	0

GRWR, graft/recipient weight ratio; LDLT, living-donor liver transplantation; MELD, Model for End-stage Liver Disease; PELD, Pediatric End-stage Liver Disease; PFIC, progressive familial intrahepatic cholestasis; SD, standard deviation; UNOS, United Network for Organ Sharing.
[a]Fibrosis scores by the METAVIR system in removed native livers.
[b]The percentage of steatosis in zero-h biopsy specimens obtained during donor operations.

grafts. The mean graft/recipient weight ratio was 2.08 ± 0.91 (range, 1.20–4.02). Histopathological analyses of biopsy specimens during the donor operation were performed in seven cases, and normal findings were confirmed. The mean operative time was 525.4 ± 57.4 min (range, 402–636 min) and the mean blood loss was 949.3 ± 833.9 mL (range, 105–2610 mL). The mean cold and warm ischemic times were 51.0 ± 29.4 min (range, 15–99 min) and 35.9 ± 11.6 min (range, 24–56 min), respectively.

Basically, the left hepatic vein or common trunk of the left and middle hepatic veins was anastomosed to the inferior vena cava in an end-to-side fashion. Portal veins were reconstructed in an end-to-end fashion, after the confirmation of enough front-flows. Hepatic arteries were anastomosed in an end-to-end fashion by using microscope. Hepaticojejunostomy were performed in twelve cases, and duct-to-duct reconstruction in two cases, as biliary reconstruction during initial LDLT. Essentially, the posterior wall was sutured with a continuous-suture technique and the anterior wall was sutured with an interrupted-suture technique, using absorbable monocryl threads (6-0 PDS II; Ethicon Inc., Somerville, NJ USA).

Immunosuppression

Immunosuppression after LDLT was started with tacrolimus and methylprednisolone. The trough level of tacrolimus was maintained at 8–15 ng/mL during the early post-operative period, based on the clinical findings in each case. Methylprednisolone was given intravenously (1 mg/kg) once daily from post-operative day (POD) 1 to POD 3 followed by 0.5 mg/kg once daily for the next three d. On POD 7, 0.3 mg/kg of methylprednisolone was given intravenously. Steroid administration was switched to oral prednisolone 0.3 mg/kg once daily on POD 8. This dose was reduced to 0.1 mg/kg at one month after LDLT. Thereafter, immunosuppressants were adjusted according to clinical course. We had already overcome ABO-incompatibility in LDLT, and our regimens for these recipients, including heparin usage, were described previously (17).

Histopathological analysis

In our institution, laboratory and ultrasound examinations were routinely performed after LDLT in all recipients. A liver needle biopsy (LNB) protocol is not employed in our institution. However, ultrasound-guided LNBs were performed, if required, based on the results of conventional liver function tests, findings of Doppler ultrasound examinations and consideration of the original diseases. All the PFIC recipients underwent LNBs after LDLT, and received histopathological follow-up by LNBs.

All liver tissues were fixed in neutral buffered formalin, embedded in paraffin, and sliced into 4 μm-thick sections. The morphological characteristics were assessed by standard hematoxylin-eosin staining, and hepatic fibrosis was evaluated by

Masson trichrome and reticulin staining. Post-transplant steatosis was calculated as the percentage of steatosis in the liver tissue (18). Macrovesicular steatosis was graded quantitatively according to the percentage of involved hepatocytes (18). Steatohepatitis was diagnosed according to the degree of steatosis, ballooning degeneration and portal/lobular inflammation. The fibrosis scores were strictly estimated based on the presence or absence of post-operative steatosis. Estimation of the hepatic venous area is important during the early phase of fibrosis progression in NASH (19), although the fibrosis in common hepatitis initially occurs in the periportal area. In the assessments of post-transplant fibrosis, we used the fibrosis scores in the NASH score for the PFIC1 recipients with steatosis, and the METAVIR score for the recipients without steatosis. The fibrosis scores in the recipients with steatosis were assigned as follows (19): 1, perivenular fibrosis; 2, perivenular and periportal fibrosis; 3, bridging fibrosis; 4, cirrhosis. The fibrosis scores in the recipients without steatosis were assigned as follows (20): 1, periportal fibrosis; 2, bridging fibrosis; 3, pre-cirrhosis; 4, cirrhosis.

Statistical analysis

The survival curves were calculated by the Kaplan–Meier method (the log-rank test). Statistical analyses were performed using SPSS software version 16.0 (SPSS Inc., Chicago, IL, USA). $p < 0.05$ was considered statistically significant.

Results

Histopathological analyses in native liver of recipients and zero-h biopsy specimens of living donor

Histopathologically, cirrhosis without steatosis (F4) was confirmed in the native livers of all recipients (Table 1).

All 14 living donors had no PFIC symptoms. All living donor showed no liver disfunction and abnormalities in image studies. Eight of 14 living donors received zero-h biopsy during living-donor operation (Cases 7–14), and no steatosis was confirmed. All of these eight living donors showed a percentage below five, at least (Table 1).

Clinical courses and outcomes after LDLT

The mean hospital stay after LDLT was 70.7 ± 42.8 d (range, 29–189 d). Digestive symptoms after LDLT were confirmed in 10 of 11 PFIC1 recipients (Cases 1–7 and 9–11), but absent in all the PFIC2 recipients. Cirrhotic findings including esophageal varix and splenomegaly after LDLT were confirmed in six PFIC1 recipients, and these recipients received endoscopic or surgical therapies for esophageal varix and splenomegaly (Cases 2, 3, 5, 7, 8, 10). One PFIC1 recipient suffered from *de novo* autoimmune hepatitis (AIH) (Case 2), and was closely followed. Among the PFIC2 recipients, one received steroid pulse and muromonab-CD3 therapies for refractory acute cellular rejection during the early post-operative period (Case 14).

The actual survival curves are shown in Fig. 1. Three of 11 PFIC1 recipients died, whereas all three PFIC2 recipients survived. One PFIC1 recipient died after rupture of the splenic artery at POD 5032 (Case 3). One PFIC1 recipient received a retransplantation on POD 4646 owing to graft loss, but she died of cardiac failure at 25 d after the retransplantation (Case 5). One PFIC1 recipient suffered from chronic rejection at six months after LDLT, and underwent a retransplantation on POD 1393. An arterioportal shunt occurred after the retransplantation, and another retransplantation was performed on POD 1986 after the initial LDLT. However, rupture of an esophageal varix occurred on POD 2005 after the initial LDLT (Case 10).

UDCA and pancreatic enzyme supplementation

In our institution, UDCA was routinely used after LDLT. Therefore, all PFIC recipients received this therapy. Pancreatic enzyme was supplied in two PFIC1 recipients after LDLT due to chronic pancreatitis (Cases 1 and 5), although PFIC2 recipients did not need pancreatic enzyme supplementation.

Histopathological findings after LDLT

The histopathological findings are shown in Table 2. The mean performance of LNBs after LDLT was 8.3 ± 5.1 times (range, 3–23 times).

Eight of 11 PFIC1 recipients showed steatosis after LDLT (Cases 1, 3, 5–9 and 11), whereas no steatosis was confirmed in the remaining three PFIC1 recipients (Cases 2, 4 and 10). Seven of the eight steatosis-positive PFIC1 recipients showed steatosis degrees of ≥80% (Cases 1, 3, 5, 6, 8, 9 and 11). The mean POD when the steatosis reached its peak was 229.6 ± 253.7 d (range, 21–736 d). Seven of the eight steatosis-positive PFIC1 recipients also showed findings of steatohepatitis (Cases 1, 3, 5, 6, 7, 9 and 11).

Overall, eight of 11 PFIC1 recipients (73%) showed steatosis with fibrosis after LDLT, whereas

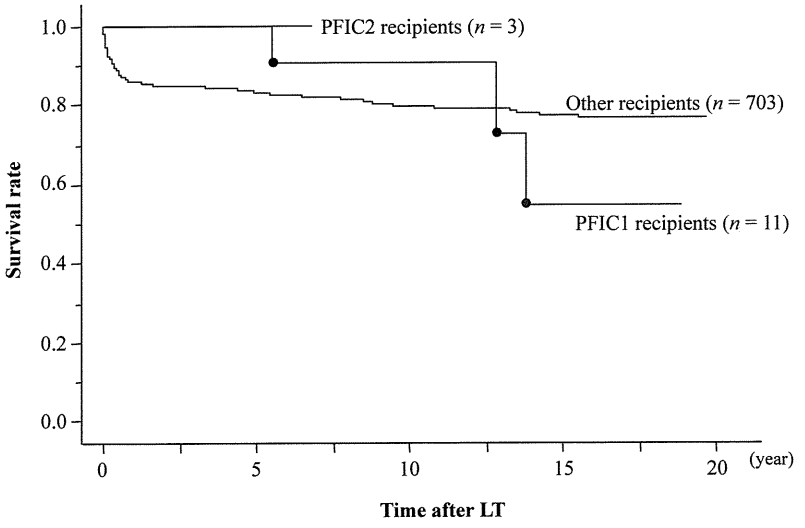

Fig. 1. Survival curves for PFIC1, PFIC2, and other recipients after living-donor liver transplantation (LDLT). The survival curves after LDLT in each of the 11 PFIC1 recipients, three PFIC2 recipients, and 703 other recipients were calculated by the Kaplan–Meier method. PFIC, progressive familial intrahepatic cholestasis.

Table 2. Histopathological findings after LDLT

Case number	PFIC	LNBs after LDLT (times)	Steatosis		Steatohepatitis	Fibrosis		Other histopathological findings after LDLT	Outcome (POD)
			LNB[a] (POD)	Percentage		LNB[a] (POD)	Score[b] (F)		
1	Type 1	10	378	90	+	2303	3	–	Alive (6884)
2	Type 1	6	–	0	–	4171	4	*De novo* AIH	Alive (6604)
3	Type 1	9	46	90	+	480	4	–	Dead (5032)
4	Type 1	4	–	0	–	–	0	–	Alive (5605)
5	Type 1	13	1305	80	+	1869	3	–	Dead (4671)
6	Type 1	8	138	80	+	468	3	–	Alive (4295)
7	Type 1	7	736	30	+	3254	4	–	Alive (4065)
8	Type 1	3	87	80	–	–	0	–	Alive (3384)
9	Type 1	11	397	90	+	991	3	–	Alive (3265)
10	Type 1	23	–	0	–	1125	4	Chronic rejection	Dead (2005)
11	Type 1	5	34	80	+	34	1	–	Alive (2028)
12	Type 2	4	–	0	–	–	0	–	Alive (2453)
13	Type 2	6	–	0	–	–	0	–	Alive (1601)
14	Type 2	7	–	0	–	–	0	Refractory ACR	Alive (500)

ACR, acute cellular rejection; AIH, autoimmune hepatitis; LDLT, living-donor liver transplantation; LNB, liver needle biopsy; NASH, nonalcoholic steatohepatitis; PFIC, progressive familial intrahepatic cholestasis; POD, postoperative day.
[a]The PODs of the peak findings are shown if the histopathological findings were positive. The earliest PODs are shown if the histopathological findings were equal.
[b]Fibrosis score: the NASH score was used for steatosis-positive recipients, while the METAVIR score was used for steatosis-negative recipients.

no fibrosis was confirmed in the PFIC2 recipients. Moreover, the steatosis and steatohepatitis that subsequently caused fibrosis were surprisingly confirmed even during the early post-operative period in these PFIC1 recipients.

Nine of 11 PFIC1 recipients showed fibrosis after LDLT (Cases 1–3, 5–7 and 9–11), whereas no fibrosis was confirmed in the remaining two PFIC1 recipients. Eight of the nine fibrosis-positive PFIC1 recipients showed F scores of ≥3 (Cases 1–3, 5–7, 9 and 10). The mean POD when the F score reached its peak was 1632.8 ± 1391.4 d (range, 34–4171 d). Among the nine F score-positive PFIC1 recipients, seven were complicated with steatosis (Cases 1, 3, 5–7, 9 and 11), and two had fibrosis without steatosis (F4) for another reason (*de novo* AIH and chronic rejection) (Cases 2 and 10). In contrast to the PFIC1 recipients, the PFIC2 recipients did not show any steatosis or fibrosis.

Total external biliary diversion (TEBD)

In one PFIC1 recipient (Case 10), TEBD was performed at retransplantation, although we had no experience of TEBD at initial LDLTs. However, we cannot confirm the effects of LDLT

accompanied by TEBD, because this patient suffered graft loss owing to an arterioportal shunt after the retransplantation.

In our institution, TEBD is performed as a hepatico-jejuno-cutaneostomy. An isolated jejunal interposition of 15–20 cm in length is made with the proper mesentery at a point 20 cm distant from Treitz's ligament. Next, hepatico-jejunostomy was made. The jejunal interposition is placed between the hepatic hilum and the skin. An end-stoma is made in the right lower quadrant of the abdominal wall.

Discussion

The combined considerations of clinical, biochemical, radiological and histological approaches including liver immunostaining and biliary lipid analyses help diagnosis of PFIC candidates. Clinical manifestations of each type of PFIC documented in these studies are summarized in Table 3.

Phenotypic findings and extrahepatic features have been described in PFIC1 (4–10). The hepatic histopathology is characterized by canalicular cholestasis and the absence of true ductular proliferation with only periportal biliary metaplasia of hepatocytes. PFIC1 is caused by mutations in the ATP8B1 gene (designated FIC1) (7). Adenosine triphosphate (ATP) is elaborated from adenosine diphosphate and phosphoric acid via ATPase, and the FIC1 gene, which encodes a P-type ATPase, is located on human chromosome 18. Some investigators have described that impaired FIC1 function results in substantial downregulation of farnesoid X receptor (FXR), a nuclear receptor involved in the regulation of BA metabolism, with subsequent downregulation of BSEP in the liver and upregulation of BA synthesis and the apical sodium bile salt (BS) transporter in the intestine (21). The possible mechanisms of BA overload in hepatocytes are highlighted in Fig. 2A. The extrahepatic features such as diarrhea, liver steatosis, and short

Table 3. Characteristic manifestations of PFIC

	PFIC1 (FIC1 deficiency) (Byler's disease)	PFIC2 (BSEP deficiency) (Byler's syndrome)	PFIC3 (MDR3 deficiency)
Inheritance	18q21-22	Autosomal recessive	Autosomal recessive
Age of onset	Neonatal period	Neonatal period	1 month–20 yr
Cholestasis	Chronic	Chronic	Chronic
Ductular proliferation	Absent	Absent	Absent
Progression to cirrhosis	Yes	Yes	Yes
Cutaneous pruritus	Severe	Severe	Moderate
Serum γ-GT activity	Normal	Normal	High
Serum cholesterol level	Normal	Normal	Normal
Serum PBA concentration	Very high	Very high	High
Bile composition	Low PBA concentration	Very low PBA concentration	Low phospholipid concentration
Gene (encoding protein)	Autosomal recessive	ABCB11 (BSEP)	ABCB4 (MDR3)
Chromosomal locus	ATP8B1 (FIC1)	2q24	7q21
Function of hereditary defect	Aminophospholipid translocase	BA secretion	Phosphatidylcholine secretion
Hepatocyte location	Canalicular membrane	Canalicular membrane	Canalicular membrane
Other sites of mRNA expression	Cholangiocytes Intestine Pancreas	None	None
Resultant functional defect	ATP-dependent Aminophospholipid translocase	ATP-dependent BA transport in bile	ATP-dependent Phosphatidylcholine translocation in bile
Histopathological findings	Canalicular cholestasis Absence of true ductular proliferation Periportal biliary metaplasia of hepatocytes Pronounced portal/lobular fibrosis Pronounced portal/lobular inflammation Hepatocellular necrosis Giant cell transformation Perturbed liver architecture	Canalicular cholestasis Absence of true ductular proliferation Severe lobular injury More pronounced lobular/portal fibrosis More pronounced lobular/portal inflammation More obvious hepatocellular necrosis More obvious giant cell transformation More perturbed liver architecture	Rare cholestasis True ductular proliferation Normal interlobular bile ducts Rare extensive portal fibrosis Mixed inflammatory infiltrate Rare biliary cirrhosis Slight giant cell transformation No biliary epithelium injury

ATP, adenosine triphosphate; BA, bile acid; BSEP, bile salt export pump; FIC1, familial intrahepatic cholestasis 1; γ-GT, gamma-glutamyltransferase; MDR3, multidrug resistant 3; PBA, primary bile acid; PFIC, progressive familial intrahepatic cholestasis.

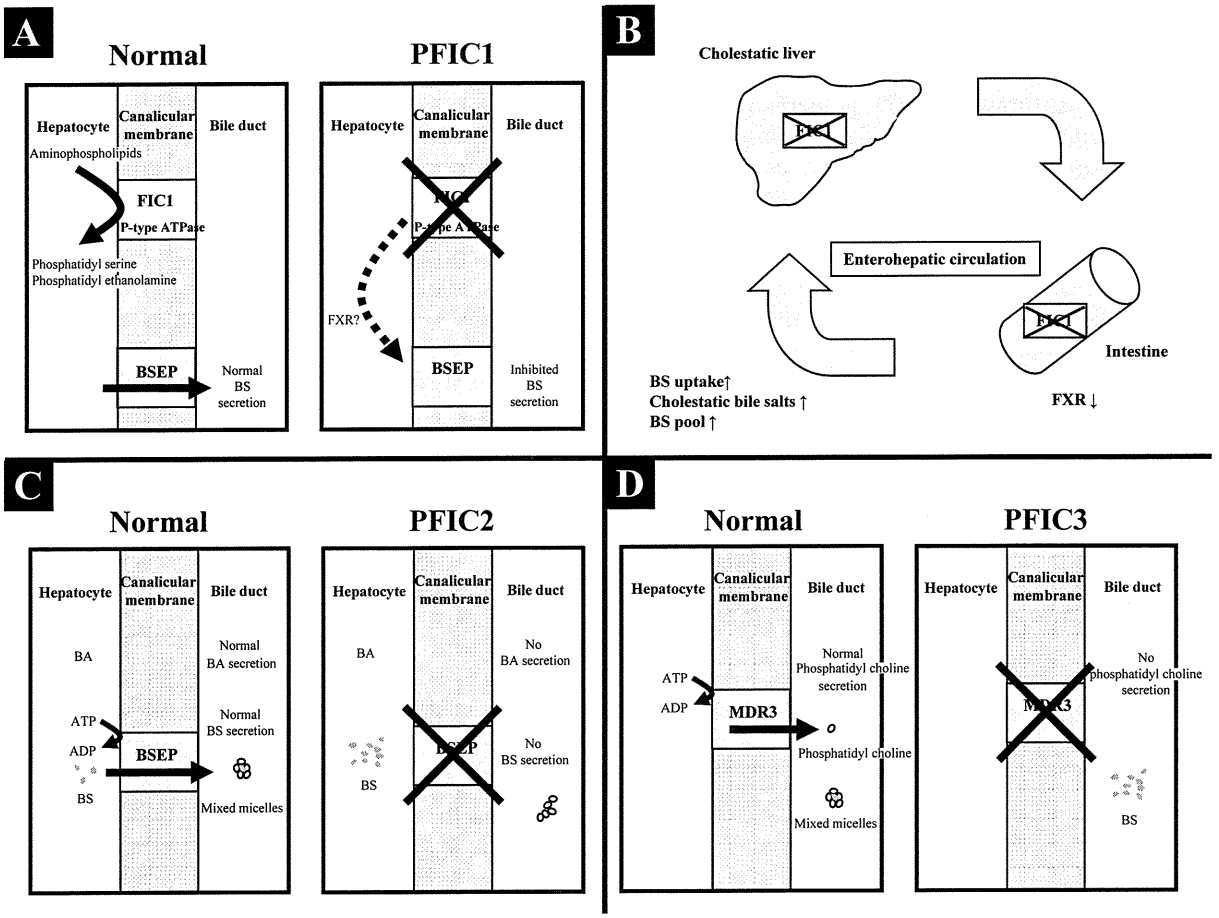

Fig. 2. Schemas of the mechanisms for the different types of progressive familial intrahepatic cholestasis (PFIC). (A) The possible mechanism of PFIC1. The FIC1 protein is located on the canalicular membrane of hepatocytes, but is mainly expressed in intrahepatic cholangiocytes. The function of the P-type ATPase is still unknown. However, it could be an aminophospholipid transporter responsible for maintaining the enrichment of phosphatidylserine and phosphatidylethanolamine on the inner leaflet of the plasma membrane. The asymmetric distribution of lipids in the membrane bilayer plays a protective role against high concentrations of bile salt (BS) in the canalicular lumen. The issue of how these mutations cause cholestasis remains unclear. It is postulated that abnormal protein function may indirectly disturb the biliary secretion of BA, thus explaining the low concentration of biliary BA. Impaired FIC1 function results in substantial downregulation of farnesoid X receptor (FXR), a nuclear receptor involved in the regulation of BA metabolism, with subsequent downregulation of bile salt export pump (BSEP) in the liver and upregulation of BA synthesis and the apical sodium BS transporter in the intestine. Eventually, these events lead to BA overload in hepatocytes. (B) Enterohepatic circulation in PFIC1. The downregulation of the cystic fibrosis transmembrane conductance regulator in cholangiocytes has been reported in PFIC1, and this downregulation could contribute to the impairment of bile secretion and explain some of the extrahepatic features. The FIC1 gene is expressed in various organs, including the liver, pancreas, small intestine and kidney, but is more highly expressed in the small intestine than in the liver. Therefore, enterohepatic cycling of BS should be considered in PFIC1. This may also explain the digestive symptoms including chronic diarrhea in PFIC1. Other extrahepatic features such as persistent short stature, deafness, and pancreatitis suggest a general cell biological function for FIC1. The downregulation of FXR in intestines is suggested in this pathway. (C) The mechanism of PFIC2. The BSEP protein, which is expressed on the hepatocyte canalicular membrane, is the major exporter of primary bile acid against extreme gradients of concentration. Mutations in this gene are responsible for decreased biliary BS secretion, which leads to decreased bile flow and accumulation of BS inside the hepatocytes, thereby resulting in severe hepatocellular damage. (D) The mechanism of PFIC3. The mechanism of the liver damage in PFIC3 is related to the absence of biliary phospholipids. The damage to the bile canaliculi and biliary epithelium probably results from continuous exposure to hydrophobic BSs, the detergent effects of which are no longer countered by phospholipids, thus leading to cholangitis. FIC1, familial intrahepatic cholestasis 1.

stature that are sometimes associated with PFIC1 do not improve or may be aggravated after successful biliary diversion or LT (1, 10). Chronic diarrhea may become intractable when biliary BS secretion is restored after LT (7, 10), while diarrhea may be favorably managed by bile adsorptive resin treatment (10). Similar to these previous reports, our data confirmed digestive symptoms after

LDLT in 10 of 11 PFIC1 recipients (91%), whereas the PFIC2 recipients showed no digestive symptoms. PFIC1 associated with severe liver steatosis and/or steatohepatitis may lead to cirrhosis over time, and to indications for retransplantation (1). However, liver steatosis and diarrhea may occur even after retransplantation (1). In LDLT, donor selection is very limited ethically, socially and medically. In Japan in particular, expansion of deceased-donor LT is not yet in sight, although the governmental legislation for organ transplantation was revised in 2009. There are no affirmations for repeated retransplantation that may collateralize the long-term outcomes of pediatric PFIC1 recipients. The outcomes of PFIC1 recipients after LDLT are still not sufficient, based on our data and a previous report (22). Our actual results for the early post-operative occurrences of steatosis and fibrosis oblige us to reconsider the timing of LDLT and to challenge some other therapies for PFIC1 patients. Previously, partial external biliary diversion (PEBD) has been documented as a surgical procedure for PFIC patients (23, 24). Some patients with PFIC1 or PFIC2 may also benefit from surgical biliary diversion (25–27). The criteria for identifying those PFIC1/2 patients who could benefit from UDCA or biliary diversion remain unclear (14). We clearly understand that LT represents the only alternative if these therapies fail (28). Although we do not have sufficient experience of PEBD for PFIC1, we now take this anticipatory surgery before LDLT into consideration, if the overall considerations including donor limitations and the patient status indicate the permissive possibility. We understand that PFIC1 patients who eventually have refractory cirrhosis will finally require LT, and we do not consider that PFIC1 contraindicates LDLT because not all of our PFIC1 recipients necessarily suffered graft losses after LDLT. Based on our results, LDLT accompanied by TEBD appears to be rather better than PEBD from the viewpoints of the etiology in PFIC1 and graft protection after LDLT.

Although PFIC1 and PFIC2 share similar laboratory findings, the initial evolution of cholestasis in PFIC2 patients is more severe than in the other PFIC types. Hepatocellular carcinoma may complicate the course. As patients with BSEP deficiency accompanied by biallelic truncating mutations have a considerable risk for hepatobiliary malignancy (15% of patients develop hepatocellular carcinoma or cholangiocarcinoma) (29, 30), close monitoring of malignancy in PFIC2 patients is justified. The histopathological findings reveal more perturbed liver architecture than PFIC1, with more pronounced lobular and portal fibrosis and inflammation. Hepatocellular necrosis and giant cell transformation are also much more pronounced in PFIC2 than in PFIC1. These differences between PFIC1 and PFIC2 probably reflect the severe lobular injury in PFIC2 (4, 9, 10). PFIC2 is caused by mutations in the ABCB11 gene (designated BSEP) (8). The BSEP gene encodes the ATP-dependent canalicular BSEP of the liver and is located on human chromosome 2. The mechanism is shown in Fig. 2C.

PFIC3 can be distinguished from the other types of PFIC because it rarely presents with cholestatic jaundice in the neonatal period, and instead occurs later in infancy and childhood and even in young adulthood. The evolution of the cholestasis is characterized as chronic icteric or anicteric. However, adolescent and young adult patients have cirrhotic symptoms owing to portal hypertension that may result in liver failure. PFIC3 is caused by genetic mutations in the ABCB4 gene (designated MDR3) located on chromosome 7. MDR3 is a phospholipid translocase involved in biliary phospholipid (phosphatidylcholine) excretion and is predominantly expressed in the canalicular membrane of hepatocytes (31). Cholestasis results from toxicity of the bile, in which detergent BSs are not inactivated by phospholipids, thus leading to bile canaliculi and biliary epithelium injuries. A schematic mechanism for PFIC3 is proposed in Fig. 2D.

The mechanism of PFIC1 is still unclear. To our knowledge, the downregulation of cystic fibrosis transmembrane conductance regulator in cholangiocytes has been reported in PFIC1, and this downregulation could contribute to the impairment of bile secretion and explain some of the extrahepatic features (32). The FIC1 gene is expressed in various organs, including the liver, pancreas, small intestine, and kidney, but is more highly expressed in the small intestine than in the liver (33). Therefore, enterohepatic cycling of BS should be considered in PFIC1, and this is a possible explanation of our experiences that PFIC1 recipients easily showed steatosis/steatohepatitis even after LDLT and that PFIC2 recipients showed no steatosis/steatohepatitis after LDLT.

Only one mutated allele or no mutation is rarely identified in a few PFIC patients (< 10%) (1). Mutations that may map to regulatory sequences of the genes are a possible explanation for these findings. A gene involved in the transcription of the PFIC genes (i.e., FXR) or in protein trafficking could also be involved (34, 35). It cannot be negated that other unidentified genes involved in bile formation may be responsible for the PFIC1/2/3 phenotypes. Furthermore, it can be

hypothesized that combined heterozygous mutations for MDR3 and BSEP lead to PFIC-like phenotypes (36). Another possible explanation is that the mutated protein may have a dominant-negative effect on the expression and/or function of the protein in a heterozygous state (37). Modifier genes and environmental influences could play roles in the expression of PFIC (3). The possibility of recurrence of PFIC after LT owing to alloimmunization of the recipient against the FIC1, BSEP or MDR3 proteins of the liver donor remains a theoretical matter of debate (1). It is hypothesized that PFIC patients with a severe mutation leading to the absence of the gene product would be immunologically naive for the FIC1, BSEP or MDR3 gene products. Moreover, alloimmunization necessarily occurs after LT. Although evidence regarding this possible hypothesis after LT has not been reported (28, 38), a case of a PFIC2 patient who experienced an unexplained severe bout of pure hepatocellular cholestasis resembling PFIC2 after deceased-donor LT has been reported (1). In the case of LDLT based on donor relationships with parents, it can be expected that the heterozygous status of the liver allograft leads to a predisposition for developing lithiasis or cholestasis favored by immunosuppressive drugs (39) that may interfere with canalicular protein function, as reported in a PFIC2 patient (1). We consider that this possibility is very rare as there is only one previous report (1), and we performed LDLT in which the donor origins were parents in 10 of 11 recipients (91%) without PFIC recurrences and a previous series of LDLT for PFIC is already documented (38). Our data and a review of the mechanisms by which previous papers have demonstrated that PFIC2 is indicated for LT, including LDLT, as a definitive therapy, similar to other diseases indicated for LT, and also that the clinical courses and outcomes after LDLT are still not sufficient for PFIC1 recipients owing to early post-operative steatosis/fibrosis. Although PFIC1 patients will require LT during the long-term progression of the disease, we suggest that LDLT for PFIC1 may be reconsidered especially with regard to the timing of LDLT under the current donor shortage. Moreover, the establishment of alternative and/or anticipatory strategies for LDLT induction is needed to improve the long-term prognosis of PFIC1.

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Authors' contributions

T. Hori wrote the paper and performed this research. M. Ueda, F. Oike, Y. Ogura, S. Sakamoto, M. Kasahara, K. Ogawa, Y. Yonekawa, K.I. Watanabe and H. Doi provided important opinions based on their specialized experiences. A. Miyagawa-Hayashino performed histopathological examinations. F. Chen, A.M.T. Baine, and L.B. Gardner helped to perform this research. Prof. S. Uemoto, Prof. Y. Takada, Prof. H. Egawa and Prof. T. Yorifuji designed this research. Prof. S. Uemoto and Prof. J.H. Nguyen supervised this research.

Ethical approval

The protocol of this study was approved by the Ethics Review Committee for Clinical Studies of Kyoto University Graduate School of Medicine.

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Short Report

Maternal uniparental isodisomy and heterodisomy on chromosome 6 encompassing a *CUL7* gene mutation causing 3M syndrome

Sasaki K, Okamoto N, Kosaki K, Yorifuji T, Shimokawa O, Mishima H, Yoshiura K-i, Harada N. Maternal uniparental isodisomy and heterodisomy on chromosome 6 encompassing a *CUL7* gene mutation causing 3M syndrome.
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We report a case of segmental uniparental maternal hetero- and isodisomy involving the whole of chromosome 6 (mat-hUPD6 and mat-iUPD6) and a cullin 7 (*CUL7*) gene mutation in a Japanese patient with 3M syndrome. 3M syndrome is a rare autosomal recessive disorder characterized by severe pre- and postnatal growth retardation that was recently reported to involve mutations in the *CUL7* or obscurin-like 1 (*OBSL1*) genes. We encountered a patient with severe growth retardation, an inverted triangular gloomy face, an inverted triangle-shaped head, slender long bones, inguinal hernia, hydrocele testis, mild ventricular enlargement, and mild mental retardation. Sequence analysis of the *CUL7* gene of the patient revealed a homozygous missense mutation, c.2975G>C. Genotype analysis using a single nucleotide polymorphism array revealed two mat-hUPD and two mat-iUPD regions involving the whole of chromosome 6 and encompassing *CUL7*. 3M syndrome caused by complete paternal iUPD of chromosome 6 involving a *CUL7* mutation has been reported, but there have been no reports describing 3M syndrome with maternal UPD of chromosome 6. Our results represent a combination of iUPDs and hUPDs from maternal chromosome 6 involving a *CUL7* mutation causing 3M syndrome.

Conflict of interest

None of the authors of this paper declares a conflict of interest.

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Key words: 3M syndrome – cullin 7 (*CUL7*) – Genome-Wide Human SNP Array 6.0 (SNP6.0) – maternal uniparental disomy of chromosome 6 (matUPD6)

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3M syndrome is a rare inherited autosomal recessive disorder characterized by pre- and postnatal growth retardation, characteristic facial features, and skeletal anomalies. Clinical features of 3M syndrome include large head circumference, broad forehead, a triangular facial outline, dolichocephaly, long philtrum, short stature, short thorax and neck, tall vertebral bodies, and slender

long bones and ribs. Males with 3M syndrome occasionally have hypogonadism and hypospadias (1–9). However, intelligence is unaffected and karyotype is normal on conventional chromosome analysis.

In patients with 3M syndrome, disease-causing mutations have been identified in the cullin 7 (*CUL7*, MIM *609577) and obscurin-like 1 (*OBSL1*, MIM *610991) genes (7–9). Mutations of *CUL7* are the major cause of 3M syndrome, accounting for 80% of previously reported cases, whereas *OBSL1* accounts for 20% of cases (8, 10).

Uniparental disomy (UPD) is the transmission pattern of either two copies of the identical chromosome (uniparental isodisomy; iUPD) or of both homologous chromosomes (uniparental heterodisomy; hUPD) from one parent with no contribution from the other parent (11). Phenotypes that are clinically associated with paternal UPD of chromosome 6 (patUPD6) and genomic imprinting have been established, but because of the rarity of maternal UPD of chromosome 6 (matUPD6), clinical features have not yet been established. Here, we report a patient with a homozygous mutation in *CUL7* due to a maternal iUPD of chromosome 6 (mat-iUPD6).

Materials and methods

Clinical report

A Japanese male patient with 3M syndrome was examined in this study. The patient was delivered by caesarean section at 36 weeks of gestation without a family history of 3M syndrome (Fig. 1a). His birth weight was 1000 g (−4.8 SD), length 33.0 cm (−6.8 SD), head circumference 30.2 cm (−1.5 SD), and Apgar score 7/9. Feeding difficulty was noted during the neonatal period. He remained in a neonatal intensive care unit for 2 months and was referred to our group because of developmental delay and muscle hypotonia at 4 months. The patient displayed anomalies including hypospadias, inguinal hernia, hydrocele testis, inverted triangular gloomy face, malar hypoplasia, long eyelashes, epicanthal folds, short nose, anteverted nares, full lips, long philtrum, pointed chin, short chest, grooved lower anterior thorax, hypermobility of joints, and slender long bones (Fig. 1a,b). Mild ventricular enlargement was observed by neuroradiological studies. His growth was severely retarded.

At 2 years 9 months, his height, weight, and head circumference were 69.3 cm (−4.6 SD), 6.8 kg (−6.7 SD), and 48 cm (−1.2 SD), respectively. His head size was disproportionately large compared to his height. Thus the patient was diagnosed as suffering from 3M syndrome. He could understand simple sentences, but could not speak nor sit alone. Partial growth hormone (GH) deficiency was noted. GH replacement therapy was started from 2 years. GH was effective without side effects. At 5 years, his height and weight were 84.8 cm (−5.9 SD) and 10 kg (−3 SD), respectively. He was moderately mentally retarded.

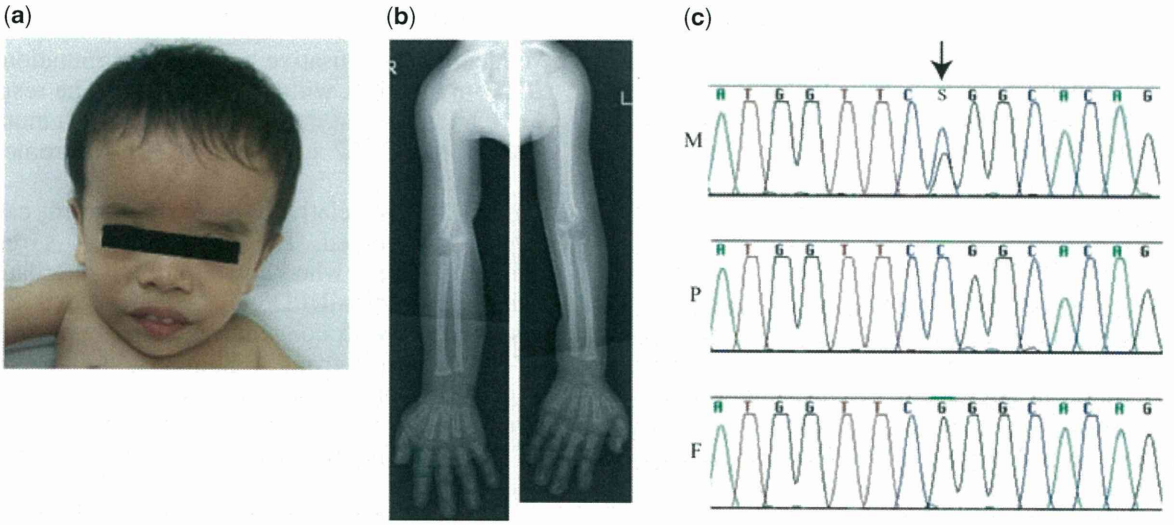

Fig. 1. Facial and skeletal features of the patient at 2 years 7 months of age. (**a**) Note the inverted triangular gloomy face, short nose, full lips, and long philtrum. (**b**) Note the slender long bones. (**c**) Electropherograms of the patient and parents. DNA sequence showing a single base change substituting cytosine for guanine, which results in p.R992P, in the patient. M, mother; P, patient; and F, father.

Conventional cytogenetic studies and FISH analysis

We obtained blood samples under written informed consent for participation in this study. Conventional cytogenetic examination of G-banded chromosomes from peripheral blood lymphocytes was performed. We also performed fluorescence *in situ* hybridization (FISH) analysis on lymphocyte metaphase spreads from the patient using two Bacterial Artificial Chromosome (BAC) clones containing *CUL7*, RP11-628J2 and RP11-653G5, as probes.

Genomic sequencing

Genomic DNA was extracted from peripheral blood following standard protocols. For mutation analysis, we designed primers to amplify all the coding exons of *CUL7* and the flanking intron sequences. Direct sequencing was carried out using a BigDye Terminator v3.1 Cycle sequencing Kit™ and separated on a Genetic Analyzer 3130*xl* (Applied Biosystems Inc., Foster City, CA). Sequence electropherograms were aligned with SEQUENCHER™ software (Gendcodes, Ann Arbor, MI) to visually inspect base alterations.

Microarray analysis

We performed genome-wide single nucleotide polymorphism (SNP) genotyping using Genome-Wide Human SNP Array 6.0 (SNP6.0) following the manufacturer's instructions (Affymetrix, Santa Clara, CA, http://www.affymetrix.com/index.affx). The data generated from Genotyping Console (GTC) 4.0 were loaded into CHROMOSOME ANALYSIS SUITE (CHAS) 1.0.1 software to display the results. We carried out UPD analyses of the patient using genotype data in trio. Genomic positions of SNPs corresponded to the March 2006 human genome (hg18).

Results

Genomic sequencing

We sequenced all 26 coding exons and flanking intronic regions of the *CUL7* gene, which spans a genomic region of approximately 16.3 kb, in the family. In the patient, we detected a homozygous missense mutation (c.2975G>C) in exon 15, which resulted in the substitution of proline for arginine at amino acid residue 992 (p.R992P) (Fig. 1c). The mother was a heterozygous carrier of the mutation, whereas the father was homozygous for the wild-type allele (Fig. 1c). The p.R992P mutation was not detected in 100 unrelated control individuals.

Conventional and molecular cytogenetic analyses

G-banding and FISH analysis at the *CUL7* locus showed a normal karyotype in the patient and the parents with no microdeletion at *CUL7* locus in the patient (data not shown).

Microarray analysis

To confirm paternity, and to find a small size deletion, we performed SNP6.0 analysis. However, no copy number variations (CNVs) were identified in the region containing both *CUL7* and *OBSL1* genes (Fig. 2a). The other variants overlap with reported regions of CNVs in the Database of Genomic Variants (http://projects.tcag.ca/variation) or were transmitted from the parents (data not shown).

To confirm matUPD6 in the patient, we examined the genotypes of the patient/father/mother trio. The results using informative markers indicated that there were two maternal heterodisomic regions (hUPD6-1 and hUPD6-2) and two maternal isodisomic regions (iUDP6-1 and iUPD6-2) in chromosome 6, respectively (Fig. 2 and Table 1). The results indicated that the patient had inherited two alleles from his mother, but none from his father, in chromosome 6. The final karyotype of this patient was 46,XY,upd(6)mat and arr 6p25.3p22.3(110,391–16,287,166)×2 htz mat,6p 22.3q12(16,290,223–65,796,893)×2 hmz mat,6q 12q25.1(65,799,990–150,517,779)×2 htz mat,6q 25.1q27(150,518,012–170,759,956)×2 hmz mat.

Discussion

We identified a causative homozygous mutation in *CUL7* in a patient with 3M syndrome. The results clearly indicate that mat-iUPD6 involving a mutant allele of the *CUL7* gene caused 3M syndrome in the patient.

matUPD6 is relatively rare and seven cases have been reported. The first case was a renal transplant patient who showed growth retardation at birth and mat-iUPD6 (12). The second case was a patient with congenital adrenal hyperplasia (CAH) resulting from a homozygous mutation in the 21-hydroxylase gene (*CYP21*), and had intrauterine growth retardation (IUGR) and mat-iUPD6 (13). The third case was a macerated male fetus from a pregnancy terminated at 23 weeks of gestation because of intrauterine death. The patient showed a mosaic trisomy 6 (14). The fourth case was a male patient with two clinical phenotypes, Klinefelter's syndrome and CAH. His karyotype was mosaic 48,XXY, +mar[30]/47,XXY[20] and

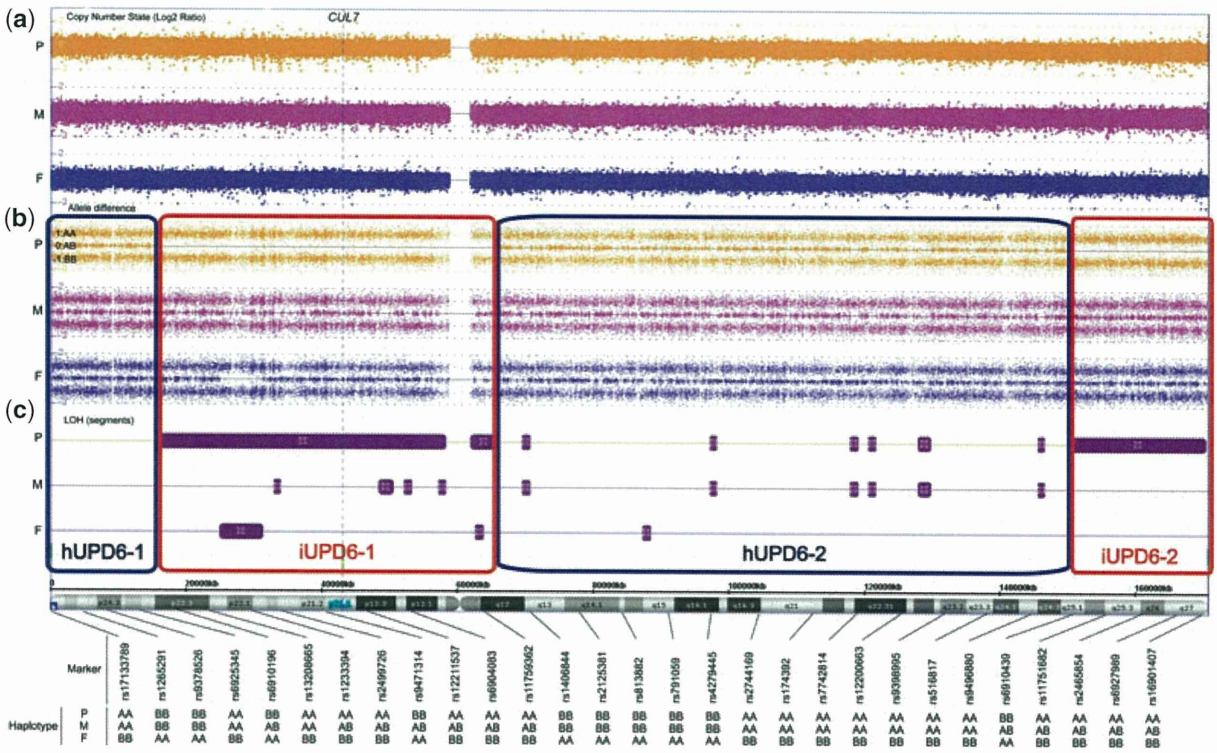

Fig. 2. SNP6.0 data. (**a**) Plots of the SNP6.0 data displayed in ChAS 1.0.1 showing the log2 ratio plot of copy number state, allele difference plot, and loss of heterozygosity (LOH) segment (purple box) (P, patient; M, mother; and F, father). (**b**) The allele difference graph represents the genotypes for each individual single nucleotide polymorphism (SNP). Dots with a value of 1, −1, and 0 represent SNPs with AA, BB, and AB genotypes, respectively. A vertical dashed line indicates the *CUL7* locus. (**c**) The LOH segment plot indicates nine LOH regions on chromosome 6. iUPD6-1 and iUPD6-2 denote the regions of uniparental isodisomy (red box). hUPD6-1 and hUPD6-2 denote the regions of uniparental heterodisomy (blue box). The genotypes on chromosome 6 indicate maternal heterodisomy or isodisomy in the affected offspring [only the uniparental disomy (UPD) markers are displayed].

Table 1. Examination of SNPs from a patient/father/mother trio[a]

			hUPD6-1	iUPD6-1	hUPD6-2	iUPD6-2
Genotype of trio (patient/father/mother)	iUPD	AA/BB/AB	0	534	0	318
		BB/AA/AB	0	576	3	304
	iUPD or hUPD	AA/BB/AA	178	543	605	272
		BB/AA/BB	196	506	563	262
Share genotype (patient/mother)	iUPD or hUPD	AA/AA	2,812	5,897	9,716	3,009
		BB/BB	2,799	5,785	9,557	2,919
	hUPD	AB/AB	1,699	19	6,384	12
		Total of share genotype	7,310	11,701	25,657	5,940
		Share genotype rate (%)	99.82	78.20	99.89	73.31
		Total SNP probe	7,323	14,963	25,684	8,103
		Start SNP	rs4959515	rs9370869	rs9354209	rs9384189
		Start position	110,391	16,290,223	65,799,990	150,518,012
		End SNP	rs9477050	rs9453156	rs7765984	rs6931065
		End position	16,287,166	65,796,893	150,517,779	170,759,956
		Size (bp)	16,176,776	49,506,671	84,717,790	20,241,945
		Cytoband	p25.3-p22.3	p22.3-q12	q12-q25.1	q25.1-q27

hUPD, uniparental heterodisomy; iUPD, uniparental isodisomy; iUPD or hUPD, UPD could not be defined as isodisomy or heterodisomy; SNP, single nucleotide polymorphism.
[a]Each row contains information on each matUPD6 inheritance block identified by trio haplotype analysis.