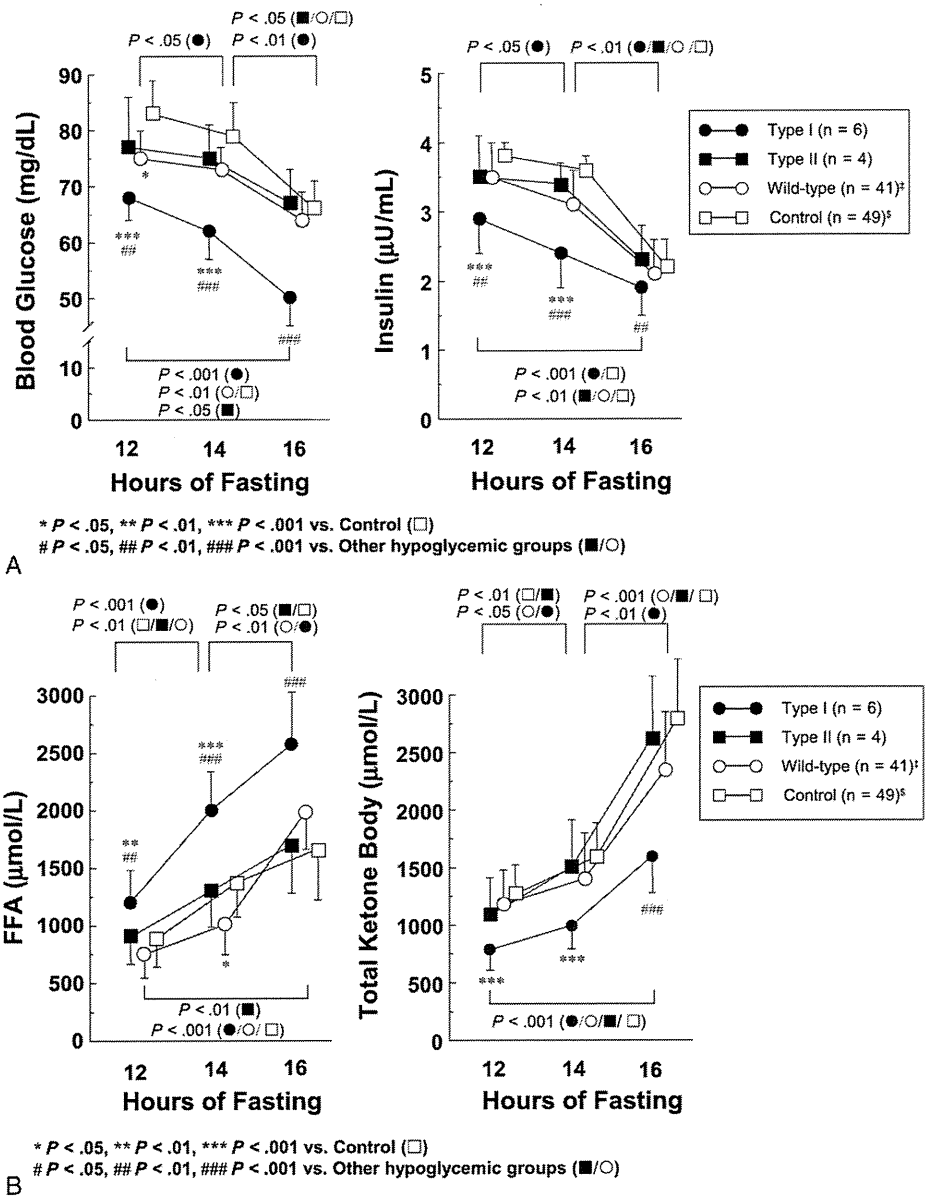

Fig. 2. Differences in BG, insulin, FFAs, and TKB concentrations at the 12-, 14-, and 16-hour fasting points. Children were not allowed to ingest any food or drink after supper on the day before blood sampling. #Data obtained from 24 of the 41 wild-type group children are presented. $Data obtained from 17 of the 49 control group children are presented.

After 16 hours of fasting, BG concentrations in the type I CD36 deficiency group were less than 50 mg/dL. Serum insulin concentrations showed results similar to those of BG concentrations (Fig. 2A, right panel).

The FFA concentrations were always significantly higher in the type I CD36 group than in the other 3 groups (Fig. 2B, left panel). The ΔFFA between 12 and 14 hours was larger in the type I CD36 deficiency group ($P < .001$) than in the other 3 groups ($P < .01$).

The TKB concentrations were always lower in the type I CD36 group than in the other 3 groups. The last 2 hours of fasting induced a great increase in TKB in all the groups. However, the ΔTKB was significantly smaller in the type I CD36 group ($P < .01$) than in the other groups ($P < .001$) (Fig. 2B, right panel).

Unlike type I CD36 group, these parameter levels in the type II CD36 group were consistently similar to those in the wild-type and control groups and did not show any significant differences.

At any fasting point, serum lipid concentrations were not significantly different among the groups. Triglyceride concentration but not other lipid concentrations exhibited

Table 2
Serum lipid concentrations at 12, 14, and 16 hours of fasting in the hypoglycemic and control groups

Group	Hypoglycemic group (n = 51)									Controls (n = 49)		
Subgroup	Type I (n = 6)			Type II (n = 4)			Wild type (n = 41)					
Hours of fasting	12	14	16	12	14	16	12	14	16[a]	12	14	16[b]
TC	152 ± 20	149 ± 22	144 ± 19	155 ± 17	151 ± 20	147 ± 22	142 ± 11	138 ± 12	137 ± 10	151 ± 13	149 ± 12	145 ± 8
TG	53 ± 15	49 ± 9	44 ± 12†	56 ± 17	49 ± 15	47 ± 15*	51 ± 12	47 ± 10	44 ± 10†	51 ± 10	47 ± 10	45 ± 8†
HDL-C	56 ± 12	56 ± 11	54 ± 9	55 ± 10	57 ± 9	55 ± 9	56 ± 10	55 ± 10	56 ± 11	56 ± 14	55 ± 13	55 ± 12
LDL-C	85 ± 13	82 ± 11	81 ± 11	89 ± 16	83 ± 14	81 ± 15	80 ± 19	77 ± 15	74 ± 15	80 ± 13	78 ± 11	77 ± 11

Values are mean ± SD (in milligrams per deciliter). At any fasting point, there were no significant differences in lipid concentrations between each 2 groups (Mann-Whitney *U* test). Triglyceride concentrations but not other lipid concentrations exhibited significant changes between the 12- and 16-hour fasting points in respective groups:*$P < .05$ and †$P < .01$ (1-factor analysis of variance test). There were no significant differences in changes in lipid concentrations (Δ values) between 2 fasting points among the groups (Mann-Whitney *U* test). TC indicates total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol.

[a] Data obtained from 24 of the 41 wild-type children were presented.

[b] Data obtained from 17 of the 49 controls were presented.

significant changes between the 12- and 16-hour fasting points in the respective groups (Table 2).

4. Discussion

The present study showed that fasting BG concentration was lower in preschool children of type I CD36 deficiency than in children of type II/wild-type CD36 hypoglycemic groups and control children.

Type I CD36 deficiency accounted for 11.8% of 51 children with histories of hypoglycemia but was not diagnosed in the 49 age-matched control children without histories of hypoglycemia. On the other hand, the prevalence of type II CD36 deficiency was not different between the hypoglycemic and control children. According to earlier reports, the prevalence of type I CD36 deficiency in the general Japanese population is 0.5% to 1.0% [9-10,15]. It is, therefore, plausible that this deficiency is highly prevalent among hypoglycemic children. However, the children groups enrolled in this study were too small to define the reliable prevalence. To gain more informative data, more extensive studies would be essential.

Besides fasting BG level, fasting insulin, TKB, and FFA concentrations in type I CD36 group were different from those in the other groups. We found that fasting insulin and TKB concentrations were significantly lower in the type I group than in the other groups, whereas the fasting FFA level was significantly higher in the type I group. These differences were more prominent as the fasting time was extended (Fig. 2A, B).

High FFA levels at the fasting state can probably be attributed to impaired FFA uptake by skeletal and heart muscles in type I CD36 deficiency. Free FA is used as an energy source in skeletal and heart muscles, as well as in the liver. Long-chain FAs enter cells either by passive diffusion or by transporter-dependent uptake. In humans, there are 3 transporters for LCFA: CD36, plasma membrane-associated FA binding protein (43 kd), and FA transport protein (FATP, 63 kd) [23–25]. Although plasma membrane-associated FA binding protein and FATP are expressed in the liver as well as skeletal and heart muscles, CD36 is expressed in skeletal and heart muscles, but not in the liver. Most serum LCFAs are bound to albumin. CD36 promotes the dissociation of LCFA from albumin, which results in increased passive diffusion of LCFA [24]. Furthermore, CD36 is coexpressed with FATP in skeletal and heart muscles [24]. This colocalization strongly suggests that the FATP-mediated uptake of LCFA is promoted by an interaction with CD36 in skeletal and heart muscles. In patients with type I CD36 deficiency, the radiolabeled LCFA analog ^{123}I-15-(*p*-idiophenyl)-(*R,S*)-methylpentadecanoic acid (BMIPP) does not accumulate in heart muscles; and BMIPP clearance from the circulation is delayed [5]. Furthermore, the oxidation rate of LCFA is reduced by 40% to 60% in the isolated hearts of CD36-deficient mice [25]. These observations agree well with our hypothesis.

In skeletal and heart muscles, impaired FA uptake in CD36 deficiency is probably compensated by shifting the energy supply from FA dominant to glucose dominant. In fact, CD36-deficient rats had high glucose oxidation rates and maintained normal myocardial functions [26]. If this is the case in humans, it would be a rationale for the increased susceptibility of CD36-deficient children to hypoglycemia in the fasting state. Similar to CD36-deficient children, CD36-deficient rats had higher serum FFA concentrations; and their BG concentrations were lower than those observed in control rats [27].

Nevertheless, the findings obtained from this study were not consistent with those in CD36-deficient adults described in earlier reports [10-13]. It has been shown that CD36 deficiency predisposes one to insulin resistance and the subsequent hyperglycemia. Most of the subjects described in such reports were older than 30 years, and young subjects have been scarcely examined. Yanai et al [14] previously reported increased insulin sensitivity in young adults with CD36 deficiency, suggesting different effects of CD36 deficiency on carbohydrate metabolism by age. To gain a

better understanding of the relationship between CD36 deficiency and insulin sensitivity, we will perform more extensive studies covering large groups of children and adolescents.

The energy demands of young children exhibiting rapid growth and development are quite high. Their glucose and FA metabolisms must be greatly activated. In fact, ketone body synthesis via FA β-oxidation is far greater in young children than in adults [19,28]. Therefore, impaired FA metabolism must have a more serious effect in children than in adults, although both have the same genetic disorder (CD36 deficiency).

The reason for the low TKB levels in type I CD36 deficiency remains to be elucidated. Ketone bodies are generated mainly in the liver and secreted into the blood circulation. The substrate of ketone bodies is acetyl–coenzyme A, which is a product of FA β-oxidation. From this context, we speculated that decreased FA β-oxidation in heart and muscles led to the limited synthesis of ketone body from acetyl–coenzyme A. In type I CD36 deficiency, hepatic uptake of BMIPP (an analog of LCFA) is nearly double [5], probably because CD36 is naturally absent in normal liver and the other transporters play significant roles in hepatic uptake of LCFA [23-25]. As a whole, ketone body synthesis along with FA β-oxidation in type I CD36 deficiency was substantially but not definitely reduced.

The results of this study suggested that type I CD36 deficiency predisposes preschool children to hypoglycemia. If this is true, affected children should avoid long-time fasting and excess exercise without sugar supplementation.

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Living-donor Liver Transplantation for Progressive Familial Intrahepatic Cholestasis

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Abstract
Background Progressive familial intrahepatic cholestasis (PFIC) results in liver cirrhosis during the disease course, although the etiology includes unknown mechanisms. Some PFIC patients require liver transplantation (LT).
Methods In this study, 11 patients with PFIC type 1 (PFIC1) and 3 patients with PFIC type 2 (PFIC2) who underwent living-donor LT (LDLT) were evaluated.
Results Digestive symptoms after LDLT were confirmed in 10 PFIC1 recipients (90.9%); 8 PFIC1 recipients showed steatosis after LDLT (72.7%), which began during the early postoperative period (71.5 ± 55.1 days). Seven of the eight steatosis-positive PFIC1 recipients (87.5%) showed a steatosis degree of ≥80%, which was complicated with steatohepatitis and resulted in fibrosis. Cirrhotic findings persisted in six PFIC1 recipients even after LDLT (54.5%), and three PFIC1 recipients finally died. The survival rates of the PFIC1 recipients at 5, 10, and 15 years were 90.9%, 72.7%, and 54.5%, respectively. In contrast, the PFIC2 recipients showed good courses and outcomes without any steatosis after LDLT.
Conclusions The clinical courses and outcomes after LDLT are still not sufficient in PFIC1 recipients owing to steatosis/steatohepatitis and subsequent fibrosis, in contrast to PFIC2 recipients. PFIC2 is good indication for LDLT. PFIC1 patients require LT during the disease course; therefore, we suggest that the therapeutic strategies for PFIC1 patients, including the timing of LDLT, under the donor limitation should be reconsidered. The establishment of more advanced treatments for PFIC1 patients is required to improve the long-term prognosis of these patients.

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Introduction

Progressive familial intrahepatic cholestasis (PFIC) refers to a heterogeneous group of autosomal recessive disorders of childhood that disrupt bile formation and present with cholestasis. PFIC is a rare disease, with an estimated incidence of 1 per 50,000–100,000 births [1]. Cholestasis of hepatocellular origin is the major sign in PFIC. The cholestasis appears within the first year of life and leads to death from liver failure at ages from infancy to adolescence [2, 3]. Although the etiology of PFIC still involves unknown mechanisms, the natural course of PFIC causes portal hypertension, liver failure, cirrhosis, carcinoma, and extrahepatic disorders.

PFIC is classified into three types as follows: (1) deficiency of familial intrahepatic cholestasis 1 (FIC1); (2) deficiency of bile salt export pump (BSEP); (3) deficiency of multidrug-resistant 3 (MDR3). Mutations in these genes

are related to the hepatocellular transport system involved in bile formation. The clinical, biochemical, radiological, and histological manifestations of each type have been described previously [1–11].

In PFIC type 1 (PFIC1) patients, cholestasis appears during the first months of life and causes recurrent episodes of jaundice that eventually become permanent. Severe pruritus is observed. The serum γ-glutamyltransferase (γ-GT) and cholesterol levels are normal, but the bile acid (BA) concentration is high. The hepatic histopathology is characterized by canalicular cholestasis and the absence of true ductular proliferation. PFIC1 is caused by mutations in the *ATP8B1* gene, which is designated FIC1 [6–12]. FIC1 is expressed in the liver, pancreas, small intestine, and kidney. The FIC1 protein is located on the canalicular membrane of hepatocytes [13–15]. FIC1 is more highly expressed in the small intestine than in the liver [12]. Taken together, these events lead to BA overload in hepatocytes, impaired bile secretion in cholangiocytes, and extrahepatic features in the intestine [1, 6, 14, 16]. Extrahepatic symptoms (persistent short stature, sensorineural deafness, watery diarrhea, pancreatitis, elevated sweat electrolyte concentration) have been confirmed in PFIC1 patients [9], and enterohepatic circulation should be considered in PFIC1.

Cholestasis with permanent jaundice is more severe in PFIC type 2 (PFIC2) patients than in those with the other PFIC types, although PFIC2 patients share similar laboratory findings with PFIC1 patients. The initial evolution of cholestasis appears during the first months of life and rapidly results in liver failure within the first few years of life. More severe pruritus is observed. The histopathological findings reveal more perturbed liver architecture than is seen in PFIC1, with more pronounced lobular and portal fibrosis and inflammation [2, 8, 9]. PFIC2 is caused by mutations in the *ABCB11* gene, which is designated BSEP [7, 17]. This gene encodes the ATP-dependent canalicular BSEP of the liver. The BSEP protein, which is expressed at the hepatocyte canalicular membrane, is the major exporter of primary BA against extreme concentration gradients. Mutations in this gene are responsible for decreased the secretion of bile salts (BSs), leading to decreased bile flow and accumulation of BSs inside the hepatocytes, which results in severe hepatocellular damage. Extrahepatic features have not been documented in PFIC2. However, hepatocellular carcinoma (HCC) and cholangiocarcinoma occurs at a considerable rate (15%) before 1 year of age [18, 19].

PFIC type 3 (PFIC3) patients show high γ-GT, normal cholesterol, and slightly elevated BA levels. PFIC3 can be distinguished from the other PFICs, because it rarely appears during the neonatal period but manifests during infancy, childhood, and even young adulthood [11, 20]. Pruritus is mild, and the evolution of cholestasis is chronic icteric or anicteric. Therapy with ursodeoxycholic acid (UDCA) may be especially effective for PFIC3 [1, 11, 21].

Regardless of the various types, PFIC patients develop hepatic failure and liver cirrhosis during the disease course. Therefore, it is currently justified that PFIC patients undergo liver transplantation (LT). Here, we present our results for PFIC patients after living-donor LT (LDLT) during two decades and discuss therapeutic strategies for PFIC patients.

Patients and methods

Patients

Since 1990, a total of 735 adult and 702 pediatric recipients underwent LT at Kyoto University Hospital. In all, 717 LDLT recipients whose ages at LDLT were <20 years were enrolled in this study. Among the LDLT recipients, 11 PFIC1 and 3 PFIC2 recipients were evaluated (Table 1); there were no LDLTs in PFIC3 patients. The Ethics Review Committee for Clinical Studies at Kyoto University Graduate School of Medicine approved the study protocol.

The 14 PFIC patients comprised five males and nine females, and their age range at LDLT was 0.6–18.2 years. The mean times from the diagnosis of PFIC to LDLT were 3.89 ± 5.63 years (range 0.21–16.3 years) for the PFIC1 recipients and 0.79 ± 0.75 years (range 0.12–1.60 years) for the PFIC2 recipients. The standard deviation (SD) values for height and body weight at LDLT were −4.5 ± 1.8 (range −7.5 to -1.1) and −2.1 ± 1.0 (range −3.5–0.3), respectively. Growth retardation was confirmed in all patients. One PFIC1 patient (case 5) had a past history of paroxysmal atrial fibrillation.

The serum total BA level was elevated to 439.1 ± 109.8 μmol/ml (range 299–600 μmol/ml), and the γ-GT level 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 (ages ≥12 years) or Pediatric End-stage Liver Disease (ages <12 years) was 12.3 ± 4.1 points (range 5–19 points). The preoperative statuses were 11 cases of at home and 3 cases of hospitalization. The United Network for Organ Sharing statuses were estimated to be 12 cases of status III and two cases of status IIB.

The donor relationships were 10 fathers, 3 mothers, and 1 grandmother. The mean donor age was 36.9 ± 7.1 years (range 28–47 years). The mean body mass index (BMI) in the donors was 22.3 ± 1.0 kg/m^2 (range 20.5–23.6 kg/m^2). One donor (case 8) was hepatitis B surface antibody (HBsAb)-positive. The ABO blood groups were

Table 1 Histopathological findings after LDLT

Case no.	Steatosis	Steatohepatitis	Fibrosis score (F)[a]	Other factors for fibrosis
PFIC type 1				
1	Severe	Yes	3	–
2	None	No	4	De novo AIH
3	Severe	Yes	4	–
4	None	No	0	–
5	Severe	Yes	3	–
6	Severe	Yes	3	–
7	Moderate	Yes	4	–
8	Severe	No	0	–
9	Severe	Yes	3	–
10	None	No	4	Chronic rejection
11	Severe	Yes	1	–
PFIC type 2				
12	None	Yes	0	–
13	None	Yes	0	–
14	None	Yes	0	–

AIH Autoimmune hepatitis, *LDLT* living-donor liver transplantation, *NASH* nonalcoholic steatohepatitis, *PFIC* progressive familial intrahepatic cholestasis

[a] The NASH score was used in steatosis-positive recipients. The Metavir score was used in steatosis-negative recipients

characterized as 11 cases identical, 2 cases compatible and 1 case incompatible (case 13). The results of lymphocyte crossmatches were negative.

Operation

There were 12 lateral-segment grafts and one case each of extended lateral-segment and left-lobe 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 operating 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 ischemia times were 51.0 ± 29.4 min (range 15–99 min) and 35.9 ± 11.6 min (range 24–56 min), respectively. Biliary reconstruction at the initial LDLT was done by hepaticojejunostomy in 12 cases and by duct-to-duct reconstruction in 2 cases (cases 8 and 14). Histopathologically, cirrhosis without steatosis was confirmed in all of the native livers.

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 postoperative period based on the clinical findings in each case. Methylprednisolone was given intravenously (1 mg/kg) once daily from postoperative day (POD) 1 to POD 3 followed by 0.5 mg/kg once daily for the next 3 days. On POD 7, methylprednisolone 0.3 mg/kg was given intravenously. Steroid administration was switched to oral prednisolone 0.3 mg/kg once daily on POD 8. Our regimens for ABO incompatibility were described previously [22, 23].

Histopathological analysis

In our institution, laboratory and ultrasonography (US) examinations are performed routinely after LDLT in all recipients. A liver needle biopsy (LNB) was performed if required based on the results of conventional liver function tests, findings of Doppler US, and consideration of the original diseases. All LNB specimens were strictly assessed by experienced pathologists.

All liver tissues were fixed in neutral-buffered formalin, embedded in paraffin, and sliced into 4 μm thick sections. The morphological characteristics were assessed after standard hematoxylin-eosin (H&E) staining, and hepatic fibrosis was reconfirmed by Masson trichrome and reticulin staining.

Posttransplant steatosis was evaluated as the percentage of hepatocytes involved in steatosis in the liver tissue [24]. Macrovesicular steatosis was graded semiquantitatively according to the percentage of involved hepatocytes as follows [24]: mild <30% of hepatocytes; moderate 30% to 60% of hepatocytes; severe >60% of hepatocytes. The diagnosis of steatohepatitis was defined according to any degree of steatosis, hepatocellular injury in the form of ballooning degeneration and/or Mallory's hyaline, mononuclear and polymorphonuclear infiltration, perisinusoidal fibrosis and portal/lobular inflammation. The fibrosis scores were strictly estimated based on the presence or absence of posttransplant steatosis. Estimation of the hepatic venous area is important at the early phase

of fibrosis progression in nonalcoholic steatohepatitis (NASH) [25], although the fibrosis in other types of hepatitis initially occurs in the periportal area. For assessing posttransplant 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 [25]: 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 [26]: 1, periportal fibrosis; 2, bridging fibrosis; 3, precirrhosis; 4, cirrhosis.

Statistical analysis

The survival rates were calculated by the Kaplan–Meier method, with a log-rank test. Statistical analyses were performed using SPSS Software Version 16.0 (SPSS, Chicago, IL, USA).

Results

Clinical course after LDLT

The mean hospital stay after LDLT was 70.7 ± 42.8 days (range 29–189 days). Viral infections and rejection, mainly during the early postoperative period, remain major complications [27]. Epstein-Barr virus and cytomegalovirus infections were detected after LDLT in 6 of 14 PFIC recipients (cases 5, 6, 8, 11–13) and were successfully treated. In all, 7 of the 14 PFIC recipients showed acute cellular rejection (ACR) after LDLT (cases 2, 4, 6, 11–14). Venous and biliary complications remain important [28, 29], and three recipients had stenosis of the hepatic vein or bile duct after LDLT (cases 1, 5, 7). These complications were successfully treated by interventional radiology or reconstruction as soon as possible after their detection.

Digestive symptoms after LDLT were confirmed in 10 of 11 PFIC1 recipients (90.9%) but were not encountered in any of the PFIC2 recipients. Cirrhotic findings including esophageal varix and splenomegaly (the longest diameter was >15 cm on imaging studies) even after LDLT were confirmed in 6 of the 11 PFIC1 recipients (54.5%). These PFIC1 recipients (cases 2, 3, 5, 7, 8, 10) underwent endoscopic or surgical therapy for esophageal varix and splenomegaly, including endoscopic injection sclerotherapy, endoscopic variceal ligation, and splenectomy. One PFIC1 recipient (case 2) suffered from de novo autoimmune hepatitis (AIH) and has been closely followed. Among the PFIC2 recipients, one recipient (case 14) received steroid pulse therapy and muromonab-CD3 therapy for refractory ACR during the early postoperative period, and the therapy was successful. The complications after LDLT are summarized in Table 2.

Histopathological findings after LDLT

Most PFIC patients underwent LNBs at intervals of 1–2 years after LDLT and histopathological follow-up according to these LNBs, although our institution does not employ a protocol biopsy. The mean number of LNBs after LDLT was 8.3 ± 5.1 times/recipient (range 3–23 times/recipient). The histopathological findings are summarized in Table 1.

Steatosis and steatohepatitis in the transplanted liver allografts

In all, 8 of 11 PFIC1 recipients exhibited steatosis after LDLT (72.7%); no steatosis was detected in the remaining 3 PFIC1 recipients. The changes in the degree of steatosis after LDLT in each case are shown in Fig. 1. Steatosis after LDLT in the steatosis-positive PFIC1 recipients seemed to begin during the early postoperative period, as the mean time to the initial confirmation of any steatosis was 71.5 ± 55.1 days after LDLT (range 21–191 days). Seven of the eight steatosis-positive PFIC1 recipients (87.5%) had ≥80% steatosis. The mean postoperative day for the steatosis to reach its peak among the steatosis-positive recipients was 229.6 ± 253.7 days (range 21–736 days). Seven of the eight steatosis-positive PFIC1 recipients had the complication of steatohepatitis (87.5%). In contrast, the PFIC2 recipients did not show any steatosis (Fig. 1).

Hepatic fibrosis in the transplanted allografts

Altogether, 9 of the 11 PFIC1 recipients exhibited fibrosis after LDLT, whereas it was not detected in the remaining 2 PFIC1 recipients. Two of the nine fibrosis-positive PFIC1 recipients (cases 2, 10) exhibited fibrosis without steatosis for other reasons (de novo AIH and chronic rejection, respectively). Only one PFIC1 recipient (case 4) had no steatosis or fibrosis, and another PFIC1 recipient (case 8) had steatosis but no fibrosis (F). Seven of the eight steatosis-positive PFIC1 recipients (87.5%) had F scores of ≥3, although one case stayed at F = 1 (case 11). The mean postoperative day for the F score to reach its peak among the eight steatosis-positive PFIC1 recipients was 1342.7 ± 1168.9 days (range 34–3254 days). The changes in the fibrosis scores after LDLT in each case are shown in Fig. 2. The initial confirmation of any fibrosis after

Table 2 Clinical courses and outcomes after LDLT

Case no.	Digestive symptoms	Complications (POD—treatment)	Outcome (POD)
1	Yes	Biliary stenosis (POD 3962—IVR)	Alive (6884)
2	Yes	ACR (moderate, PODs 100 and 2592—SPT)	Alive (6604)
		De novo AIH (POD 913—steroid)	
		Esophageal varices (POD 2546—EVL)	
3	Yes	Esophageal varices (POD 1624—EVL, EIS)	Dead (5032)
		Splenomegaly (POD 2595—splenectomy)	
		Rupture of splenic artery (POD 5032—hemostasis)	
4	Yes	Intraperitoneal bleeding (PODs 4 and 5—hemostasis)	Alive (5605)
		ACR (mild, PODs 13 and 2595—SPT)	
		Bad compliance of medicine and alcohol drinking	
5	Yes	EBV infection (POD 21—acyclovir)	Dead (4671)
		Bad compliance of medicine	
		Esophageal varices (POD 3529—EVL)	
		Splenomegaly (POD 3864—splenectomy)	
		Fatal dysrhythmia, myocarditis after re-LDLT on POD 4646 (POD 4671)	
		Biliary stenosis (POD 124—reconstruction)	
6	Yes	Cytomegalovirus infection (POD 136—ganciclovir)	Alive (4295)
		ACR (moderate, POD 140—SPT)	
		Intraperitoneal bleeding (POD 2—hemostasis)	
7	Yes	Stenosis of hepatic vein (POD 191—IVR)	Alive (4065)
		Splenomegaly (POD 1806—splenectomy)	
		Biliary stenosis (POD 1836—IVR)	
		Biliary stenosis (POD 48—reconstruction)	
8	No	Cytomegalovirus infection (POD 65—ganciclovir)	Alive (3384)
		Esophageal varices (POD 720—EIS)	
9	Yes	–	Alive (3265)
10	Yes	Chronic rejection (POD 182—Re-LDLT on POD 1393)	Dead (2005)
		Arterioportal shunt (POD 1825—Re-LDLT on POD 1986)	
		Rupture of esophageal varices (POD 2004—hemostasis)	
11	11	Cytomegalovirus infection (POD 33—ganciclovir)	Alive (2028)
		ACR (mild, POD 23—SPT)	
12	No	ACR (mild, POD 13—SPT)	Alive (2453)
		EBV infection (POD 27—acyclovir)	
		Cytomegalovirus infection (POD 34—ganciclovir)	
13	No	ACR (moderate, POD 14—SPT)	Alive (1601)
		EBV and EBV hepatitis (POD 34—acyclovir)	
		Cytomegalovirus infection (POD 103—ganciclovir)	
14	No	Refractory ACR (severe, PODs 7, 14, and 24—SPT and muromonab-CD3)	Alive (500)

The postoperative days (PODs) are shown as the days after the initial LDLT

ACR Acute cellular rejection, *EBV* Epstein-Barr virus, *EIS* endoscopic injection sclerotherapy, *EVL* endoscopic variceal ligation, *IVR* interventional radiology, *LNB* liver needle biopsy, *SPT* steroid pulse therapy

LDLT in the eight steatosis-positive PFIC1 recipients was 327.8 ± 353.4 days (range 34–932 days). As an example, the histopathological findings in case 6 are shown in Fig. 3. In contrast, the PFIC2 recipients did not exhibit any fibrosis (Fig. 2), although one recipient (case 14) temporarily showed an F score of 1 at PODs 39 and 47 owing to refractory ACR that was successfully treated.

Treatment for PFIC recipients after LDLT

All the PFIC1 recipients received UDCA therapy. Therapy with a BA adsorptive resin for PFIC1 recipients has been introduced in our institution [18], and 7 of 11 PFIC1 patients (cases 1, 2, 5–7, 9, 11) received this treatment combined with supplementations of pancreatic enzymes,

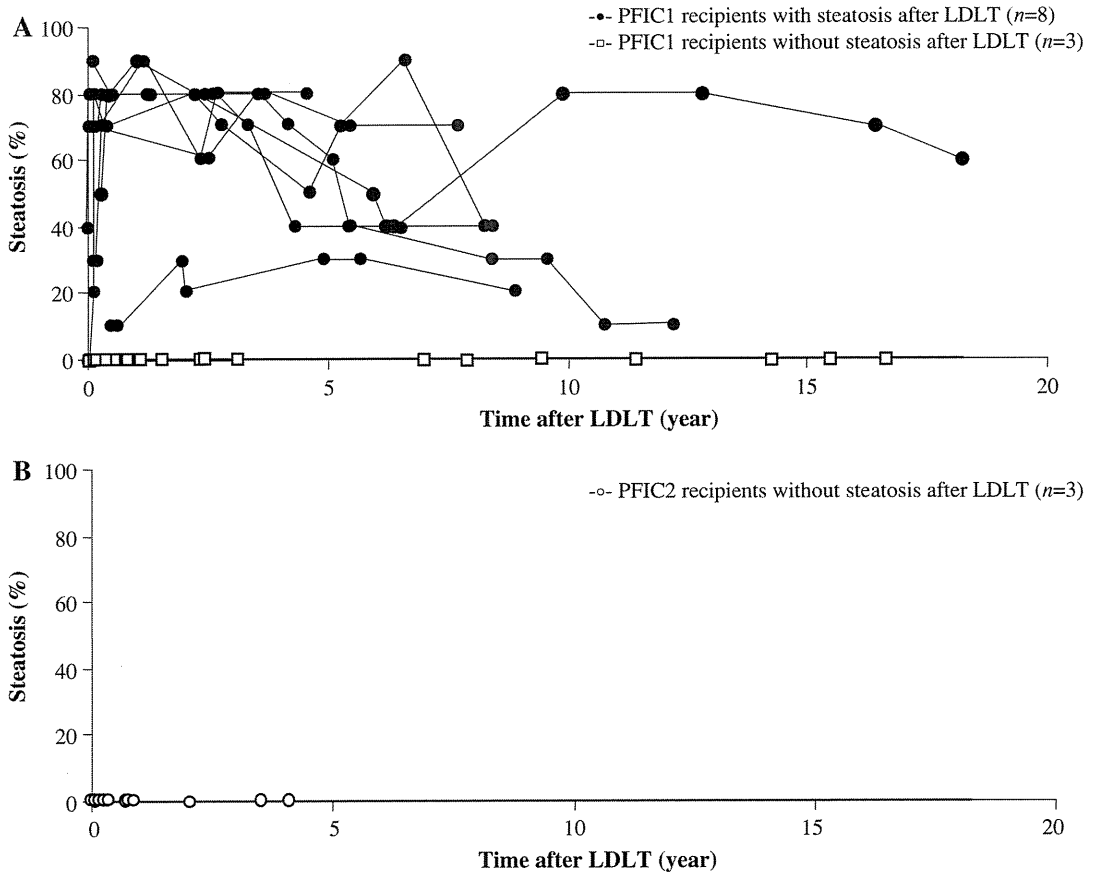

Fig. 1 Time course of steatosis in allografts after living donor liver transplantation (LDLT). **a** Temporal changes in the degree of steatosis after LDLT in progressive familial intrahepatic cholestasis type 1 (*PFIC1*) recipients. Eight PFIC1 recipients presented with steatosis after LDLT, and three PFIC1 recipients did not. Seven of the eight steatosis-positive recipients had the complication of steatohepatitis. *Filled circles* and *open squares* represent the degree of steatosis in PFIC1 recipients with and without steatosis after LDLT, respectively. **b** Temporal changes in the degree of steatosis after LDLT in PFIC type 2 (*PFIC2*) recipients. None of the three PFIC2 patients presented with steatosis after LDLT. *Open circles* represent the degree of steatosis in the PFIC2 recipients

protease inhibitors, bicarbonate, and fat-soluble vitamins. Positive or subtle effects against digestive symptoms were confirmed in all cases, although the symptoms persisted. Regarding the degree of steatosis and the fibrosis scores in the six steatosis-positive PFIC1 recipients who received these combined therapies (cases 1, 5–7, 9, 11), all of the recipients showed temporary responses to these treatments. However, in the histopathological findings of the latest LNBs, the degree of steatosis and the fibrosis scores for these six patients persisted at 46.7% ± 28.0% (range 10–80%) and 3.0 ± 1.1 (range 1–4), respectively. No specific treatment against steatosis were necessary in the three PFIC2 recipients.

Outcomes and survival rates after LDLT in the PFIC1, PFIC2, and other recipients

The mean observation periods were 11.9 ± 4.5 years for the PFIC1 recipients and 4.2 ± 2.7 years for the PFIC2 recipients. In all, 3 of the 11 PFIC1 recipients died, whereas all three PFIC2 recipients survived (Table 2). It should be noted that all three PFIC1 recipients with poor outcomes also had cirrhotic findings even after LDLT. One PFIC1 recipient (case 3) died after rupture of the splenic artery at POD 5032. Another PFIC1 recipient (case 5) underwent retransplantation on POD 4646 owing to graft loss but died from cardiac failure 25 days after the retransplantation. The third PFIC1 recipient (case 10) suffered from chronic rejection at 6 months after the LDLT and underwent retransplantation on POD 1393. Thereafter, an arterioportal shunt after the retransplantation caused graft loss, and yet another retransplantation was performed on POD 1986 after the initial LDLT. However, the esophageal varix ruptured on POD 2005 after the initial LDLT. The survival rates of the PFIC1 recipients at 5, 10, and 15 years after LDLT were 90.9%, 72.7%, and 54.5%, respectively. All three PFIC2 recipients survived. The survival rates of the other 703 recipients at 5, 10, 15, and

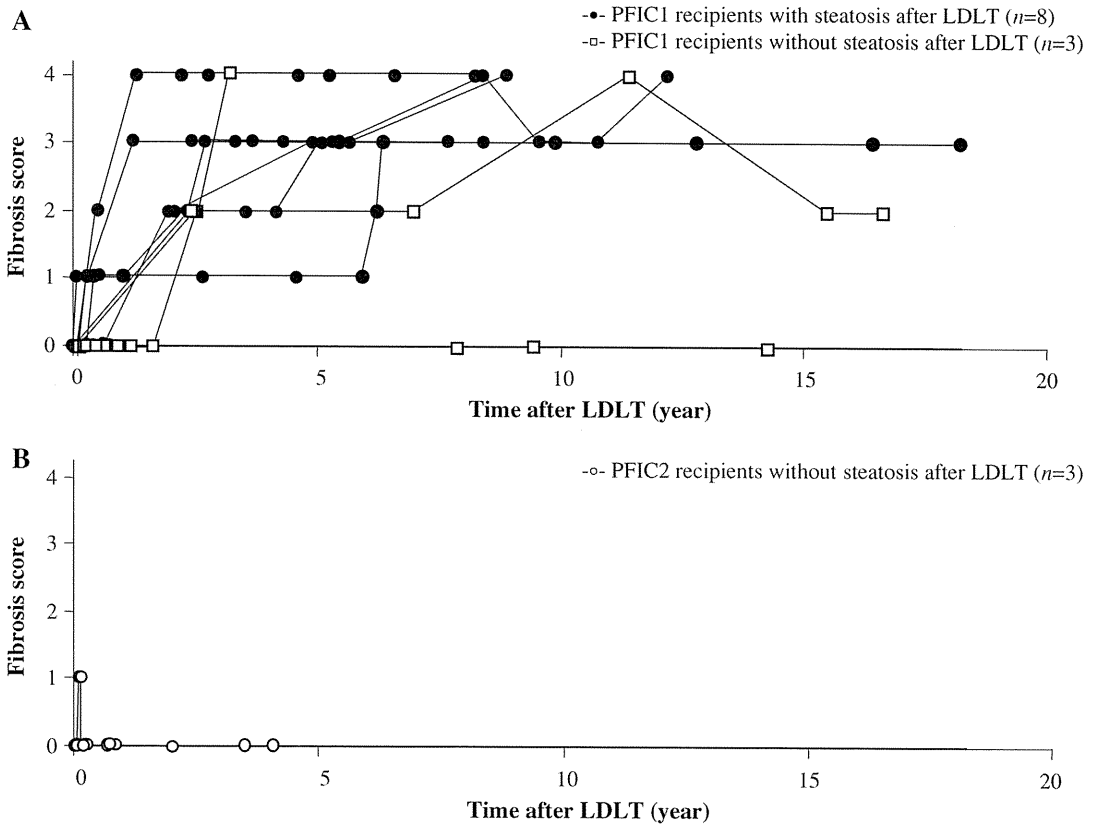

Fig. 2 Time course of fibrosis in allografts after LDLT. **a** Temporal changes in the scores for hepatic fibrosis after LDLT in PFIC1 recipients. Eight PFIC1 recipients with steatosis after LDLT subsequently developed positive fibrosis, and seven of these eight recipients had fibrosis (F) scores of ≥3. Among the three PFIC1 recipients without steatosis after LDLT, one recipient (case 4) showed no fibrosis, and two recipients (cases 2 and 10) had F scores of 4 due to reasons other than steatosis [de novo autoimmune hepatitis (AIH) and chronic rejection, respectively]. *Filled circles* and *open squares* represent the scores for hepatic fibrosis in the PFIC1 recipients with and without steatosis after LDLT, respectively. **b** Temporal changes in the scores for hepatic fibrosis after LDLT in PFIC2 recipients. All three PFIC2 patients had F scores of 0, although one recipient (case 14) temporarily had an F score of 1 at postoperative days (PODs) 39 and 47 owing to refractory acute cellular rejection (ACR), which was successfully treated. *Open circles* represent the scores for hepatic fibrosis after LDLT in the PFIC2 recipients

20 years after LDLT were 83.3%, 79.9%, 77.4%, and 76.5%, respectively.

Discussion

Although our PFIC1 recipients who received UDCA therapy showed only temporary effects and their steatosis and fibrosis persisted, therapy with UDCA (20–30 mg/kg/day) is considered for the initial therapeutic management of PFIC, especially in PFIC3 patients [1, 11, 21], although this therapy cannot be stopped in female patients during pregnancy [30]. We have no experience of LDLT for PFIC3 patients. However, some previous reports have documented PFIC3 recipients who underwent LDLT [31, 32], and LT was required at a mean age of 7.5 years in those patients [1]. We understand that even PFIC3 recipients require LT owing to resultant cirrhosis [33]. In our institution, the PFIC2 recipients maintained good graft conditions and showed excellent outcomes. We suggest that early LDLT may have a sufficient advantage for PFIC2 patients.

Steatosis is categorized in nonalcoholic fatty liver disease [25, 34]. Although steatosis itself is considered to be nonprogressive, steatosis with a developed fibroinflammatory counterpart can develop into cirrhosis [35, 36]. Continuous fat accumulation in hepatic cells is an initial step in the processes that result in necroinflammation and fibrosis in steatohepatitis [25, 37, 38]. Currently, oxidant stress, free fatty acids, lipid peroxidation products, and ATP depletion are focused on as factors that may induce cell injury and subsequent fibrosis in the fatty liver [39, 40]. Our results demonstrated that PFIC1 patients may have persistent steatosis progression even during the early

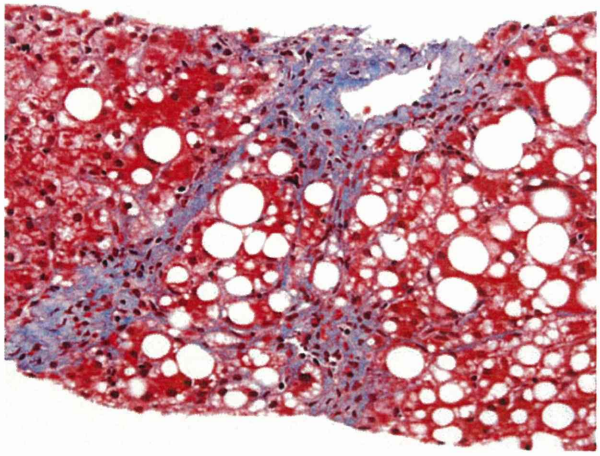

Fig. 3 Histopathological findings of steatosis and subsequent fibrosis after LDLT. A representative section from case 6 shows fibrosis with an F score of 3 at POD 468. In this case, 70% steatosis complicated by severe steatohepatitis was confirmed at POD 69 by hematoxylin-eosin staining (not shown), and the degree of steatosis worsened to 80% at POD 138. Subsequently, this case resulted in hepatic fibrosis with an F score of 3. (Masson trichrome and reticulin)

postoperative period after LDLT and that steatohepatitis after LDLT can be associated with subsequent fibrosis and allograft failure.

The extrahepatic features in PFIC1 patients do not improve or may be aggravated after LT [1, 9]. Chronic diarrhea may become intractable when biliary BS secretion is restored after LT [6, 9, 16], although diarrhea may be favorably managed by certain medications [9, 16]. Similar to these previous reports, our results confirmed digestive symptoms after LDLT in PFIC1 recipients but not in PFIC2 recipients. The clinical courses of our PFIC1 recipients were not satisfactory, and some of our PFIC1 recipients suffered from cirrhotic findings even a long time after the LDLT. The hyperdynamic state in cirrhotic recipients cannot be restored immediately, even after normalization of the portal pressure by LDLT [41–43]. We suggest that continuous graft damage including fibrosis in the PFIC1 recipients disturbed the restoration of their peculiar hemodynamics and that the persistence of these systemic hemodynamics may have resulted in fatal complications, such as rupture of dilated vessels, even a long time after the LDLT.

The outcomes of LDLT in our PFIC1 recipients are still not sufficient, nor were they in a previous report [44]. Donor selection for LDLT is limited ethically, socially, and medically, although repeated retransplantation can augment the long-term survival of pediatric PFIC1 patients. Our findings for the early postoperative occurrence of steatosis and fibrosis oblige us to reconsider the timing of LDLT and to challenge some other therapies for PFIC1 patients. Partial external biliary diversion (PEBD) has been

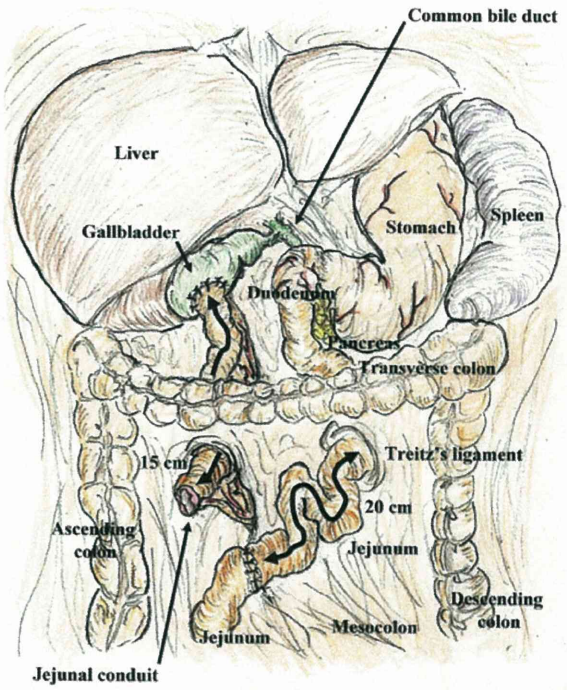

Fig. 4 Surgical technique for partial external biliary diversion (PEBD) in our institution. PEBD was performed as a cholecystojejunocutaneostomy. An isolated jejunal interposition 15 cm in length was made with the proper mesentery at a point 20 cm distant from Treitz's ligament. Next, the proximal side of this interposition was anastomosed to the body of the gallbladder in a side-to-end manner. The jejunal interposition was placed between the gallbladder and the skin; and end-stoma was made in the right lower quadrant of the abdominal wall

documented as a possibility for PFIC patients [45, 46]. Some patients with PFIC may benefit from PEBD [47], although its effects remain controversial [45, 46]. The criteria for identifying PFIC patients who could benefit from UDCA or PEBD are unclear [48], although nasobiliary drainage and gene mutations are reported to select potential responders to PEBD [48]. LT represents the only alternative if these therapies fail [49].

After our experience with the 11 PFIC1 recipients described here, in 2009 we introduced PEBD as an anticipatory surgery before LDLT in a female PFIC1 patient aged 1.8 years (Fig. 4). We are closely following this case, and her clinical symptoms, which include itching, bad temper, agrypnia, and digestive symptoms. They fortunately diminished during the first year after PEBD. The histopathological findings in follow-up LNBs revealed that the liver damage has not progressed based on the intraoperative LNB findings. Although we have not had sufficient experiences of PEBD for PFIC1, we now consider this anticipatory surgery before LDLT if the overall considerations, including the donor limitation and patient status,

indicate its possibility. We do not believe that PFIC1 contraindicates LDLT because not all of our PFIC1 recipients necessarily suffered graft losses after LDLT. However, we hope that optimal control by PEBD and possible procrastination with a stable status until LDLT may contribute to the long-term quality of life in PFIC1 patients under the donor limitation situation. On the other hand, we performed total external biliary diversion (TEBD) in one PFIC1 recipient at retransplantation (case 10), although we had no experience with TEBD at the initial LDLT. We cannot confirm the effects of LDLT accompanied by TEBD because this recipient suffered graft loss owing to an arterioportal shunt after the retransplantation.

Only one mutated allele or no mutation is identified in a few PFIC patients (<10%) [1]. Mutations that may map to regulatory sequences of the genes is a possible explanation for this observation. A gene related to the transcription of PFIC genes or protein trafficking could also be involved [50]. It cannot be negated that other unidentified genes involved in bile formation may be responsible for the PFIC phenotypes. The mutated protein may have a dominant-negative effect on the expression and/or function of the protein in a heterozygous state [51]. Modifier genes and environmental influences could play roles in the expression of PFIC [52]. The possibility of PFIC recurrence after LT owing to alloimmunization of the recipient against the FIC1, BSEP, and MDR3 proteins of the donor remains a theoretical matter of debate. 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, and MDR3 gene products [1]. In LDLT based on donor relationships with parents, it can be expected that the heterozygous status of the liver allograft will lead to a predisposition for developing lithiasis or cholestasis favored by immunosuppressive drugs that may interfere with canalicular protein function [53]. We think that this possibility is rare because we performed LDLT in which the donor origins were parents in 10 of 11 cases without PFIC recurrences, and this possible hypothesis was not reported in previous series [49].

Some investigators have documented that more advanced strategies, including cell transplantation, gene therapy, or specific targeted pharmacotherapy, may represent alternative therapies for all PFIC types in the future [48]. Our own results and a review of the mechanisms in previous articles have demonstrated that LT, including LDLT, may have advantages in PFIC2 patients as a definitive therapy and that the clinical courses and outcomes after LDLT are still not sufficient in PFIC1 patients owing to postoperative steatosis/fibrosis. As PFIC1 patients do require LT during the disease course, we suggest that the therapeutic strategies for PFIC1 patients, including the timing of LDLT under the donor limitation, should be reconsidered. The LDLT should not be performed in PFIC1 patients until effective interventions can be made to correct the metabolic defects, although PFIC2 is good indication for LDLT. The establishment of more advanced treatments for PFIC1 patients is required to improve the long-term prognosis.

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Conflict of interest None of the authors has a conflict of interest.

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ORIGINAL ARTICLE

Cross-sectional study of bone metabolism with nutrition in adult classical phenylketonuric patients diagnosed by neonatal screening

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Abstract The mechanism underlying the development of osteopenia or osteoporosis in longstanding phenylketonuria (PKU) remains to be clarified. We investigated the details of bone metabolism in 21 female and 13 male classical PKU patients aged 20–35 years. Vitamin D (VD), parathyroid hormone (PTH), bone turnover markers, and daily nutrient intake were examined. The patients had lower daily energy and protein intake than did the age-matched controls (22 women, 14 men), but their respective fat, VD, and calcium intake did not differ. Serum 1,25-dihydroxy VD and 25-hydroxy VD levels in female and male patient groups were significantly higher and lower than those in respective control groups (females, $P < 0.001$; males, $P < 0.05$ and $P < 0.01$, respectively). Serum intact PTH levels were significantly higher in the female patient group ($P < 0.05$). Urinary calcium levels in the patient groups were significantly higher than those of the control subjects (females, $P < 0.001$; males, $P < 0.05$). Bone resorption markers were significantly higher in patients than in controls, although bone formation markers were not different. Patient serum levels of osteoprotegerin-inhibiting bone resorption were significantly lower (females, $P < 0.001$;

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males, $P < 0.01$). None of the bone parameters correlated significantly with serum phenylalanine or nutrient intake. PKU patients exhibited lower VD status and more rapid bone resorption despite normal calcium–VD intakes.

Keywords Classical phenylketonuria · Osteopenia · Adult · Vitamin D · Bone resorption

Introduction

Phenylketonuria (PKU) is caused by a deficiency of phenylalanine hydroxylase. This disorder is transmitted in a manner of autosomal recessive inheritance [1]. Neurological impairment and mental retardation, as consequences of inappropriately high plasma phenylalanine (Phe) levels, are often severe problems in affected subjects [1–4]. Therefore, PKU children receive phenylalanine-restricted diets almost exclusively to maintain appropriate plasma Phe levels. Nevertheless, high risk for osteopenia or osteoporosis has been shown in long-standing PKU [5–11]. Details of bone metabolism in PKU remain to be clarified, but abnormalities in bone turnover have been reported [6, 9–11]. Recently, Modan-Modes et al. [12] reported that the peak bone mass is lower in adult PKU patients who adhere to phenylalanine-restricted diets, which are rich in energy, protein, and calcium, than in patients not adhering to such a diet [12]. Sufficient knowledge related to this subject is necessary for handling bone diseases in PKU.

This study aimed to obtain fundamental data for establishing an optimal treatment strategy for bone disease in PKU. We investigated the bone metabolism, including vitamin D (VD), parathyroid hormone (PTH), bone turnover markers, and nutritional status, of adult classical PKU patients lacking phenylalanine hydroxylase activity.

Materials and methods

Subjects

We enrolled 34 classical PKU patients (21 women, 13 men) who were 20 to 35 years old. Mass screening at around 5 days of age had revealed hyperphenylalaninemia of more than 20 mg/dl in these patients. Until the age of 15 years, after the diagnosis of classical PKU, they had received phenylalanine-restricted diets with phenylalanine-free milk. Thereafter, the restrictions of phenylalanine varied among the patients. Some patients were almost free of diet restrictions (9 women, 4 men). Others received mildly restricted or less restricted diets. Results show that their serum Phe levels were 10–33 mg/dl (mean ± SD, 22.3 ± 4.5 mg/dl). The patients showed no overt developmental or psychomotor disturbance. None had a history of immobilization or disease-limiting movement.

As controls, we enrolled 36 healthy volunteers (22 women, 14 men) who were 19 to 40 years old. No significant difference was found in age, body mass index, liver function test, or sex hormone levels between female PKU patients and the female controls or between male PKU patients and the male controls (Table 1).

Study design

Quantities of daily energy, protein, fat, calcium, and vitamin D intake were calculated based on a detailed 3-day dietary history. Affected patients or their parents were required to record the amounts of food and drink consumed during the 3 days preceding the clinic visit.

We measured blood levels of intact PTH, 25-hydroxy VD, and 1,25-dihydroxy VD. To evaluate bone remodeling and turnover, we measured the blood and urinary markers for bone formation and resorption [13–15]. Blood bone alkaline phosphatase (BAP) and osteocalcin (OCN) were measured as bone formation markers. As bone resorption markers, the blood pyridinoline cross-linked telopeptide domain of type I collagen (ICTP), urinary deoxypyridinoline (D-Pyr), and urinary *N*-telopeptide of type I collagen (NTx) were measured. Furthermore, blood osteoprotegerin (OPG) was measured because OPG is a major inhibitor of bone resorption [16]. To evaluate calcium (Ca) and phosphorus (P) excretion in urine, urinary Ca/creatinine (Cr) ratio and P/Cr were determined.

Venous blood samples were collected after overnight fasting. They were centrifuged and stored at −80°C until analyses. Urine samples were collected at 9:00–10:00 a.m. and frozen until analyses. This study protocol was approved by the relevant institutional review boards. All patients provided written informed consent before enrollment in the study.

Assays

Serum intact PTH level was determined using a radioimmunoassay (RIA) kit from the Nichols Institute (Quest Diagnostics, Geneva, Switzerland). Serum 1,25-hydroxy VD and 25-hydroxy VD levels were determined using RIA kits from Immunodiagnostic Systems Holdings plc (Boldon, UK) and Diasorin, Inc. (Stillwater, MN, USA), respectively,. Serum OCN levels were determined using an RIA kit (BGP-IRMA; Mitsubishi Kagaku Iatron, Tokyo, Japan). Serum BAP levels were determined using an enzyme-linked immunosorbent assay (ELISA) kit (Osteo-Links-BAP; Quidel, San Diego, CA, USA). The serum ICTP level was measured using an RIA kit from Orion Diagnostica (Espoo, Finland). Urinary D-Pyr and NTx

were determined using ELISA kits from Quidel Corp. and Inverness Medical Innovation (Chiba, Japan), respectively. An ELISA kit from Biomedia (Vienna, Austria) was used to determine the serum OPG level. Serum estradiol and testosterone levels were determined using enhanced chemiluminescence immunoassay Estradiol-kit and Testosterone-kit (Biocheck, Foster City, CA, USA).

Urinary Ca, P, and Cr levels were determined using routine autoanalyzer methods.

Statistical analyses

Data are presented as mean ± SE. Differences between values of patients and those of controls were estimated using Student's t test. The relationship between each pair of parameters was estimated using Pearson's correlation test. All P values less than 0.05 were considered statistically significant.

Results

Daily energy and nutrient intakes

Average daily energy and protein intakes in female and male patient groups were significantly lower than those in the respective control groups ($P < 0.01$ and $P < 0.05$, respectively). Average daily fat, Ca, and VD intakes in the patient groups were not different from those in respective control groups (see Table 1). Serum levels of total protein, albumin, and lipids, as well as liver function tests, also did not differ between patient and control groups.

Bone parameters

Intact PTH levels in the 21 female PKU patients were significantly higher than those in the female controls ($P < 0.05$). Their 1,25-dihydroxy VD and 25-hydroxy VD levels were significantly higher and lower, respectively, than those in the female controls ($P < 0.001$) (Table 2).

Regarding serum bone formation markers, no significant differences in serum BAP and OCN levels were found between these two female groups. In contrast, bone resorption markers were significantly higher in female patients ($P < 0.001$). Serum ICTP and urinary D-Pyr and NTx levels in the female PKU patients were significantly higher than those in the female controls ($P < 0.001$). Serum OPG levels in female patients were significantly lower than those in the female controls ($P < 0.001$).Their urinary Ca excretion was significantly higher than that of the female controls ($P < 0.001$), but P excretion was similar to the control level (Table 2).

The 13 male PKU patients showed intact PTH levels similar to those of the male controls. Their 1,25-dihydroxy VD levels were significantly higher than those of the male controls ($P < 0.05$); the 25-hydroxy VD levels were significantly lower than those of the control subjects ($P < 0.01$) (Table 2).

No significant differences were found in serum BAP and OC levels between the male patients and controls. In contrast, all bone resorption markers were significantly higher than those of the respective control subjects ($P < 0.01$). Serum OPG levels in male patients were significantly lower than those in the male controls ($P < 0.01$). Their urinary Ca excretion was significantly higher than that of control subjects ($P < 0.05$), but P excretion was similar to that of control subjects (see Table 2).

Correlations between serum phenylalanine level or daily nutrient intake and bone parameters and those among parameters

In both female and male PKU patient groups, the serum Phe level showed no significant correlation with any bone parameter (Table 3). Moreover, average daily nutrient and energy intakes showed no significant correlation with these parameters (data not shown).

In the female PKU group, significant correlation was found between two bone resorption markers ($P < 0.01$). Particularly, the correlation between D-Pyr and ITCP was strongest ($r = 0.841$, $P < 0.001$). Here, BAP showed significant correlations with all bone resorption markers ($P < 0.05$ or $P < 0.01$), although OCN was correlated significantly with only one resorption marker, NTx ($P < 0.01$). Urinary Ca excretion was correlated significantly with intact PTH and 1,25-dihydroxy VD levels (Table 3). In contrast, urinary P excretion was not correlated with any other parameter (data not shown).

The male PKU group also showed a significant correlation for each of the two bone resorption markers ($P < 0.01$, $P < 0.001$). Particularly, the correlation between D-Pyr and ITCP was strongest ($r = 0.957$, $P < 0.001$). Actually, BAP, but not OCN, showed significant correlation with D-Pyr and NTx levels (D-Pyr, $P < 0.05$; NTx, $P < 0.01$). The urinary Ca and P excretions were not correlated significantly with any other parameter in this group.

Discussion

Evidence is accumulating that PKU patients are at risk for osteopenia and bone fractures [4–9, 12, 13]. Bone marker abnormalities have been described in recent reports [6, 9–11]. Particularly, increases in bone resorption marker levels

Table 1 Characteristics of phenylketonuric patients and healthy age-matched controls

	Female patients ($n = 21$)	Female controls ($n = 22$)	Male patients ($n = 13$)	Male controls ($n = 14$)
Age (years)	27.1 ± 3.2	27.9 ± 5.1	26.9 ± 3.3	30.3 ± 4.5
Body mass index (kg/m^2)	22.5 ± 1.5	23.7 ± 2.2	24.7 ± 2.4	23.5 ± 2.3
Daily energy intake (kcal/day)	1601 ± 275**	2052 ± 243	2015 ± 215*	2452 ± 243
Daily protein intake (g/day)	66 ± 15**	79 ± 10	72 ± 17*	88 ± 11
Daily natural protein intake (g/day)	40 ± 11***	79 ± 10	47 ± 10***	88 ± 11
Daily fat intake (g/day)	55 ± 21	52 ± 16	59 ± 17	56 ± 16
Daily Ca intake (mg/day)	1212 ± 385	1130 ± 375	1122 ± 386	1055 ± 333
Daily vitamin D intake (IU/day)	127 ± 26	112 ± 23	112 ± 23	112 ± 21
Total protein (g/dl)	7.2 ± 0.3	7.5 ± 0.3	7.3 ± 0.3	7.6 ± 0.3
Albumin (g/dl)	4.3 ± 0.2	4.6 ± 0.2	4.4 ± 0.2	4.7 ± 0.2
Alanine aminotransferase (IU/l)	9 ± 2	11 ± 3	10 ± 2	12 ± 3
Aspartate aminotransferase (IU/l)	18 ± 3	18 ± 4	18 ± 2	18 ± 3
Total cholesterol (mg/dl)	150 ± 13	163 ± 14	154 ± 13	173 ± 17
Triglycerides (mg/dl)	81 ± 20	82 ± 23	85 ± 21	86 ± 20
Low-density lipoprotein cholesterol (mg/dl)	81 ± 8	88 ± 10	81 ± 8	88 ± 10
High-density lipoprotein cholesterol (mg/dl)	50 ± 3	53 ± 4	54 ± 4	56 ± 4
Estradiol (pg/ml)	59 ± 16	55 ± 18	37 ± 6	40 ± 5
Testosterone (ng/dl)	33 ± 12	31 ± 15	555 ± 61	578 ± 77

Data are presented as mean ± SE

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus controls

have been documented in PKU patients [6, 9, 11]. Nevertheless, bone metabolism in PKU has not been studied sufficiently. Accordingly, clinical management to prevent development and progression of bone disease in PKU patients remains to be established.

This study found increased bone resorption markers together with increased urinary Ca excretion in adult PKU patients. However, their blood levels of bone formation markers were similar to those of control subjects. Serum ICTP level, and urinary D-Pyr and NTx levels in patient groups were 1.5 times higher than the respective control levels without increases in the bone formation markers. Such discrepancies between bone resorption and formation marker levels, together with increased urinary Ca, strongly support the inference that PKU patients are at high risk for osteopenia or osteoporosis in later life.

In preliminary evaluations, we measured the lumbar spine bone mineral density (BMD) at L1–L4 levels using dual-energy X-ray absorptiometry (QDR-4500; Hologic, Waltham, MA, USA) in 10 of the 21 female patients and 7 of the 13 male patients. The BMD values were, respectively, 0.823–0.993 g/cm^2 (mean ± SD, 0.856 ± 0.041 g/cm^2) and 0.936–1.152 g/cm^2 (1.042 ± 0.064 g/cm^2). The values were 81–97% (91% ± 3%) and 90–101% (94% ± 2%) of the mean of age-matched and sex-matched healthy controls. The SD scores were −1.7 to −0.1 (−0.7 ± 0.3) and −1.3 to +0.2 (−0.4 ± 0.2), respectively. The patients tended to have lower BMD at the lumbar spine. The results were consistent with those described in other reports [5–8, 11, 12].

In our PKU patients, serum 25-hydroxy VD levels were decreased, although serum 1,25-dihydroxy VD levels were increased. We inferred that the 1,25-hydroxy VD level was increased compensatively via an action of the PTH to activate 1α-hydroxylase [17, 18]. A significant increase in serum iPTH levels was found only in female patients, not in male patients.

We were unable to detect differences in VD and Ca intake between PKU patients and controls. The control serum Phe level, which is determined mainly by the intake, was also not correlated with any bone metabolism parameter in the patients. It would therefore be impossible to explain the lower VD status according to Ca, VD, or Phe intake. The underlying mechanism for their lower vitamin D status remains unclear.

Modan-Modes et al. [12] reported that the peak bone mass is decreased in adult PKU patients who adhere to phenylalanine-restricted diets, which are rich in VD, protein, and minerals involving Ca, compared to those not adhering to such diets. Results of this study also support the contention that sufficient nutrient intake might not necessarily result in normal mineral accumulation in bones of PKU patients.

Bone remodeling is regulated by cross-talk reactions between osteoblasts and osteoclasts [19, 20]. Earlier reports

Table 2 Bone markers of control and patient groups

	Female patients ($n = 21$)	Female controls ($n = 22$)	Male patients ($n = 13$)	Male controls ($n = 14$)
Intact PTH (pg/ml)	37.5 ± 2.4*	32.3 ± 3.5	36.5 ± 3.8	32.7 ± 3.7
1,25 $(OH)_2$ vitamin D (pg/ml)	58.4 ± 2.7***	41.6 ± 3.1	50.6 ± 2.0*	39.9 ± 2.7
25 (OH) vitamin D (ng/ml)	18.7 ± 1.3***	27.6 ± 2.1	22.2 ± 1.7**	30.0 ± 2.6
Bone alkaline phosphatase (U/l)	22.7 ± 2.2	21.7 ± 2.5	28.5 ± 2.7	25.4 ± 2.7
Osteocalcin (ng/ml)	5.6 ± 0.7	5.9 ± 0.5	5.4 ± 1.0	5.5 ± 0.6
U-D-Pyr (nmol/mmol creatinine)	7.3 ± 0.5***	4.9 ± 0.4	5.2 ± 0.5**	3.8 ± 0.6
U-NTx (nmol/mmol creatinine)	47.8 ± 6.1***	31.7 ± 5.1	54.7 ± 12.1**	38.3 ± 10.5
ICTP (ng/ml)	4.6 ± 0.2***	3.0 ± 0.2	4.3 ± 0.3**	3.0 ± 0.2
Osteoprotegerin (pmol/l)	3.3 ± 0.3***	4.7 ± 0.4	3.1 ± 0.2**	4.3 ± 0.2
U-Ca/Cr (mmol/mmol creatinine)	0.46 ± 0.08***	0.33 ± 0.08	0.42 ± 0.10*	0.34 ± 0.07
U-P/Cr (mmol/mmol creatinine)	1.33 ± 0.23	1.23 ± 0.28	1.41 ± 0.22	1.35 ± 0.26

Data are presented as mean ± SE

Intact PTH, intact parathyroid hormone; U-D-Pyr, urinary deoxypyridinoline; U-NTx, urinary *N*-telopeptides of type collagen; ICTP, pyridinoline cross-linked telopeptide domain of type I collagen

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus controls

Table 3 Correlation among bone parameters, including vitamin D and PTH, in 21 female patients (bold letters) and 13 male patients (plain letters)

	Phe	BAP	OCN	Intact PTH	1,25 $(OH)_2$ VD	25 (OH) VD	U-Ca/Cr	U-P/Cr	U-D-Pyr	U-NTx	ICTP
Phe	/	−0.068	−0.225	0.326	0.391	−0.262	0.227	0.131	−0.127	−0.200	0.111
BAP	0.240	/	0.331	0.155	0.303	0.138	0.332	0.296	0.612**	0.607**	0.511*
OCN	−00.502	0.210	/	0.244	0.239	0.217	0.414*	0.319	0.309	0.634**	0.374
Intact PTH	00.326	−0.166	−0.534	/	0.333	−0.090	0.443*	−0.178	0.329	−0.007	0.167
1,25 $(OH)_2$ VD	0.564	0.275	−0.138	−0.152	/	0.250	0.528**	−0.111	0.252	0.187	0.269
25 (OH) VD	−0.565	−0.612*	−0.047	0.231	−0.392	/	0.308	−0.091	−0.054	0.091	−0.145
U-Ca/Cr	0.237	0.289	0.195	−0.196	0.506	−0.184	/	−0.279	0.206	0.164	0.102
U-P/Cr	−0.299	0.105	0.007	−0.157	−0.399	0.166		/	0.100	0.233	0.149
U-D-Pyr	−0.089	0.653*	0.537	−0.652*	0.260	−0.471	0.351	0.277	/	0.679**	0.841***
U-NTx	−0.228	0.743**	0.381	−0.619*	0.157	−0.519	0.250	0.312	0.826**	/	0.650**
ICTP	0.082	0.729*	0.459	−0.469	0.350	−0.621*	0.395	0.333	0.957***	0.795**	/

Presented data are *r* values

Phe, phenylalanine; BAP, bone alkaline phosphatase; OCN, osteocalcin; Intact PTH, intact parathyroid hormone; D-Pyr, urinary deoxypyridinoline; U-NTx, urinary N-telopeptides of type I collagen; ICTP, pyridinoline cross-linked telopeptide domain of type I collagen

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

have described that many biological substances participate in cross-talk reactions [19–22]. Particularly, an effect of OPG inhibiting the signal transduction between osteoblasts and osteoclasts, leading to suppressed bone resorption, has been documented [16, 22]. Serum OPG levels in the PKU patients were significantly lower than those in healthy controls. Accordingly, the predominance of bone resorption over bone formation in our patients was explained, at least in part, by the lowered OPG levels.

Yannicelli et al. [23] reported that phenylalanine per se affected the bone mineral status in animal models. In our patients, the serum phenylalanine level showed no significant correlation with any bone marker level. It therefore appears unlikely that serum phenylalanine level represents a major determinant factor of bone status in PKU patients.

The bone metabolism in our patients might be attributable to their dietary pattern and cholesterol metabolism. For many patients, the natural protein content was assumed to be lowered, in various degrees, in the diets. They ingested low-phenylalanine proteins and specialized milk, in which some proportion of natural amino acids was replaced by phenylalanine-free amino acids. It has been

suggested that cholesterol production is often reduced in PKU [24, 25]. In fact, the serum cholesterol level was not increased, even in our patients taking plenty of lipids. Considering that vitamin D production is linked to cholesterol production, the lowered vitamin D status in our patients is likely to be associated with such limited cholesterol production. Future studies will be undertaken to investigate the bone metabolism of PKU in terms of dietary patterns and cholesterol metabolism.

The contribution of oxidative stress to bone diseases has been demonstrated [21, 22]. Enhancement of oxidative stress in PKU has been reported recently [26–28]. Therefore, it seems plausible that the promoted bone resorption in PKU is also, in part, attributable to the enhanced oxidative stress. This issue must be addressed in future studies.

Overall, the data gained from this study suggest a decreased vitamin D status and the predominance of bone resorption over bone formation in adult classical PKU patients. These findings appeared to be more prominent for female patients than for male patients. First, we expected that estrogen-inhibiting OPG production was deeply associated with osteopenia in the female group. However, sex hormone levels did not differ between this group and the control group. Many factors other than sex hormones, such as exercise, lifestyle, and dietary patterns, might influence bone metabolism in PKU patients, particularly female patients [29–31].

Additional investigations must be undertaken to detect the determinant factors of bone metabolism status in PKU.

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