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Steatogenesis in adult-onset type II citrullinemia is associated with down-regulation of PPARα

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ABSTRACT

SLC25A13 (citrin or aspartate–glutamate carrier 2) is located in the mitochondrial membrane in the liver and its genetic deficiency causes adult-onset type II citrullinemia (CTLN2). CTLN2 is one of the urea cycle disorders characterized by sudden-onset hyperammonemia due to reduced argininosuccinate synthase activity. This disorder is frequently accompanied with hepatosteatosis in the absence of obesity and ethanol consumption. However, the precise mechanism of steatogenesis remains unclear. The expression of genes associated with fatty acid (FA) and triglyceride (TG) metabolism was examined using liver samples obtained from 16 CTLN2 patients and compared with 7 healthy individuals. Although expression of hepatic genes associated with lipogenesis and TG hydrolysis was not changed, the mRNAs encoding enzymes/proteins involved in FA oxidation (carnitine palmitoyl-CoA transferase 1α, medium- and very-long-chain acyl-CoA dehydrogenases, and acyl-CoA oxidase 1), very-low-density lipoprotein secretion (microsomal TG transfer protein), and FA transport (CD36 and FA-binding protein 1), were markedly suppressed in CTLN2 patients. Serum concentrations of ketone bodies were also decreased in these patients, suggesting reduced mitochondrial β-oxidation activity. Consistent with these findings, the expression of peroxisome proliferator-activated receptor α (PPARα), a master regulator of hepatic lipid metabolism, was significantly down-regulated. Hepatic PPARα expression was inversely correlated with severity of steatosis and circulating ammonia and citrulline levels. Additionally, phosphorylation of c-Jun-N-terminal kinase was enhanced in CTLN2 livers, which was likely associated with lower hepatic PPARα. Collectively, down-regulation of PPARα is associated with steatogenesis in CTLN2 patients. These findings provide a novel link between urea cycle disorder, lipid metabolism, and PPARα.

Abbreviations: ACAA1, acetyl-CoA acyltransferase 1; ACACA, acetyl-CoA carboxylase α; ACACB, acetyl-CoA carboxylase β; ACADM, medium-chain acyl-CoA dehydrogenase; ACADVL, very-long-chain acyl-CoA dehydrogenase; ACLY, ATP citrate lyase; ACOX1, acyl-CoA oxidase 1; ACSL1, acyl-CoA synthetase long-chain family member 1; AGC, aspartate–glutamate carrier; ALT, alanine aminotransferase; APOB, apolipoprotein B; ASS, argininosuccinate synthase; AST, aspartate aminotransferase; BMI, body mass index; CAC, carnitine–acylcarnitine carrier; CAT, catalase; CD, citrin deficiency; CoA, coenzyme A; CPT1A, carnitine palmitoyl-CoA transferase 1α; CTLN2, adult-onset type II citrullinemia; CYBB, cytochrome b-245, β polypeptide; CYP4A11, cytochrome P450, family 4, subfamily A, polypeptide 11; DGAT, diacylglycerol O-acyltransferase; FA, fatty acid; FABP1, fatty acid-binding protein 1; FASN, fatty acid synthase; IL, interleukin; JNK, c-Jun-N-terminal kinase; LIPC, hepatic lipase; MDA, malondialdehyde; MTTP, microsomal triglyceride transfer protein; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NF-κB, nuclear factor kappa B; NFKBIA, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor α; NICCD, neonatal intrahepatic cholestasis caused by citrin deficiency; NOX4, NADPH oxidase 4; PCR, polymerase chain reaction; p-JNK, phosphorylated JNK; PPAR, peroxisome proliferator-activated receptor; RXR, retinoid X receptor; SOD, superoxide dismutase; SREBF1, sterol regulatory element-binding transcription factor 1; TG, triglyceride; TGFB1, transforming growth factor β1; TNF, tumor necrosis factor α; US, ultrasonography; XDH, xanthine dehydrogenase

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1. Introduction

Urea cycle disorder can cause hepatic steatosis. It was reported that liver pathology in 10 children with defects of ureagenesis, such as carbamoyl-phosphate synthase deficiency and ornithine transcarbamoylase deficiency, showed diffuse microvesicular steatosis mimicking Reye's syndrome [1]. Additionally, suppression of urea cycle enzymes was observed in juvenile visceral steatosis mice [2]. These findings suggest a possible relationship between urea cycle disorder and disruption of lipid metabolism in the liver. However, the mechanism by which hepatosteatosis develops in the patients having urea cycle disorder has not been investigated.

Citrin [SLC25A13, also known as aspartate–glutamate carrier 2 (AGC2)], encoded by the *SLC25A13* gene, is a liver-type carrier located in the inner mitochondrial membrane and plays an important role in ureagenesis and gluconeogenesis [3–6]. Citrin deficiency (CD, also known as SLC25A13 deficiency or AGC2 deficiency) is an autosomal recessive disorder caused by mutations in the *SLC25A13* gene on chromosome 7q21.3, leading in many cases to neonatal intrahepatic cholestasis (NICCD; OMIM#605814) and adult-onset type II citrullinemia (CTLN2; OMIM#603471) [7]. Although CD had been regarded as a hereditary disease in East Asia [8,9], there are some recent reports of non-Asian CD patients from US, UK, France, Czech Republic, Canada, and Israel, revealing that CD is a pan-ethnic disorder [10–12]. Humans having *SLC25A13* mutations are sometimes asymptomatic probably due to unknown metabolic adaptation mechanisms. However, sudden onset of hyperammonemia and neuropsychiatric symptoms, such as unconsciousness and abnormal behavior, due to decreased argininosuccinate synthase (ASS) activity and impaired urea cycle in the liver, may lead to the diagnosis of CTLN2 [5,13,14]. The period of first appearance of neurological symptoms is various and some patients undergo first onset of the symptoms at the age of more than 50 years [15]. CTLN2 patients have unique dietary habits that like an affinity for fat-rich foods and dislike sweets and ethanol [16]. Ethanol consumption, glycerol infusion, and correction of fat-rich diet may trigger/aggravate hyperammonemia and encephalopathy in the patients with CD [17,18]. Some CTLN2 patients have experienced pancreatitis and been pointed out liver dysfunction and hypertriglyceridemia prior to appearance of neuropsychiatric symptoms [14,19,20]. These diverse clinical features make the correct and early diagnosis of CD/CTLN2 complicated.

Similarly to other urea cycle disorders, CD is frequently accompanied by hepatosteatosis. Hepatic fibrosis and increased incidence of hepatocellular carcinoma were also reported in CD patients [21,22]. Since hepatic steatosis sometimes precedes the appearance of neurological abnormalities, some CD patients may be misdiagnosed as having non-alcoholic fatty liver disease (NAFLD) [14,20]. Liver histology of CTLN2 patients demonstrated macrovesicular and microvesicular steatosis, infiltration of inflammatory cells in the lobular and portal areas, and hepatocyte ballooning that mimics simple steatosis or steatohepatitis [14,20]. We previously reported that CD-related fatty liver showed lower prevalence of accompanying obesity, lower body mass index (BMI), higher prevalence of history of pancreatitis, and higher serum concentrations of pancreatic secretory trypsin inhibitor compared with conventional NAFLD livers without the *SLC25A13* mutations [20]. However, the precise mechanism on how hepatic steatosis develops in CD/CTLN2 patients remains unclear.

To address it, the expression levels of the genes associated with fatty acid/triglyceride (FA/TG) metabolism were examined in CTLN2 patients having hepatic steatosis. We found that peroxisome proliferator-activated receptor α (PPARα) and its downstream FA β-oxidation were significantly down-regulated in the livers of CTLN2 patients. Additionally, hepatic PPARα expression was in inverse proportion to severity of steatosis and circulating ammonia and citrulline levels. These findings provide new insights regarding the association between urea cycle, PPARα signaling, and hepatosteatosis.

2. Materials and methods

2.1. Ethics

This study was carried out in accordance with the 1975 World Medical Association Helsinki Declaration and was approved by the Shinshu University School of Medicine ethics committee. Informed consent was obtained from all patients.

2.2. Patients

Sixteen CTLN2 patients (8 men and 8 women, 38 ± 12 years), who had been admitted to Shinshu University Hospital between 1998 and 2012 and confirmed the presence of *SLC25A13* mutations, were examined in this study. For comparison, 7 healthy living donors for liver transplantation (4 men and 3 women, 40 ± 8 years), who had been admitted to Shinshu University Hospital between 2008 and 2012, were analyzed as normal controls. All of these healthy individuals satisfied the following criteria: 1) the absence of past history of liver disease and regular intake of alcohol and drugs; 2) the absence of obesity, diabetes, hypertension, and hyperlipidemia; 3) normal liver function tests; 4) normal liver histology; and 5) the absence of *SLC25A13* mutations.

2.3. SLC25A13 gene mutation analysis

DNA was extracted from peripheral blood using standard methods [7–9]. Sixteen *SLC25A13* mutations reported previously were tested by the polymerase chain reaction (PCR)-restriction fragment length polymorphism method and/or multiple methods using Genescan/SNaPshot [7–9].

2.4. Laboratory examination

At the time of admission, blood samples were obtained in a fasting state and complete blood counts and blood chemistries were determined by standard methods [23,24]. Free FAs and ketone bodies were measured enzymatically (http://www.srl-group.co.jp, SRL, Tokyo, Japan). Hepatic ASS activity was quantified in 11 CTLN2 patients as described previously [25]. Serum concentrations of malondialdehyde (MDA), a typical oxidative stress marker, were measured using a lipid peroxidation colorimetric assay kit purchased from Oxis International (Beverly Hills, CA, USA).

2.5. Histological analysis

Liver samples in CTLN2 patients were obtained by percutaneous ultrasonography (US)-guided biopsy (9 patients) or living donor liver transplantation (7 patients). Liver samples in healthy liver transplantation donors were obtained at the time of pre-operative percutaneous US-guided biopsy. Fragments of liver tissue (5–7 mm) were immediately frozen in liquid nitrogen and stored at −80 °C until RNA extraction. The remaining tissues were fixed in 10% neutral formalin, embedded in paraffin, cut at 4 μm thickness, and stained with the hematoxylin and eosin or Azan–Mallory method [23,26]. Histological findings were assessed by an independent experienced pathologist in a blinded fashion and scored according to the staging/grading system proposed by Kleiner et al.[27]. The NAFLD activity score was calculated as the unweighted sum of the scores for steatosis (0–3), lobular inflammation (0–3), and ballooning (0–2). The diagnosis of steatohepatitis was made by the presence of macrovesicular steatosis, hepatocyte ballooning, and lobular inflammation [23,26].

2.6. Quantitative mRNA analysis

Total liver RNA was extracted using a RNeasy Mini Kit (QIAGEN, Hilden, Germany), and 2 μg of RNA was reverse-transcribed using oligo-dT primers and SuperScript II reverse transcriptase (Invitrogen

Table 1
Clinical characteristics of subjects enrolled in this study.

	Normal	Mild steatosis	Severe steatosis	*P* value		
	(N = 7)	(N = 10)	(N = 6)	Normal vs mild	Normal vs severe	Mild vs severe
Age (years)	40 (27–50)	42 (19–52)	33 (21–52)	0.845	0.568	0.515
Male	4 (57%)	6 (60%)	2 (33%)	1.000	0.592	0.608
Height (m)	1.60 (1.53–1.75)	1.63 (1.41–1.75)	1.60 (1.41–1.75)	0.922	0.615	0.786
Weight (kg)	54.7 (53.0–75.0)	50.4 (42.0–68.3)	46.5 (34.0–49.5)	0.121	0.008	0.247
BMI (kg/m^2)	22.2 (21.1–23.9)	19.0 (16.2–23.6)	15.9 (14.8–21.5)	0.034	0.013	0.118
Waist (cm)	76 (67–85)	72 (68–76)	60 (58–69)	0.346	0.029	0.025
WBC (/μl)	5370 (3020–7120)	3865 (2620–10,420)	3745 (2300–5610)	0.435	0.190	0.386
Hb (g/dl)	14.0 (12.3–16.0)	13.4 (9.5–15.5)	11.9 (10.5–14.3)	0.438	0.346	0.067
PLT ($\times 10^4$/μl)	24.0 (19.9–34.1)	19.4 (6.8–25.7)	18.8 (8.6–25.6)	0.107	0.347	1.000
TP (g/dl)	6.8 (6.1–7.5)	6.7 (5.9–7.2)	6.1 (5.6–7.0)	0.375	0.132	0.192
ALB (g/dl)	4.1 (4.0–4.5)	4.0 (3.1–4.5)	3.5 (3.1–3.7)	0.697	0.057	0.007
T-BIL (mg/dl)	0.60 (0.35–0.81)	0.82 (0.31–1.42)	0.95 (0.52–1.70)	0.663	0.186	0.356
AST (IU/l)	20 (13–21)	40 (18–123)	69 (20–205)	0.010	0.022	0.832
ALT (IU/l)	19 (11–22)	40 (20–132)	66 (20–126)	0.009	0.024	0.761
ALP (IU/l)	180 (81–290)	297 (90–800)	411 (70–521)	0.153	0.259	0.828
GGTP (IU/l)	35 (10–69)	103 (24–376)	185 (21–343)	0.212	0.190	0.914
AMY (IU/l)	71 (50–90)	101 (42–177)	59 (23–103)	0.355	0.848	0.196
BUN (mg/dl)	15 (8–15)	15 (13–23)	16 (13–28)	0.439	1.000	0.659
CRE (mg/dl)	0.6 (0.4–0.7)	0.5 (0.3–0.8)	0.6 (0.3–0.8)	0.632	0.785	0.823
TG (mg/dl)	100 (50–149)	98 (53–684)	223 (78–918)	0.494	0.189	0.525
FFA (μEq/l)	696 (500–802)	801 (530–1170)	818 (632–1056)	0.526	0.391	0.745
TC (mg/dl)	156 (137–189)	203 (112–259)	149 (113–216)	0.321	0.775	0.699
LDL-C (mg/dl)	89 (78–122)	90 (37–163)	65 (32–163)	0.908	0.464	0.396
FBS (mg/dl)	99 (89–106)	94 (77–129)	101 (78–145)	0.958	0.667	0.813
NH3 (μg/dl)	25 (20–32)	172 (39–239)	162 (35–224)	0.003	0.008	0.813
Citrulline (nmol/ml)	20 (15–23)	215 (86–498)	373 (200–1542)	0.003	0.013	0.952
Hepatic ASS activity (units/mg protein)	–	0.005 (0.001–0.024)	0.002 (0.0003–0.007)	–	–	0.300

Results are expressed as median (range) or number (percentage). Comparisons between groups were made using the Kruskal–Wallis test followed by the Mann–Whitney *U*-test with Bonferroni's correction or the Fisher's exact probability test. Hepatic argininosuccinate synthase (ASS) activities were measured in 11 CTLN2 patients (6 mild and 5 severe steatosis patients) as described previously [25]. Normal value of hepatic ASS activity was 0.033 ± 0.012 units/mg protein [32]. A *p* value of less than 0.05 was underlined. BMI, body mass index; WBC, white blood cell; Hb, hemoglobin; PLT, platelet; TP, total protein; ALB, albumin; T-BIL, total bilirubin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; GGTP, γ-glutamyltransferase; AMY, amylase; BUN, blood urea nitrogen; CRE, creatinine; TG, triglyceride; FFA, free fatty acid; TC, total cholesterol; LDL-C, low-density-lipoprotein cholesterol; FBS, fasting blood sugar; NH3, ammonia.

Corporation, Carlsbad, CA, USA) as described previously [23]. The levels of mRNAs were quantified by real-time PCR using a SYBR Premix Ex Taq™ II (Takara Bio, Otsu, Japan) on a Thermal Cycler Dice TP800 system (Takara Bio). The primer sequences are shown in Supplementary Table 1, whose specificity was confirmed by nucleotide blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Hepatic mRNA levels were determined using the ΔΔCt method as described previously [28], normalized to those of *18S* ribosomal RNA, and then expressed as fold changes relative to those of control livers.

2.7. Immunoblot analysis

Preparation of whole liver lysates was carried out as described previously [29]. Protein concentration was measured colorimetrically with BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA). Whole liver lysates (20 μg of protein) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis [30]. Three samples from each group were loaded into each electrophoresis assay and all samples were examined. After electrophoresis, proteins were transferred to nitrocellulose membranes and incubated with primary antibodies (1:200 dilution) against medium-chain and very-long-chain acyl-coenzyme A (CoA) dehydrogenase (ACADM and ACADVL, respectively), acyl-CoA oxidase 1 (ACOX1), PPARα and δ (PPARA and PPARD, respectively), sterol regulatory element-binding protein 1 (SREBF1), c-Jun-N-terminal kinase (JNK), and phosphorylated JNK (p-JNK) purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA), followed by alkaline phosphatase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Bands of β-actin and histone H1 were used as loading controls for ACADM/ACADVL/ACOX1/JNK/p-JNK and PPARA/PPARD/SREBF1, respectively. Band intensities were measured densitometrically, normalized to those of loading control, and subsequently expressed as fold changes relative to those of normal control livers.

2.8. Assays for DNA-binding activity of PPARs

The DNA-binding activity of nuclear PPARα, PPARδ, and PPARγ was determined using PPARα, PPARδ, and PPARγ Transcription Factor Assay kits (Cayman Chemical, Ann Arbor, MI, USA), respectively. These assays are based on an enzyme-linked immunosorbent assay using PPAR response element-immobilized microplates and specific PPAR antibodies, thus offering similar results to those from the conventional radioactive electrophoretic mobility shift assay. DNA-binding assays were carried out according to the manufacturer's instructions using 50 μg of protein prepared as described elsewhere [31]. Results are expressed as fold changes relative to those of normal livers.

2.9. Statistical analysis

Statistical analyses were performed using SPSS software version 17.0 for Windows (SPSS, Chicago, IL, USA) and Stat Flex version.6 for Windows (ARTECH, Osaka, Japan). Clinical parameters were expressed as median (range) or number (percentage) and the others values were presented as mean ± SEM. For parametric variables, comparisons between groups were made using the one-way ANOVA with Bonferroni's correction. For nonparametric continuous variables, the Kruskal–Wallis test was used and followed by the Mann–Whitney *U*-test with Bonferroni's correction. Categorical variables were analyzed by the Fisher's exact probability test. Correlation coefficients were calculated using Spearman's rank

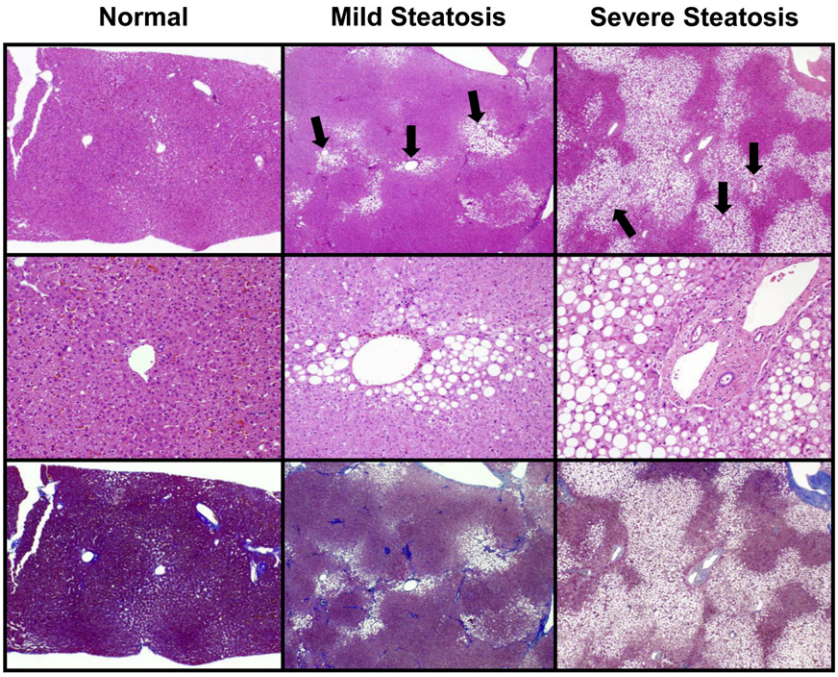

Fig. 1. Representative liver histology of CTLN2. Samples were stained with the hematoxylin and eosin (upper and middle rows) or Azan–Mallory method (lower row). Original magnification;×20 (upper and lower rows) and × 100 (middle row), respectively. Arrows indicate central vein.

correlation analysis. A *p* value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. CTLN2 patients having hepatic steatosis exhibit a lean phenotype

All liver samples from 16 CTLN2 patients had steatosis. Ten patients were histologically diagnosed as having mild steatosis and the remaining 6 patients had severe steatosis. Clinical features of normal control group (n = 7) and CTLN2 patients with mild steatosis (n = 10) and severe steatosis (n = 6) are summarized in Table 1. In the mild steatosis group, BMI was lower than that of the control group. In the severe steatosis group, body weight, BMI, and waist circumference were significantly lower than those in the control group. In CTLN2 patients, serum levels of aspartate and alanine aminotransferases (AST and ALT, respectively), ammonia, and citrulline were significantly higher than those of the control group. Hepatic ASS activities were reduced in CTLN2 patients as reported previously [32], but there were no significant differences between the mild and severe steatosis groups.

Representative liver histology is demonstrated in Fig. 1 and histological findings are summarized in Table 2. Steatotic hepatocytes were present mainly around central vein and the grade of steatosis and NAFLD activity score were significantly different between the groups. In CTLN2 patients, the grade of lobular inflammation was significantly higher than that of the control group. Only 3 patients (19%) were diagnosed as having steatohepatitis. The stage of fibrosis was not statistically different among the groups.

The degree of steatosis was correlated with serum levels of ALT ($r = 0.415$, $P = 0.031$) and citrulline ($r = 0.638$, $P = 0.002$) and was inversely correlated with body weight ($r = -0.580$, $P = 0.004$), BMI ($r = -0.617$, $P = 0.002$), waist circumference ($r = -0.675$, $P < 0.001$), and serum albumin concentrations ($r = -0.626$, $P = 0.002$) (Supplementary Fig. 1). These results corroborate previous observations that CTLN2-related fatty liver is not accompanied by obesity and visceral fat accumulation.

3.2. Hepatic FA/TG metabolism is disrupted in CTLN2 patients

To examine the mechanism of steatogenesis in CTLN2 patients, hepatic mRNA levels of genes associated with FA uptake, transport and activation were determined. The mRNAs encoding *CD36*, FA-binding protein 1 (*FABP1*), and long-chain acyl-CoA synthetase (*ACSL1*) were significantly decreased in the CTLN2 groups compared with the control group (Fig. 2A). The *CD36*, *FABP1*, and *ACSL1* mRNAs in the severe steatosis CTLN2 group were lower than those in the mild steatosis CTLN2 group (Fig. 2A). FAs are catabolized through β-oxidation in the mitochondria, peroxisomes, and ω-oxidation in

Table 2
Histological findings of livers.

	Normal	Mild steatosis	Severe steatosis	*P* value		
	(N = 7)	(N = 10)	(N = 6)	Normal vs mild	Normal vs severe	Mild vs severe
NAFLD activity score	0 (0–0)	2 (1–3)	4 (3–5)	<0.001	<0.001	<0.001
Steatosis (%)	0 (0–0)	20 (5–30)	70 (60–80)	–	–	–
Steatosis 0/1/2/3	7/0/0/0	0/10/0/0	0/0/1/5	0.004	0.043	0.015
Lobular inflammation 0/1/2/3	7/0/0/0	4/6/0/0	0/6/0/0	0.035	<0.001	0.234
Ballooning 0/1/2	0/0/0	8/2/0	5/1/0	0.925	0.861	0.884
Fibrosis 0/1A/1B/1C/2/3/4	7/0/0/0/0/0/0	5/3/0/2/0/0/0	2/2/0/1/0/1/0	0.937	0.928	1.000

The steatosis/lobular inflammation/ballooning/fibrosis grades were scored according to the criteria proposed by Kleiner et al.[27]. Results are expressed as median (range) or number. A *p* value of less than 0.05 was underlined.

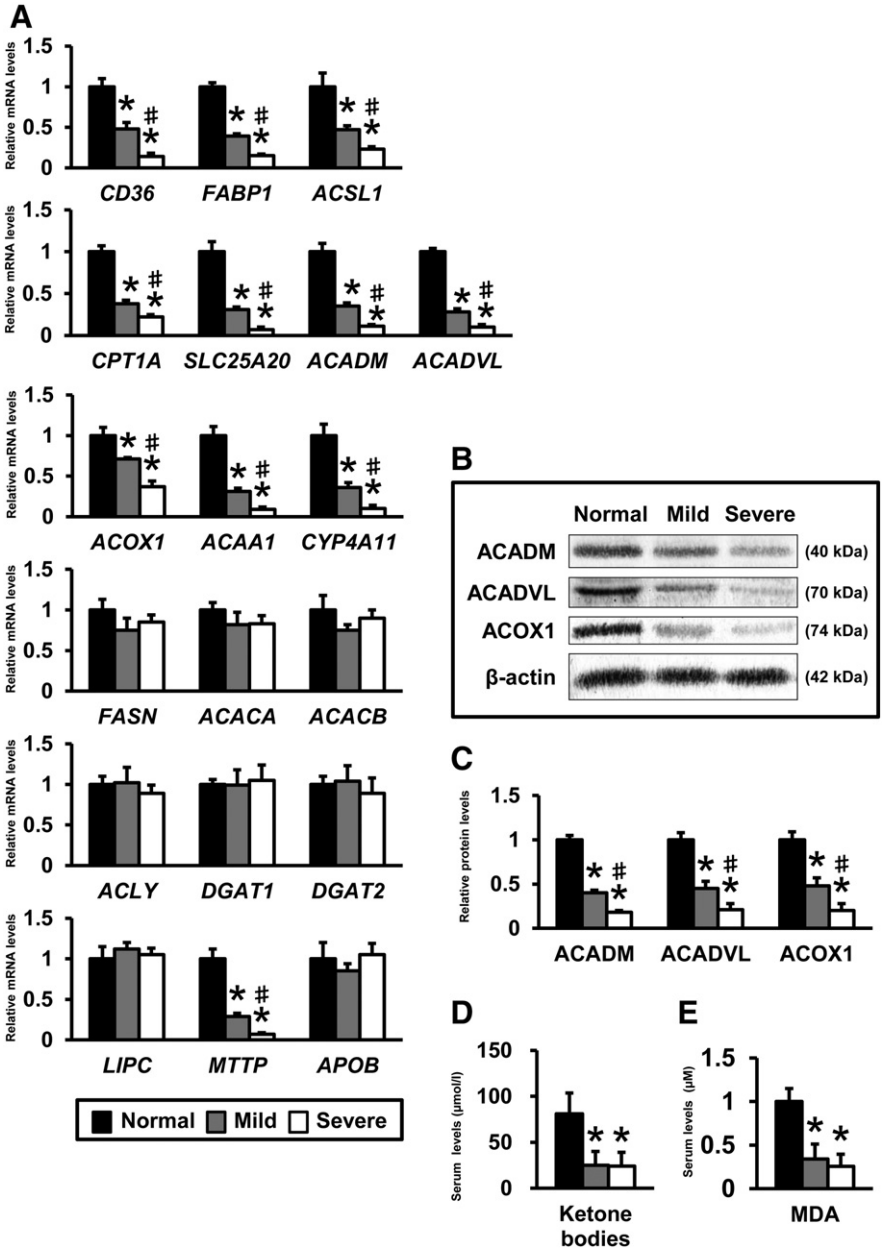

Fig. 2. Down-regulation of hepatic FA β-oxidation enzymes and MTTP in CTLN2. (A) The mRNAs encoding genes associated with FA/TG metabolism. Hepatic mRNA levels were normalized to those of *18S* ribosomal RNA and then expressed as fold changes relative to those of the normal group. (B) Immunoblot analysis of ACADM, ACADVL and ACOX1. A representative immunoblot is shown. β-actin was used as the loading control. (C) Band intensities were measured densitometrically, normalized to those of β-actin, and then expressed as fold changes relative to those of normal livers. (D and E) Serum levels of ketone bodies (D) and malondialdehyde (MDA, E). Results were expressed as mean ± SEM. Comparisons between groups were made using the one-way ANOVA with Bonferroni's correction. Black bar, normal controls (n = 7); Gray bar, mild steatosis CTLN2 group (n = 10); White bar, severe steatosis CTLN2 group (n = 6). $^{*}P < 0.05$ compared with normal controls; $^{\#}P < 0.05$ compared with mild steatosis group.

microsomes. The mRNA levels of genes encoding carnitine palmitoyl-CoA transferase 1A (*CPT1A*), carnitine-acylcarnitine carrier (CAC, encoded by *SLC25A20*), *ACADM*, and *ACADVL*, which are enzymes/proteins responsible for mitochondrial β-oxidation, were significantly decreased in CTLN2 groups and further decreased in the severe steatosis group (Fig. 2A). Similar changes were observed in the mRNAs encoding peroxisomal β-oxidation enzymes, such as ACOX1 (*ACOX1*) and acetyl-CoA acyltransferase 1 (*ACAA1*), and a microsomal ω-oxidation enzyme cytochrome P450 4A11 (*CYP4A11*) (Fig. 2A).

There were no significant changes in the mRNA encoding enzymes associated with lipogenesis, such as FA synthase (*FASN*), acetyl-CoA carboxylases α and β (*ACACA* and *ACACB*, respectively), ATP citrate lyase (*ACLY*), and diacylglycerol-O-acyltransferases 1 and 2 (*DGAT1* and *DGAT2*, respectively), among the 3 groups (Fig. 2A). Although the mRNA levels encoding apolipoprotein B (*APOB*) and hepatic lipase (*LIPC*) were not altered among the groups, the mRNAs encoding microsomal TG transfer protein (*MTTP*), a key protein transporting TG from the liver as very-low-density lipoprotein, were significantly suppressed in CTLN2 livers and further decreased in CTLN2 livers showing severe steatosis (Fig. 2A).

The mRNA levels of the genes down-regulated in CTLN2 livers were inversely correlated with the severity of steatosis (Supplementary Fig. 2). These results suggest that down-regulation of FA oxidation-associated enzymes and MTTP is associated with TG accumulation in the livers of CTLN2.

The changes in FA oxidation were assessed further by immunoblot analyses revealing significant reductions in the expression of ACADM, ACADVL and ACOX1 in the liver of CTLN2 patients that are consistent

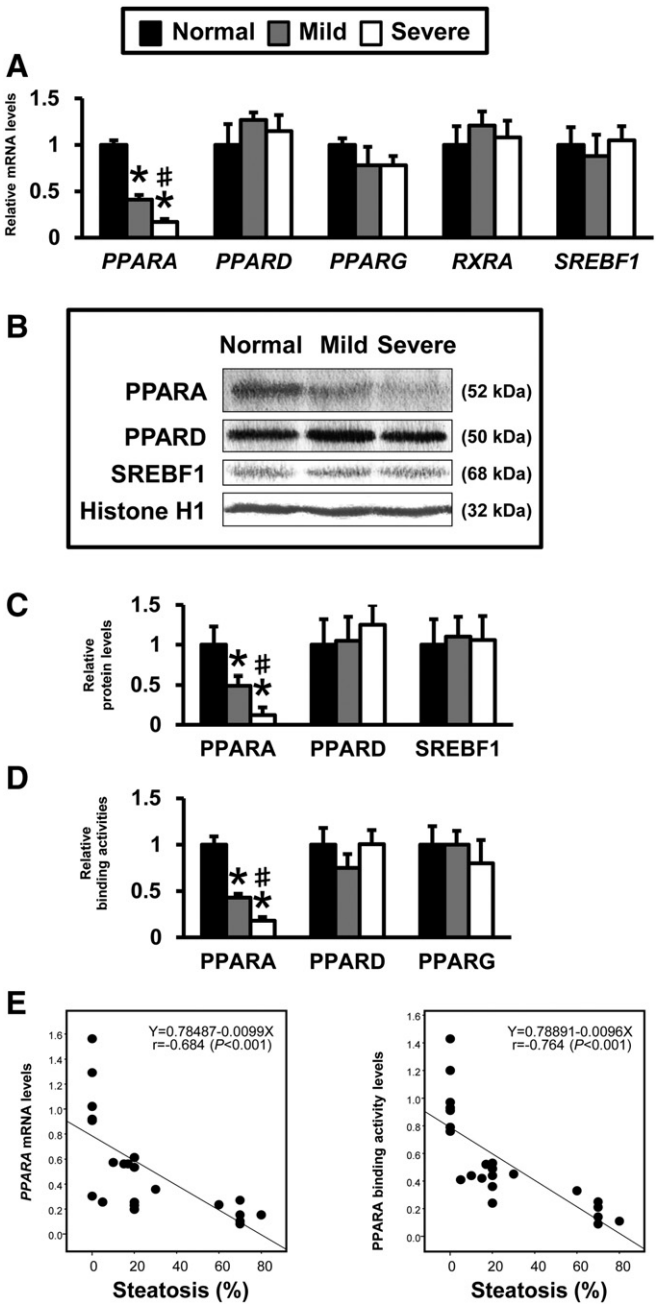

Fig. 3. Down-regulation of hepatic PPARα in CTLN2. (A) Hepatic mRNA levels of *PPARs/RXRA/SREBF1*. The same samples used in Fig. 2 were adopted. (B) Immunoblot analysis of PPARs and SREBF1. A representative immunoblot is shown. Histone H1 was used as the loading control. (C) Band intensities were measured densitometrically, normalized to those of histone H1, and then expressed as fold changes relative to those of normal livers. (D) PPARα DNA-binding activity based on an enzyme-linked immunosorbent assay. Binding activity levels were expressed as fold changes relative to those of normal livers. (E) Correlation between *PPARA* mRNA, PPARα DNA-binding activity, and severity of steatosis. Results were expressed as mean ± SEM. Comparisons between groups were made using the one-way ANOVA with Bonferroni's correction. Black bar, normal controls (n = 7); Gray bar, mild steatosis CTLN2 group (n = 10); White bar, severe steatosis CTLN2 group (n = 6). $^{*}P < 0.05$ compared with normal controls; $^{\#}P < 0.05$ compared with mild steatosis group. Correlation coefficients were calculated using Spearman's rank correlation analysis.

with the changes in mRNAs (Fig. 2B and C). Serum levels of ketone bodies, which are indicators of mitochondrial β-oxidation activity, sharply declined in the CTLN2 patients (Fig. 2D). FA oxidation is coupled with oxidative stress generation. Consistent with down-regulation of FA oxidation, circulating MDA levels were significantly reduced in CTLN2 patients (Fig. 2E). Neither reductions in mRNAs encoding oxidative stress-generating enzymes, such as xanthine dehydrogenase (*XDH*) and NADPH oxidases (*CYBB* and *NOX4*), nor inductions in mRNAs encoding oxidative stress-eliminating enzyme, including catalase (*CAT*) and superoxide dismutases (*SOD1* and *SOD2*), were seen (Supplementary Fig. 3A). Serum levels of ketone bodies and MDA were inversely correlated with the degree of steatosis (Supplementary Fig. 3B). Collectively, these results demonstrate that marked impairment of FA oxidation is associated with the development of hepatic steatosis in the CTLN2 patients.

3.3. Hepatic PPARα is down-regulated in CTLN2 patients

Since the expression of the genes involved in FA/TG metabolism is regulated by several transcriptional factors, such as PPARs, retinoid X receptor α (RXRα), and SREBFs, the expression of these factors was assayed. Although the *PPARD*, *PPARG*, *RXRA*, and *SREBF1* mRNAs were not different among the groups, *PPARA* mRNA was significantly decreased in the CTLN2 groups compared with the controls. The decreases were more marked in the severe steatosis group (Fig. 3A). Immunoblot analysis and DNA-binding activity assays further revealed that PPARα was functionally deficient in these patients (Fig. 3B–D). *PPARA* mRNA levels and PPARα-binding activities were inversely correlated with the degree of steatosis ($r = -0.684$, $P < 0.001$ and $r = -0.764$, $P < 0.001$, respectively) (Fig. 3E).

It was reported that PPARα regulates the transcriptional levels of several genes, such as *CD36*, *FABP1*, *ACSL1*, *CPT1A*, *SLC25A20*, *ACADM*, *ACADVL*, *ACOX1*, *ACAA1*, *CYP4A11*, and *MTTP*[31,33–37], all of which are down-regulated in the livers of CTLN2 patients. Indeed, a strong correlation was detected between *PPARA* mRNA levels and its target genes (Supplementary Fig. 4). Therefore, down-regulation of hepatic PPARα and the ensuing disruption of FA/TG metabolism were considered as a mechanism of steatogenesis due to CD.

3.4. Relationship between clinical parameters and hepatic PPARα expression

Hepatic *PPARA* mRNA levels were significantly correlated with body weight ($r = 0.711$, $P < 0.001$), BMI ($r = 0.651$, $P < 0.001$), waist circumference ($r = 0.600$, $P = 0.005$), and serum albumin concentrations ($r = 0.569$, $P = 0.006$) (Fig. 4A). Interestingly, *PPARA* mRNAs were inversely correlated with circulating levels of ammonia ($r = -0.503$, $P = 0.031$) and citrulline ($r = -0.492$, $P = 0.037$) (Fig. 4A). However, there were no significant correlations between *PPARA* mRNA and serum AST or ALT levels. These results suggest a close relationship between down-regulation of PPARα and clinical features in CTLN2.

3.5. Factors associated with down-regulation of hepatic PPARα in CTLN2 patients

The causes of PPARα down-regulation in CTLN2 patients were assessed. Inflammatory signaling, especially nuclear factor kappa B (NF-κB) signaling, can suppress PPARα activity. Lobular inflammation was detected in CTLN2 livers and increases in mRNAs of tumor necrosis factor α (*TNF*) and the decreases in mRNAs for NF-κB inhibitor α (*NFKBIA*) were seen in the severe steatosis group, but not in the mild steatosis group (Fig. 4B). There were no meaningful changes in mRNAs encoding other pro-inflammatory mediators, such as interleukin (IL) *1B*, *IL6*, and transforming growth factor β1 (*TGFB1*) (Fig. 4B). Recently, it was documented that activation of JNK down-regulates PPARα [38]. Immunoblot analysis revealed increased p-JNK in steatotic livers of CTLN2 (Fig. 4C and D). Therefore, JNK activation is one of the possible molecular mechanisms that could suppress the PPARα activity caused by CTLN2.

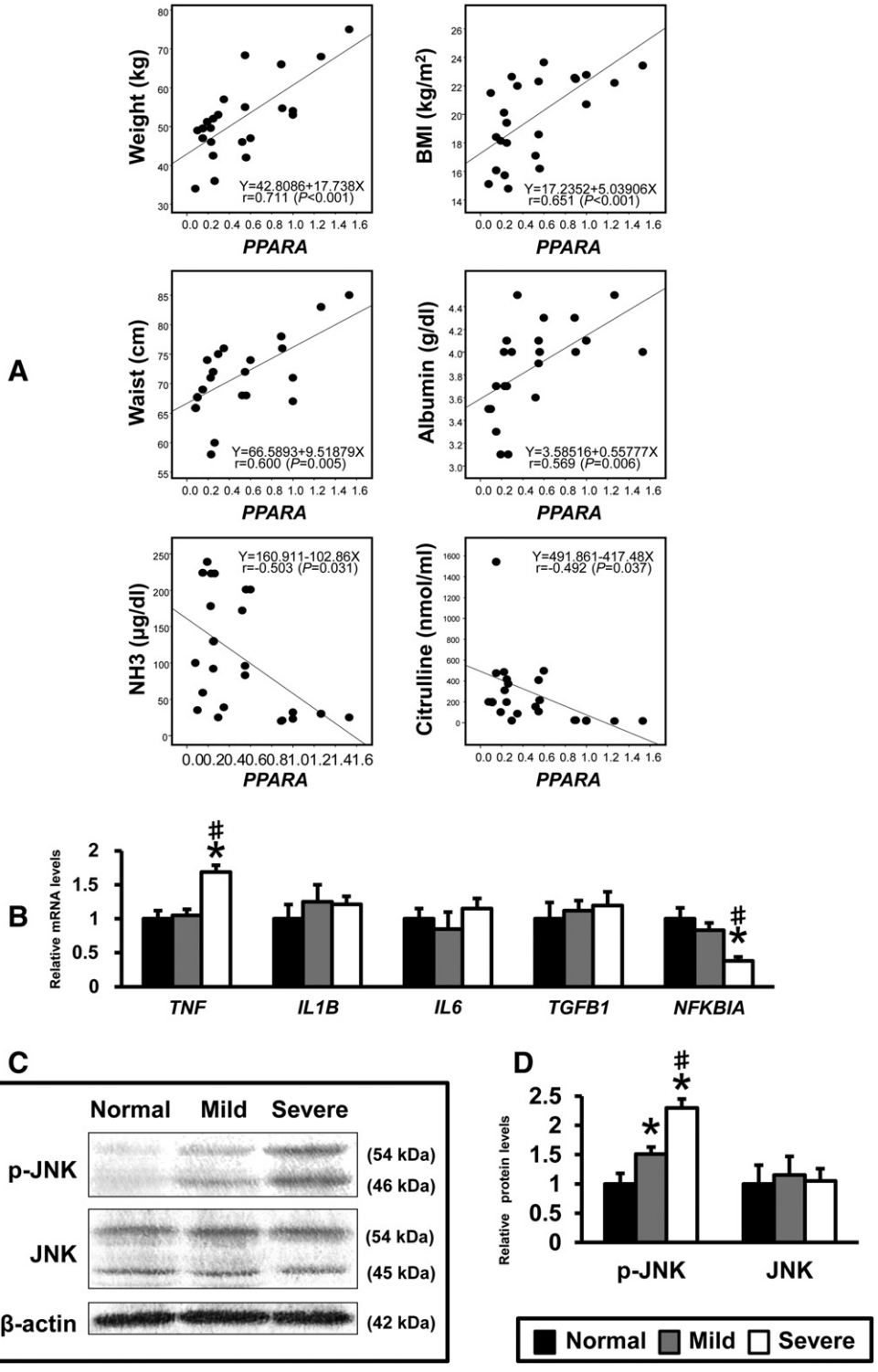

Fig. 4. Factors associated with down-regulation of PPARα in CTLN2. (A) Correlation between *PPARA* mRNA levels and clinical parameters. Correlation coefficients were calculated using Spearman's rank correlation analysis. (B) Hepatic mRNA levels of genes associated with inflammation. The same samples used in Fig. 2 were adopted. (C) Immunoblot of JNK. The same samples used in Fig. 2 were subjected to immunoblot analysis. (D) Band intensities were measured densitometrically, normalized to those of β-actin, and then expressed as fold changes relative to those of normal livers. Results were expressed as mean ± SEM. Comparisons between groups were made using the one-way ANOVA with Bonferroni's correction. Black bar, normal control (n = 7); Gray bar, mild steatosis CTLN2 group (n = 10); White bar, severe steatosis CTLN2 group (n = 6). $^{*}P < 0.05$ compared with normal controls; $^{\#}P < 0.05$ compared with mild steatosis group.

4. Discussion

In the present study, the mechanism of steatogenesis accompanied by CTLN2 was examined and significant down-regulation of genes encoding FA oxidation enzymes and MTTP was found in the patients' liver. Serum concentrations of ketone bodies, an indicator of mitochondrial β-oxidation activity, were also significantly decreased in CTLN2 patients and were inversely correlated with the severity of steatosis. Furthermore, PPARα, the main regulator of FA oxidation, was markedly suppressed in these patients, likely due to JNK activation. These results

indicate that down-regulation of PPARα is associated with hepatic steatosis in CTLN2 patients.

The transcriptional levels of several FA-metabolizing enzymes/proteins are directly regulated by PPARα [33,39–42]. Mice lacking the *Ppara* gene demonstrate constitutively low expression of FA-oxidizing enzymes and significant decreases in FA oxidation activity, leading to severe steatosis and hypoketonemia by fasting [43]. Down-regulation of PPARα and its target genes, such as *FABP1*, was also reported in human non-alcoholic steatohepatitis (NASH), which is consistent with the results of the current study [44]. The fact that *Mttp* mRNA levels are not decreased in *Ppara*-null mice [41] suggests that the contribution of PPARα to MTTP expression is not as strong as that of the FA-oxidizing enzymes, such as ACADM and ACADVL.

The precise molecular mechanisms on how PPARα is down-regulated in the livers of CTLN2 patients need to be discussed. A recent report demonstrated that forced overexpression of JNK by adenovirus suppressed the PPARα mRNA levels in cultured cardiac myocytes [38]. Additionally, pre-treatment with a JNK inhibitor to cultured cardiac myocytes inhibited TNFα-induced PPARα down-regulation without affecting inflammatory signaling [38]. These findings suggest that JNK activation is a possible mechanism for PPARα down-regulation in CTLN2 patients. FA accumulation due to PPARα down-regulation may activate JNK probably through a direct effect on FA and/or endoplasmic reticulum stress [45,46], leading to a vicious cycle of down-regulating PPARα and further lowering FA oxidation activity. Enhancement of inflammatory signaling found in CTLN2 livers with severe steatosis might be also associated with reductions in PPARα activity to some degree. Additionally, it was documented that hepatic expression of enzymes involved in ureagenesis, such as carbamoyl-phosphate synthase, ornithine transcarbamoylase, and ASS, was suppressed by PPARα activation in mice [47]. These findings suggest that down-regulation of PPARα may be an adaptive response to urea cycle disruption.

CTLN2 is one of the major urea cycle disorders [7,13]. Urea cycle disorders are sometimes accompanied by hepatic steatosis [1,48]. As far as we know, this is the first report demonstrating a link between a urea cycle disorder, hepatic steatosis, and down-regulation of FA oxidation and PPARα. To convert 1 mol of $NH4+$ to urea, 3 mol of ATP is required. Mitochondrial β-oxidation can generate ATP more efficiently than glycolysis, but hepatic ATP levels may already be low in CTLN2 livers due to a severe decline in PPARα signaling and mitochondrial β-oxidation activity. Although down-regulation of PPARα might be compensation for urea cycle disruption at first, the persistent and significant down-regulation may be coupled with further decreases in hepatic ATP contents and impairment of the urea cycle, resulting in hyperammonemia. The peculiar fondness of fat-rich diet seen in CTLN2 patients [13,16] might be an adaptive response to obtain more ATP through FA oxidation. Based on the results in this study, strategies to enhance mitochondrial β-oxidation activity and increase hepatic ATP levels may be beneficial for moderating metabolic abnormalities in CTLN2.

The present study provides several points that need further investigation. First, the molecular mechanism of PPARα down-regulation caused by CD/CTLN2, especially the involvement of JNK signaling, should be determined using suitable mouse models or cell culture experiments. Second, the effect of PPARα agonist on CTLN2-related fatty liver needs to be evaluated. Indeed, a beneficial effect of bezafibrate on CTLN2-related hypertriglyceridemia has been reported [49]. Lastly, the method detecting the alterations in PPARα-regulated pathways may provide new clues to more accurately evaluate the severity of CTLN2. Untargeted metabolomic approaches using serum/urine would be a promising tool [50].

In conclusion, hepatic PPARα and FA oxidation are significantly down-regulated in CTLN2 patients. This study demonstrates a novel mechanism of steatogenesis caused by CD and proposes a connection between urea cycle disorder and disruption of PPARα signaling and lipid metabolism in the liver.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2014.12.011.

Conflict of interest

The authors have declared that no conflict of interest exists.

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