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Mechanism of the development of nonalcoholic steatohepatitis after pancreaticoduodenectomy

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ABSTRACT

Background and aim: It is recognized that nonalcoholic fatty liver disease (NAFLD), including nonalcoholic steatohepatitis (NASH), may develop after pancreaticoduodenectomy (PD). However, the mechanism of NASH development remains unclear. This study aimed to examine the changes in gene expression associated with NASH occurrence following PD.
Methods: The expression of genes related to fatty acid/triglyceride (FA/TG) metabolism and inflammatory signaling was examined using liver samples obtained from 7 post-PD NASH patients and compared with 6 healthy individuals and 32 conventional NASH patients.
Results: The livers of post-PD NASH patients demonstrated significant up-regulation of the genes encoding CD36, FA-binding proteins 1 and 4, acetyl-coenzyme A carboxylase α, diacylglycerol acyltransferase 2, and peroxisome proliferator-activated receptor (PPAR) γ compared with normal and conventional NASH livers. Although serum apolipoprotein B (ApoB) and TG were decreased in post-PD NASH patients, the mRNAs of ApoB and microsomal TG transfer protein were robustly increased, indicating impaired TG export from the liver as very-low-density lipoprotein (VLDL). Additionally, elevated mRNA levels of myeloid differentiation primary response 88 and superoxide dismutases in post-PD NASH livers suggested significant activation of innate immune response and augmentation of oxidative stress generation.
Conclusions: Enhanced FA uptake into hepatocytes and lipogenesis, up-regulation of PPARγ, and disruption of VLDL excretion into the circulation are possible mechanisms of steatogenesis after PD.
General significance: These results provide a basis for understanding the pathogenesis of NAFLD/NASH following PD.

Abbreviations: ACACA, acetyl-CoA carboxylase α; ACACB, acetyl-CoA carboxylase β; ACADM, medium-chain acyl-CoA dehydrogenase; ACOX1, acyl-CoA oxidase 1; ALT, alanine aminotransferase; ApoB, apolipoprotein B; AST, aspartate aminotransferase; BMI, body mass index; CAT, catalase; CoA, coenzyme A; CPT1A, carnitine palmitoyl-CoA transferase 1α; CT, computed tomography; CYBB, cytochrome b-245 β polypeptide; CYP, cytochrome P450; DGAT, diacylglycerol acyltransferase; FA, fatty acid; FABP, fatty acid-binding protein; FASN, fatty acid synthase; γGT, gamma-glutamyltransferase; HADHA, hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase α; HBV, hepatitis B virus; HCV, hepatitis C virus; HOMA-IR, homeostasis model assessment for insulin resistance; LPS, lipopolysaccharide; LXR, liver X receptor; MCD, methionine- and choline-deficient diet; MTTP, microsomal triglyceride transfer protein; MYD88, myeloid differentiation primary response 88; NAFLD, nonalcoholic fatty liver disease; NAS, NAFLD activity score; NASH, nonalcoholic steatohepatitis; PD, pancreaticoduodenectomy; PPAR, peroxisome proliferator-activated receptor; PPARGC, PPARγ co-activator; qPCR, quantitative polymerase chain reaction; ROS, reactive oxygen species; RXR, retinoid X receptor; SCD, stearoyl-CoA desaturase; SOD, superoxide dismutase; SREBF1, sterol regulatory element-binding transcription factor 1; TG, triglyceride; TGFB1, transforming growth factor β1; TLR, Toll-like receptor; TNF, tumor necrosis factor α; US, ultrasonography; VLDL, very-low-density lipoprotein.

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1. Introduction

The prevalence of nonalcoholic fatty liver disease (NAFLD) is increasing worldwide. In general, NAFLD is closely linked with overnutrition, visceral fat accumulation, and obesity. Nonalcoholic steatohepatitis (NASH) is a serious subtype of NAFLD that may progress to cirrhosis, hepatocellular carcinoma, and hepatic failure [1–3]. Therefore, understanding the pathogenesis of NASH is important for the development of proper preventive and therapeutic strategies. The initial step of NASH development is accumulation of triglycerides (TGs) into hepatocytes. Sources of intrahepatic TG are non-esterified fatty acids (FAs) released from white adipose tissue and absorbed from the small intestine, as well as those newly synthesized from citrate. FAs are further metabolized to acetyl-coenzyme A (CoA) mainly through mitochondrial β-oxidation or esterified to TG, which is either stored in hepatocytes or incorporated into very-low-density lipoprotein (VLDL) and released into the circulation. Therefore, disruption of these metabolic pathways causes hepatosteatosis. Enhanced inflammatory signaling and cellular stress injure steatotic hepatocytes and activate Kupffer cells and stellate cells, resulting in steatohepatitis [4–6].

The pancreas plays a central role in the absorption of essential nutrients, such as fat, amino acids, and fat-soluble vitamin. It is well known that NAFLD/NASH may develop after pancreatic resection [7–9]. We previously reported clinical characteristics of NAFLD developed after pancreaticoduodenectomy (PD) [7]. Most of these patients were diagnosed as having steatohepatitis by liver biopsy, but were lean and had lower levels of serum albumin, total cholesterol, apolipoprotein B (ApoB), and insulin compared with conventional NASH patients [7]. Hepatic steatosis following PD was ameliorated by intensifying oral supplementation of pancreatic enzymes [7], revealing a close link between steatogenesis, pancreatic exocrine insufficiency, and malabsorption/maldigestion. These results are in agreement with the recent reports from the other groups [8,9] and suggest that the mechanism of steatogenesis after PD is different from that of conventional NAFLD/NASH accompanying obesity and insulin resistance. However, the mechanism of post-PD NAFLD/NASH occurrence has not been evaluated.

In the present study, the expression of genes associated with FA/TG metabolism, inflammation, and oxidative stress, which are key contributors of NASH development, was examined using liver samples obtained from post-PD NASH patients and compared with healthy individuals and conventional NASH patients. The livers of post-PD NASH exhibited significant increases in the mRNAs related to intrahepatic FA uptake and FA/TG synthesis. The mRNAs encoding ApoB and microsomal TG transfer protein (MTTP) were increased regardless of reduced circulating ApoB and TG, suggesting impairment of TG excretion from the liver. Additionally, hepatic mRNAs of myeloid differentiation primary response 88 (MyD88, encoded by *MYD88*) and superoxide dismutase (SOD) 1 and 2 (encoded by *SOD1* and *SOD2*, respectively), which are associated with innate immunity and oxidative stress, respectively, were augmented. These results propose possible mechanisms of post-PD NASH development caused by pancreatic exocrine insufficiency and malabsorption/malnutrition.

2. Material and methods

2.1. Patients

2.1.1. Post-PD NASH patients

The detailed patients' selection criteria were described previously [7]. Briefly, 80 patients who underwent PD (Whipple's procedure) between January 2001 and December 2006 at Showa Inan General Hospital and Iida Municipal Hospital without regular alcohol consumption were examined. These patients were all negative for hepatitis B virus (HBV) surface antigen and anti-hepatitis C virus (HCV) antibody and did not have detectable hepatic steatosis before PD. Eight patients died within 6 months after PD and 12 were unavailable for repeated abdominal computed tomography (CT) examinations for more than 6 months afterwards. The presence of newly appearing hepatic steatosis was judged as a liver-to-spleen attenuation ratio of less than 0.9 in unenhanced abdominal CT. In 13 patients developing NAFLD after PD, 8 patients received percutaneous liver biopsy and were diagnosed as having steatohepatitis [7]. Liver samples from 7 patients were available for mRNA analysis.

2.1.2. Conventional NASH patients

Liver samples were obtained from 32 NASH patients who underwent a liver biopsy at Shinshu University or its affiliated hospitals between April 2006 and March 2008. NASH was suspected by the following criteria: (1) the detection of steatosis by abdominal ultrasonography (US); (2) the absence of regular intake of alcohol or drugs; (3) negative results for HBV surface antigen and anti-HBV core and anti-HCV antibodies; and (4) the absence of other types of chronic liver disease, such as autoimmune liver disease, hereditary hemochromatosis, Wilson's disease, α1-antitrypsin deficiency, and citrin deficiency. The diagnosis of NASH was confirmed by liver histology.

2.1.3. Normal controls

Normal livers were obtained from 6 healthy liver transplantation donors at the time of pre-operative liver biopsy who 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; and (4) normal liver histology [10,11].

2.1.4. Clinical data collection

Body height and weight were determined by nursing staff unaware of the subjects' medical information. The presence of obesity was defined as having a body mass index (BMI) of more than 25 kg/m^2 based on criteria released by the Japan Society for the Study of Obesity. The diagnosis of the presence of hypertension, diabetes, and hyperlipidemia is made based on the criteria described previously [10–12]. Blood samples were obtained at the time of liver biopsy following overnight fasting for 8–10 h. Laboratory data, such as aspartate and alanine aminotransferase (AST and ALT, respectively) and γ-glutamyltransferase (γGT), were measured by standard methods using automated analyzers. The homeostasis model assessment for insulin resistance (HOMA-IR) value was calculated as described elsewhere [10–12].

2.2. Liver biopsy and histological evaluation

Liver samples were obtained from 2 different sites in the same lobe using a 14-gauge needle by percutaneous US-guided biopsy [7,10,11]. Fragments of liver tissue (5–7 mm) were immediately frozen with a RNA stabilization solution (RNAlater® solution, Life Technologies, Grand Island, NY, USA) in liquid nitrogen and stored at −80 °C until RNA extraction. The remaining specimens were fixed in 10% neutral formalin, cut in 4-μm thickness, and stained using the hematoxylin and eosin or Azan–Mallory method. Histological findings were assessed in a blinded fashion by an independent pathologist and scored according to the staging/grading system proposed by Kleiner et al. [13]. As a minor modification, Mallory bodies were scored as none to rare (0), few (1), or many (2). The NAFLD histological activity score (NAS) was calculated as the unweighted sum of the scores for steatosis (0–3), lobular inflammation (0–3), and ballooning (0–2). The histological diagnosis of NASH was made by the presence of macrovesicular steatosis and hepatocyte ballooning.

2.3. mRNA analysis

Total RNA was extracted from frozen liver samples of healthy individuals (n = 6), conventional NASH (n = 32), and post-PD NASH

(n = 7) using a RNeasy Mini Kit (Qiagen, Tokyo, Japan) and cDNA was generated by SuperScript II reverse transcriptase (Gibco BRL, Paisley, Scotland). Quantitative PCR (qPCR) was performed by use of SYBR green PCR kit and ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with the primer pairs summarized in Supplementary Table 1. All mRNA levels were determined using the ΔΔCt method as described previously [10,11]. The mRNA levels of target genes were normalized to those of *18S* ribosomal RNA and expressed as fold changes relative to those of normal livers.

2.4. Ethics

This study was approved by the ethical committee of Showa Inan General Hospital, Iida Municipal Hospital, and Shinshu University School of Medicine and adheres to the principles of the Declaration of Helsinki. Informed consent was obtained from all patients.

2.5. Statistical analysis

Statistical analyses were performed using Prism 6 for Windows (GraphPad Software Inc., La Jolla, CA, USA). Clinical parameters were expressed as a number (percentage) or median (range). Comparisons between multiple groups were made using the one-way ANOVA test with Bonferroni's correction for continuous variables and the Chi square or Fisher's exact probability test for categorical variables. A *P* value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Post-PD NASH patients exhibit malnutrition

Clinical features of post-PD NASH patients were compared with healthy individuals. Serum AST, ALT, and γGT concentrations were increased, but none had obesity and hyperlipidemia in post-PD NASH patients (Supplementary Table 2). Additionally, serum levels of albumin and ApoB were significantly lower in these patients (Supplementary Table 2). These differences became more marked when post-PD NASH patients were compared with conventional NASH patients. BMI, circulating albumin, total cholesterol, TG, ApoB, and HOMA-IR were lower in the post-PD NASH patients (Supplementary Table 2). Histological findings revealed similar degree of steatosis, inflammation, ballooning, fibrosis, and NAS between the two NASH groups (Supplementary Table 3).

3.2. Up-regulation of genes associated with FA uptake in post-PD NASH

In order to explore the mechanism of steatogenesis in post-PD NASH, hepatic expression of genes associated with FA uptake from blood into hepatocytes was examined. The levels of mRNA encoding CD36, FA-binding protein 1 (FABP1), and FABP4 were significantly elevated in post-PD NASH group compared with normal control and conventional NASH groups (Fig. 1A). These results demonstrate that up-regulation of the genes involved in FA uptake may be associated with steatogenesis after PD.

3.3. Up-regulation of lipogenic genes in post-PD NASH

The expression of genes related to lipogenesis was measured. Acetyl-CoA carboxylase α and β (ACACA and ACACB, respectively) convert acetyl-CoA into malonyl-CoA, and FA synthase (FASN) catalyzes the formation of palmitate from acetyl-CoA and malonyl-CoA. In addition to these enzymes, stearoyl-CoA desaturase (SCD) is linked with *de novo* FA synthesis. Diacylglycerol acyltransferase 1 (DGAT1) and 2 (DGAT2) are rate-limiting enzymes of TG synthesis. Among these genes, the mRNA levels of genes encoding ACACA and DGAT2 were significantly higher in post-PD NASH group compared with normal control and conventional NASH groups (Fig. 1B).

3.4. Expression of genes related to FA oxidation

Among the enzymes involved in peroxisomal β-oxidation [acyl-CoA oxidase 1 (ACOX1)], mitochondrial β-oxidation [carnitine palmitoyl-

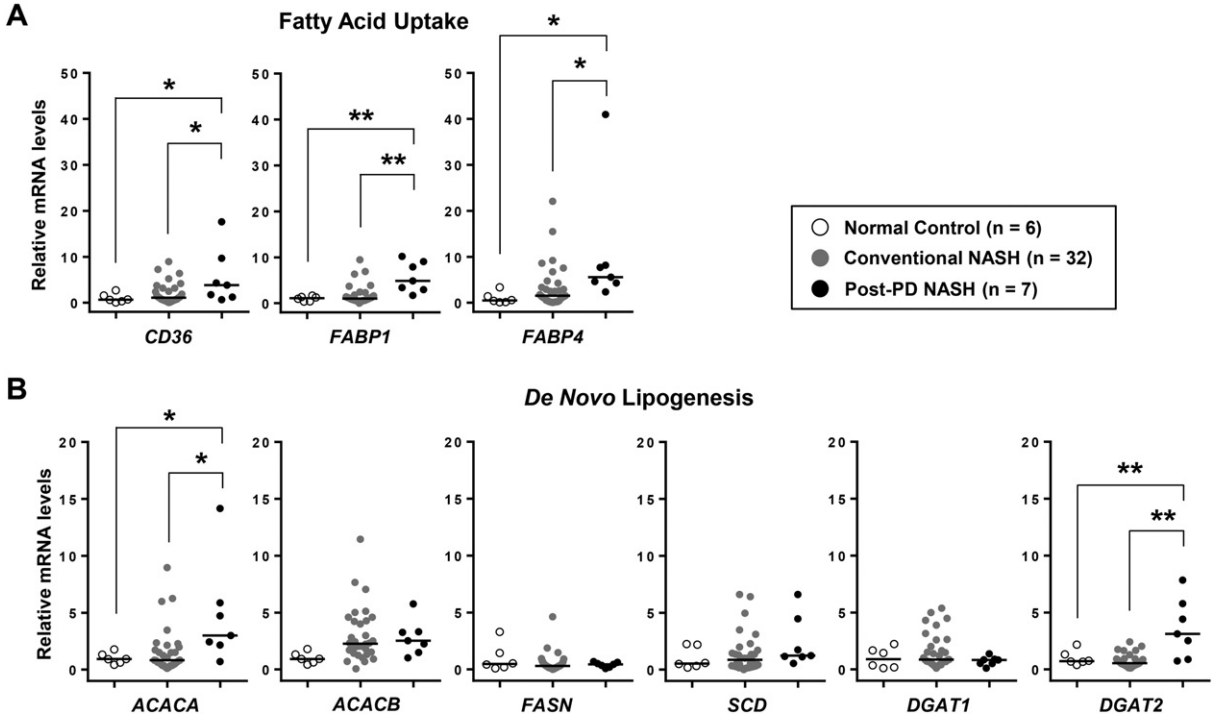

Fig. 1. Hepatic expression of genes encoding enzymes/proteins involved in fatty acid uptake (A) and *de novo* lipogenesis (B). Bars express the median. $^{*}P < 0.05$, $^{**}P < 0.01$.

CoA transferase 1α (CPT1A), medium-chain acyl-CoA dehydrogenase (ACADM), and hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase α (HADHA)], and microsomal ω-oxidation [cytochrome P450 (CYP) 2E1 and 4A11] [14,15], the levels of mRNA encoding CPT1A and ACADM were elevated in post-PD NASH patients compared with normal controls, but there were no significant differences in *ACOX1* mRNA levels (Fig. 2A). The up-regulation of *CPT1A* and *ACADM* mRNAs appeared to be a compensatory response to hepatic fat accumulation.

3.5. Up-regulation of genes associated with VLDL formation/secretion in post-PD NASH

While serum concentrations of ApoB, a major component of VLDL particle, were significantly reduced (Supplementary Table 2), hepatic mRNAs encoding APOB were robustly increased in the patients having post-PD NASH compared with normal individuals (Fig. 2B). Additionally, the mRNA levels of *MTTP*, a protein transferring TG from liver to blood as VLDL, were significantly increased in the post-PD NASH patients compared with normal individuals and conventional NASH patients regardless of low serum TG levels. These results indicate disruption of VLDL synthesis and/or secretion in post-PD NASH livers.

3.6. Up-regulation of PPARγ in post-PD NASH

The expression of enzymes/proteins involved in hepatic lipid metabolism is regulated by nuclear receptors and transcription factors, such as peroxisome proliferator-activated receptor α (PPARA) and γ (PPARG), liver X receptor α (LXRA), and sterol regulatory element-binding protein 1c (SREBF1). Although there were no meaningful differences in the expression of genes encoding retinoid X receptor α (RXRA), SREBF1, LXRA, and PPARγ co-activator 1β (PPARGC1B), significant increases in *PPARA* and *PPARG* mRNA levels were detected in post-PD NASH livers compared with normal controls and conventional NASH livers (Fig. 3). Since PPARγ induces the expression levels of CD36 and FABP4 leading to hepatic adipogenesis [16,17], activation of the PPARγ-mediated pathway may contribute to the steatogenesis after PD.

3.7. Up-regulation of MYD88 in post-PD NASH

Since inflammatory signaling promotes progression from steatosis to steatohepatitis, the expression of pro-inflammatory cytokine genes was assessed. The mRNA levels of genes encoding tumor necrosis factor α (TNFα, encoded by *TNF*) and its receptors (TNFRSF1A and TNFRSF1B), and transforming growth factor β1 (TGFB1) were not different between the groups (Fig. 4A). The expression of genes involved in innate immune system was also measured. While there were no significant differences in the mRNA levels of Toll-like receptor 4 (*TLR4*) and *CD14*, the *MYD88* mRNAs were significantly increased in post-PD NASH livers compared with control and conventional NASH livers (Fig. 4B). The *TLR2* mRNA levels were also elevated in post-PD NASH livers compared with conventional NASH livers (Fig. 4B). MyD88 is a critical modulator of lipopolysaccharide (LPS)- and TNFα-mediated signaling [18]. These results suggest significant activation of MyD88-mediated signaling in post-PD NASH livers.

3.8. Up-regulation of SOD in post-PD NASH

Oxidative stress is another key promoter of NASH development. Among oxidative stress-related genes, the mRNAs encoding SOD1 and SOD2 were significantly augmented in post-PD NASH livers compared with conventional NASH livers (Fig. 5). The mRNA levels of genes encoding catalase (CAT), glutathione peroxidase 1 (GPX1), and NADPH oxidase 2 (CYBB) were not altered in post-PD NASH livers. Since the mRNA levels of *SOD1/2* are induced in response to oxidative stress [19], these results suggest greater oxidative stress in post-PD NASH livers compared with conventional NASH livers.

4. Discussion

NAFLD/NASH may develop after PD, but the mechanism of steatogenesis is not understood. The present study revealed significant up-regulation of PPARγ and its downstream genes associated with FA uptake into hepatocytes, such as *CD36* and *FABP4*, and genes involved in lipogenesis in the post-PD NASH livers. Marked increases in hepatic

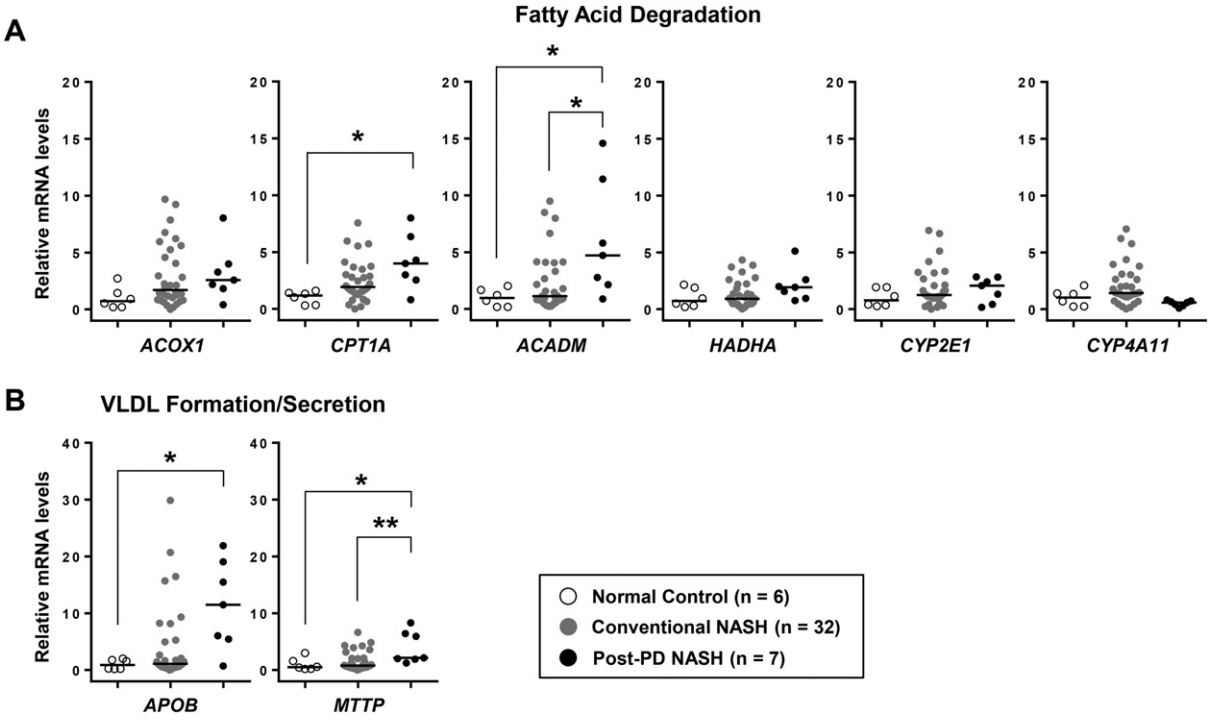

Fig. 2. Hepatic expression of genes encoding enzymes/proteins involved in fatty acid degradation (A) and VLDL formation/secretion (B). Bars express the median. $^{*}P < 0.05$, $^{**}P < 0.01$.

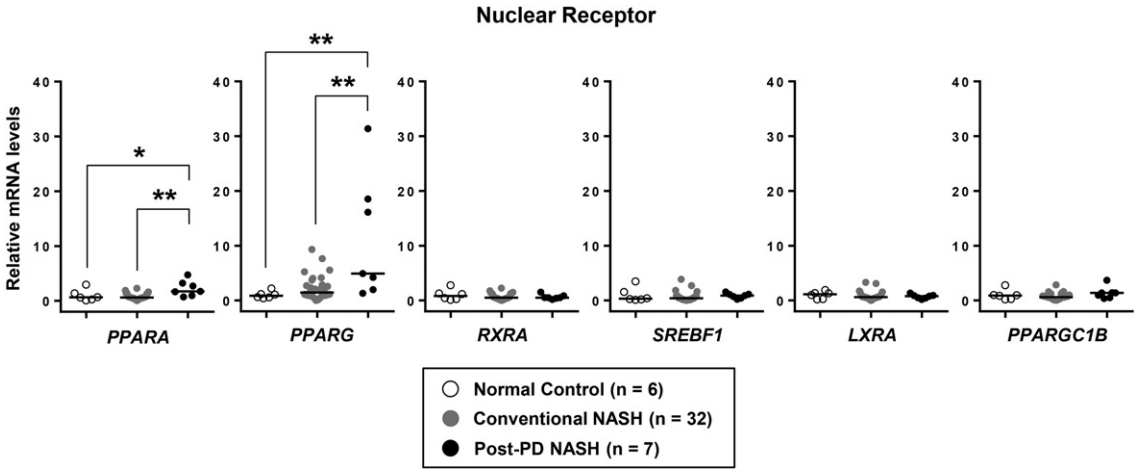

Fig. 3. Hepatic expression of genes encoding nuclear receptors. Bars express the median. $^{*}P < 0.05$, $^{**}P < 0.01$.

APOB/MTTP expression but reduced serum ApoB/TG concentrations suggested disruption of VLDL synthesis/secretion. Therefore, enhanced FA uptake and lipogenesis, up-regulation of PPARγ, and impairment of TG export from the liver are possible mechanisms of steatogenesis after PD. In addition, up-regulation of MyD88 and antioxidant genes was also observed in post-PD NASH livers. These results provide novel information regarding the pathogenesis of post-PD NAFLD/NASH.

The livers of post-PD NASH demonstrated marked up-regulation of PPARγ and its target genes, such as *FABP4* and *CD36*. PPARγ is a key regulator of adipogenesis that is mainly expressed in white adipose tissue. While hepatic basal expression of PPARγ is relatively low, forced PPARγ expression in hepatocytes using *Pparg*-encoding adenovirus led to severe TG accumulation and hepatic adipogenesis [16], revealing that aberrant PPARγ expression in the liver can cause steatosis. Activation of PPARγ and up-regulation of its target genes were reported in human NAFLD with moderate-to-severe steatosis [20]. The other study showed that the mRNA levels of *FABP4* and *CD36* were correlated with liver fat percentage in NAFLD patients [21]. It is intriguing that the activation of PPARγ was more marked in post-PD NASH livers compared with conventional NASH, suggesting greater contribution of PPARγ-mediated pathway to the pathogenesis of post-PD NASH.

Increased expression of *ACACA* and *DGAT2* mRNAs was also associated with hepatic TG accumulation after PD. It was documented that hepatic mRNA levels of *ACACA* tended to be higher in NAFLD patients with moderate-to-severe steatosis compared with non-NAFLD individuals [20]. While the expression of *ACACA* is regulated by SREBF1 and

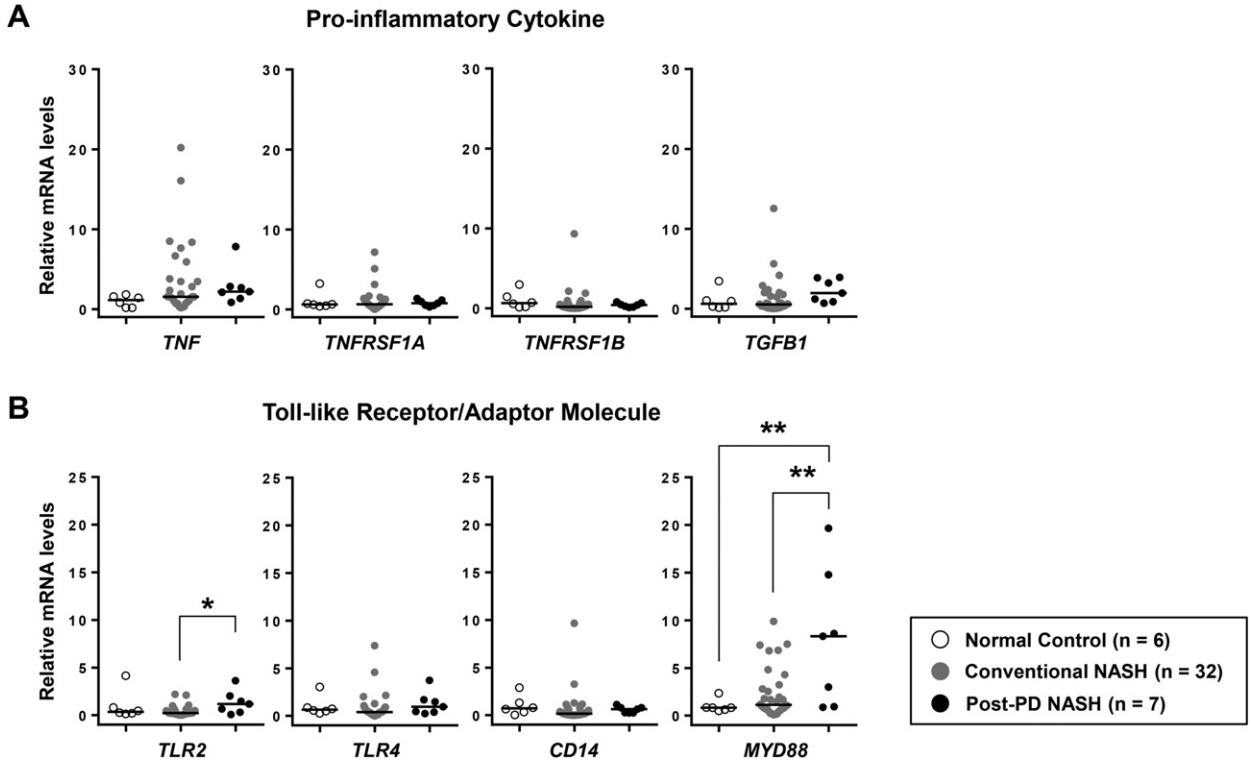

Fig. 4. Hepatic expression of genes encoding pro-inflammatory cytokines (A) and toll-like receptor-related molecules (B). Bars express the median. $^{*}P < 0.05$, $^{**}P < 0.01$.

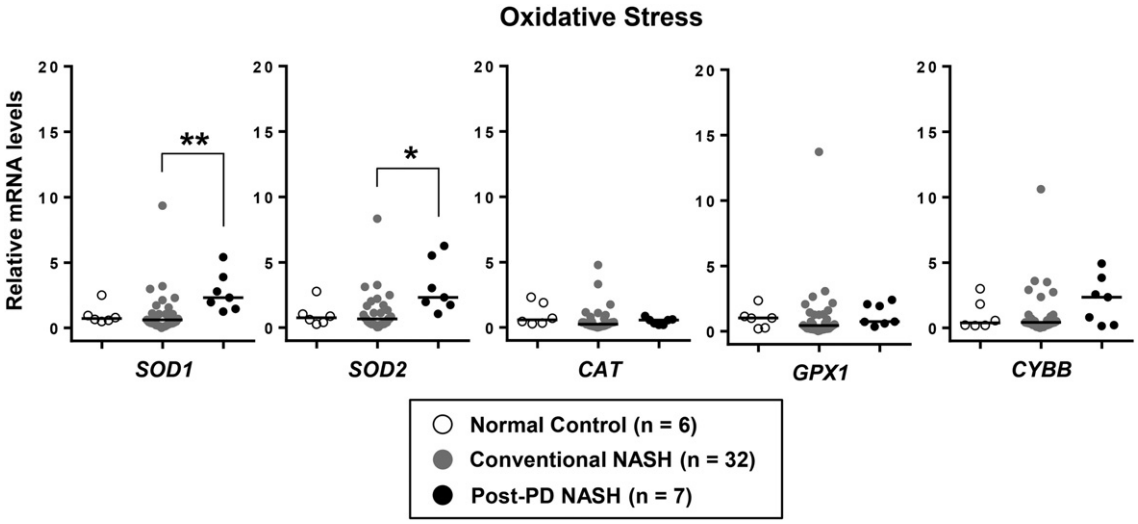

Fig. 5. Hepatic expression of genes encoding oxidative stress-related enzymes. Bars express the median. $^{*}P < 0.05$, $^{**}P < 0.01$.

its upstream LXRA, there were no increases in *SREBF1/LXRA* mRNAs in post-PD NASH. Up-regulation of *ACACA* mRNAs might occur through SREBF1-independent mechanism.

Serum TG levels reflect the amount of TG involved in VLDL, and serum ApoB levels mainly indicate the contents of ApoB in VLDL. While serum VLDL concentrations could not be measured in this study, decreased serum TG/ApoB and increased hepatic fat contents and *MTTP/APOB* expression led us to consider that VLDL formation/secretion is disrupted in post-PD NASH livers. Impaired VLDL secretion is sometimes linked with NAFLD development in humans [22]. Disruption of TG secretion from the liver might be a common mechanism of malnutrition-related NAFLD.

PPARα activation enhances mitochondrial β-oxidation activity accelerating FA degradation in the liver [14]. Additionally, down-regulation of PPARα is associated with steatogenesis in humans [11]. While the expression of *PPARA* and its target genes, such as *CPT1A* and *ACADM*, was increased in post-PD NASH livers, these changes are likely an adaptation to severe hepatic fat accumulation. Indeed, some kinds of FA can activate PPARα [23].

Our previous study demonstrated several similarities of phenotypic changes between humans having post-PD NASH and mice fed a methionine- and choline-deficient diet (MCD). Increased FA uptake, up-regulated PPARγ expression, impaired VLDL secretion, and compensatory induction of β-oxidation enzymes were documented in the mouse livers of MCD-induced NASH [24–27]. There is a view that MCD feeding is not suitable for studying the mechanism of human NASH because of the lack of obesity and insulin resistance. However, the murine MCD model largely reproduces the pathologies of post-PD NASH in humans.

Increased MyD88 expression was found in post-PD NASH livers, but not in conventional NASH, suggesting a major role of MyD88-mediated pathway, which is activated by LPS [18], for the development of post-PD NASH. Gut bacterial overgrowth might occur after PD due to intestinal hypomobility, decreased secretion of gastric juice, or blind loops. Additionally, malnutrition due to pancreatic exocrine insufficiency may induce intestinal mucosal atrophy leading to bacterial translocation [28–30]. Therefore, up-regulation of MyD88 may reflect continuous portal endotoxinemia and indicate an important role of gut-liver axis for NASH development after PD. Attenuating intestinal bacterial overgrowth and mucosal atrophy might be beneficial for post-PD NASH.

In response to enhanced oxidative stress generation, oxidative stress-related transcription factors, such as NF-E2-related factor 2, are activated and anti-oxidant genes are induced [31]. As shown in Fig. 2A, the expression of β-oxidation enzymes is enhanced in steatotic hepatocytes to degrade surplus FA, resulting in increased generation of reactive oxygen species (ROS). LPS and lipid peroxides can activate Kupffer cells and stellate cells, augmenting ROS production in these cells and thus injuring hepatocytes. The results of the present study suggest greater contribution of oxidative stress to the pathogenesis of post-PD NASH compared with conventional NASH.

The major limitation of this study is small cohort size that is derived from lower incidence of post-PD NAFLD/NASH compared with conventional NAFLD/NASH in the general population and difficulty to obtain liver samples by percutaneous liver biopsy. Although this study is likely preliminary, the results may be of great significance in understanding the pathogenesis of post-PD NASH and will serve as a foundation for more comprehensive studies in the future.

In this study, post-PD NASH patients were older compared with the normal controls and conventional NASH patients (Supplementary Table 2). We could not compare gene expression between age-matched patients because of the small cohort size. It was reported that PPARγ levels were decreased with age [32,33], but post-PD NASH livers showed higher hepatic PPARγ levels compared with normal and conventional NASH livers. Therefore, aging presumably does not give the great impact on hepatic PPARγ expression in the post-PD NASH patients.

While pancreatic enzyme supplementation can ameliorate hepatosteatosis after PD [7], the changes in gene expression after the treatment could not be assessed in this study. Further studies are needed to address this issue. Additionally, the mechanism on how hepatic PPARγ is induced after PD or under hyponutritional state also deserves future investigation for understanding the association between PPAR and nutrients.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbacli.2015.02.001.

Transparency document

The Transparency document associated with this article can be found, in the online version.

Conflict of interest

The authors have declared that no conflict of interest exists.

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Contribution

Study design; Naoki Tanaka

Acquisition of data; Tadanobu Nagaya, Naoki Tanaka, Takefumi Kimura, Hiroyuki Kitabatake, Naoyuki Fujimori, Michiharu Komatsu, Takahiro Yamaura, Takeji Umemura, Kenji Sano, and Akira Horiuchi

Analysis of data; Tadanobu Nagaya, Kimura Takefumi, and Naoki Tanaka

Supervision; Akira Horiuchi, Toshifumi Aoyama, Frank J. Gonzalez, and Eiji Tanaka

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