学位論文

表題

PTPRD遺伝子多型は非アルコール性脂肪性肝疾患の発症・進展に関与する

専攻名

旭川医科大学大学院医学系研究科
博士課程医学専攻

著者名

中嶋 駿介
Polymorphism of Receptor-Type Tyrosine-Protein Phosphatase Delta gene in the
development of non-alcoholic fatty liver disease

Shunsuke Nakajima¹, Hiroki Tanaka², Koji Sawada¹, Hidemi Hayashi³, Takumu
Hasebe¹, Masami Abe³, Chitomi Hasebe³, Mikihiro Fujiya¹ and Toshikatsu Okumura¹

1. Division of Gastroenterology and Hematology/Oncology, Department of Medicine,
Asahikawa Medical University, Asahikawa, Japan

2. Department of Legal Medicine, Asahikawa Medical University, Asahikawa, Japan

3. Department of Gastroenterology, Asahikawa Red Cross Hospital, Asahikawa, Japan

Correspondence

To whom all correspondence should be addressed;

Koji Sawada, M.D. Ph.D.

Division of Gastroenterology and Hematology/Oncology, Department of Medicine,
Asahikawa Medical University, Asahikawa, Japan
2-1 Midorigaoka-higashi, Asahikawa, Hokkaido 078-8510, Japan

Tel: +81-166-68-2462

Fax: +81-166-68-2469

e-mail: k-sawada@asahikawa-med.ac.jp

**Declaration of conflict of interest:** The authors do not have any disclosure to report.

**Acknowledgments:** This work was supported, in part, by Health Labour Sciences Research Grant (Research on Hepatitis: 2015) and by Grants-in-Aid for Young Scientists (B) provided by the Ministry of Education, Culture, Sports, Science, and Technology, in Japan.
Abstract

**Background and Aim:** Some single nucleotide polymorphisms (SNPs) are associated with the development of non-alcoholic fatty liver disease (NAFLD). As one of the genetic factors, *PNPLA3* rs738409 (I148M) is important to associate with pathogenesis of NAFLD. Since other SNPs remain unclear in Japan, we performed high-throughput sequencing, which targeted more than 1,000 genes to identify a novel genetic variant in Japanese patients with NAFLD.

**Methods:** The present study in 36 NAFLD patients and 27 healthy volunteers (HVs) was performed. A high-throughput sequencer was used to detect the gene variations. Candidate genes were validated by TaqMan SNP genotyping assay in 53 NAFLD patients and 41 HVs. To investigate the function of candidate gene, biochemical analyses were performed in cultured hepatocytes and liver tissues.

**Results:** *EXO1* rs1047840, *PTPRD* rs35929428, *IFNAR2* rs2229207, *CPOX* rs1131857, *IL23R* rs1884444, *IL10RA* rs2228055, and *FAM3B* rs111988437 were identified as candidate genetic variants and *PTPRD* rs35929428 was only extracted as a SNP predicting to cause protein dysfunction. In validation analysis, *PTPRD* rs35929428 associated with the development of NAFLD (p=0.015, OR=5.00, 95%CI: 1.33-18.70). In addition, *PTPRD* rs35929428 was associated with Fib-4 index and with hepatic fat
droplets. Biochemical analyses indicated that *PTPRD* rs35929428 promoted dephosphorylation of tyrosine 705 Signal Transducer and Activator of Transcription 3 (STAT3) (Tyr 705) in hepatocytes.

**Conclusion:*** *PTPRD* rs35929428 was a novel SNP in patients with NAFLD. Through exacerbation of the dephosphorylation of STAT3 (Tyr 705) in hepatocytes, *PTPRD* rs35929428 might play a role in hepatic lipid accumulation and fibrosis, followed by the development of NAFLD.

**Key words** Non-alcoholic fatty liver disease. *PTPRD* rs35929428. Single nucleotide polymorphism. STAT3. Fib-4 index
Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease.\(^1,2\) NAFLD starts with hepatosteatosis, and can progress to non-alcoholic steatohepatitis (NASH), cirrhosis and hepatocellular carcinoma (HCC).\(^1,2\) In the process of the development of NAFLD, fat droplets accumulated, inflammation, and fibrosis were induced by several hits which were dysregulation of adipocytokine,\(^3\) lipotoxicity,\(^4\) Toll-like receptors,\(^5\) gut-microbiota,\(^6\) and iron overload.\(^7\) While the etiology of NAFLD has not been completely understood, the development of NAFLD has been strongly related to energy homeostasis and genetic predisposition.\(^8\) Genome-wide association studies have recently identified a number of loci conferring risk for NAFLD. Among these, the rs738409 C>G (I148M) variant of the patatin like phospholipase domain containing 3 (PNPLA3) gene is the most well validated association, influencing degree of steatosis, grade of inflammation, stage of fibrosis, and risk of HCC\(^9-11\) also in Japan. Especially, this SNP was strongly associated with Matteoni type 4 NAFLD, suggesting a useful marker associated with hepatic fibrosis.\(^12\) However, the detailed mechanism underlying this genetic association remains incompletely understood,\(^10\) thereby less contribution for elucidating the pathogenesis of NAFLD. As well as PNPLA3, several SNPs associated with progression of NAFLD are...
reported. On the other hand, the genetic determinant predisposition except for \(PNPLA3\) remains in Japan. The present study aimed to identify a novel genetic variant in Japanese patients with NAFLD using a high-throughput sequencing which targeted more than 1,000 genes, and to clarify a possible mechanism how the genetic variant involved in the pathogenesis of NAFLD.

**Material and Methods**

**Subjects and clinical diagnosis**

This study was approved by the institutional review board of Asahikawa Medical University. Written informed consent was obtained from all enrolled patients, all of whom were Japanese. NAFLD patients and healthy volunteers (HVs) were recruited from December 2012 to July 2014 at Asahikawa Medical University and Japanese Red Cross Asahikawa Hospital as an estimation group. The NAFLD diagnosis was according to the guideline defined by the Japanese clinical practice guidelines. The methods of collecting HVs were as follow. 1) No history for hepatitis B virus (HBV), hepatitis C virus (HCV), fatty liver, and other liver diseases. 2) No history of liver dysfunction. 3) No history of alcohol abuse and obesity. Physical examination, ultrasound screening, medical history review, questionnaire-based survey for diet,
smoking and alcohol intake, as well as biochemical laboratory tests were performed. Fib-4 index was calculated.\(^{15}\) Cases with known causes of steatosis, e.g. heavy alcohol intake (> 20 g/day), the use of medications/herbals known to contribute to hepatic steatosis were excluded. The subjects with HBV were included if hepatitis B surface (HBs) antigen was positive or HBV DNA positive even if HBs antigen was negative when the level of Hepatitis B core (HBc) antibody was high. All patients tested in this study were HBs antigen negative. HBc antibody was positive in 11 patients. Among them, the level of HBc antibody was high in 1 patient. However, HBV DNA was negative in the patient. The subjects with HCV were included if HCV antibody was positive or HCV RNA was positive. If HCV antibody was low level, the level of HCV RNA was elucidated. The level of HCV antibody was low in 1 patient. However, HCV RNA was negative in the patient. Patients with high likelihood to have other known liver diseases including autoimmune disease and primary biliary cirrhosis based on aforementioned information were also excluded.

**Primer design for custom amplicon sequencing**

We designed multiple primer sets which targeted the exons of 1,031 genes (total of 12,609 amplicons) using the Ion AmpliSeq™ Designer software program
(https://www.ampliseq.com/browse.action) (Life Technologies, Carlsbad, CA, USA),
and these primer sets were provided as five primer pools. These genes were mainly
involved in inflammation, energy metabolism, carcinogenesis and metal
metabolism/transport described in our recent publication that examined the autoimmune
pancreatitis susceptibility in Japanese peoples. The 12,609 amplicons and the targeted
lesions are described.

Sample preparation for amplicon sequencing

Peripheral blood samples were processed for mononuclear cell isolation by
Ficoll gradient centrifugation. The genomic DNA was then extracted and purified using
DNeasy Blood & Tissue Kits (Qiagen, Venlo, Netherlands). The DNA concentrations
were determined by a QubitTM Fluorometer (Life Technologies, Carlsbad, CA, USA).
The quality of the genomic DNA was assessed by agarose gel electrophoresis.

Data analysis for gene variations

All sequencing data were mapped on a human reference genome sequence
(GRCh37/hg19) using the Torrent Suite Software program (Life technologies). The
gene variants were then detected by a Torrent Variant Caller plug-in for the software program (Life technologies). The variant information for each sample was imported into the CLC Genomics Workbench software system (CLC bio, Aarhus, Denmark), and Fisher’s exact test was performed to determine the significance of the differences among samples. DNA sequencing was performed according to the previous study.  

TaqMan SNP genotyping assay

The SNP genotyping in each DNA samples was evaluated by TaqMan SNP genotyping assay in the estimation group. By using TaqMan probes identified EXO1 rs1047840, IL23R rs1884444, CPOX rs1131857, IL10RA rs2228055, PTPRD rs35929428, IFNAR2 rs2229207, and FAM3B rs111988437, quantitative real-time PCR (7300 Real-time PCR system; Applied Biosystems) was performed for evaluating SNP genotype according to TaqMan SNP Genotyping assay protocol.

Validation analysis by TaqMan SNP genotyping assay

As a validation group, 17 NAFLD patients and 14 HVs were added to the estimation group. In total, 53 NAFLD patients and 41 HVs were analyzed in the
validation group. In the validation group, the candidate gene that was identified in the estimation group was evaluated by TaqMan SNP genotyping assay.

**Histopathological evaluation and immunohistochemistry**

The biopsy specimens stained with hematoxylin and eosin were evaluated by the classification of Matteoni\(^\text{17}\) and Brunt.\(^\text{18}\) The degree of steatosis was evaluated by amount of fat droplets as observed under the microscope as followed; 0: <5%, 1: <10%, 2: <34%, 3: <67%, 4: >67%\(^\text{12}\). Immunohistochemistry was performed by rabbit polyclonal anti-PTPRD (GeneTax) and rabbit monoclonal anti-phospho Signal Transducer and Activator of Transcription 3 (pSTAT3) (Tyr 705) (Cell–Signaling). As a secondary antibody, anti-rabbit IgG horseradish peroxidase-conjugated (R&D SYSTEMS) was used. PTPRD and pSTAT3 positive cells were analyzed by image J software.

**Transfection of PTPRD R995C and Interleukin-6 treatment**

We incorporated the cDNA that encoded human wild type PTPRD or PTPRD R995C in gene expression vector pBApo–CMV Pur (Takara). These vectors were transfected by Lipofectamine 3000 (Thermo Fisher Scientific) to Huh7 cells that was
derived from Japanese hepatocellular carcinoma and express PTPRD detected by TaqMan probe (data not shown), and then we established stable expression cell strain by treatment with puromycin. Therefore, treatment with puromycin can select Huh7 which transfected wild type PTPRD or PTPRD R995C. Recombinant interleukin–6 (IL–6) (0.5 ng/mL) added to the medium. After 3 hours, protein was extracted by RIPA buffer.

**Western blotting analysis**

Protein expression of STAT3 and pSTAT3 (Tyr 705) in the Huh7 cells were investigated by western blotting. Proteins were reacted overnight at 4°C with either rabbit monoclonal anti-STAT3 (Cell–Signaling), rabbit monoclonal anti-pSTAT3 (Tyr 705) (Cell–Signaling) or actin (BD) and reacted with secondary antibody horseradish peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG (R&D SYSTEMS) for 1 h.

**Statistical analysis**

In the amplicon sequencing analysis, the candidate gene variations were filtered using the P-values from Fisher’s test. The candidate genes with both of the P-values of < 0.05 and accompanying with amino acids substitution were selected. The
results of sequencing analysis and TaqMan SNP assay were compared by Cohen’s kappa. Cohen’s kappa > 0.8 were considered to be reliable of the diagnosis by two methods. Other dates are expressed as median and ranges. Man-Whitney U test or Fisher’s exact test was used in two groups and analysis of variance was used in more than two groups. Values of P < 0.05 were considered statistically significant.

Results

Characteristic of enrolled patients with NAFLD and HVs in the estimation group

A total of 63 participants, including 36 patients with NAFLD and 27 HVs as the estimation group were enrolled in this study. In the 36 patients, 16 patients were male and 20 were female, and the median age was 60.5 years (range; 28–80). Among them, 23 patients performed liver biopsy and 5 patients diagnosed as liver cirrhosis. Matteoni type 3/4 NAFLD were 10 and 7 patients, respectively. In the 27 HVs, the median age was 29.0 years (range, 20–49). The age in the HVs was significantly younger than that in the patients with NAFLD (p<0.01), while the gender ratio was not significantly different between the two groups.

Identification of genetic variations in patients with NAFLD
We first profiled the difference of genetic variants frequencies between in the patients with NAFLD and the HVs. Then 7 SNPs were extracted by Fishers’ exact test when $p$ value less than 0.05 accompanied by amino acid substitution were regarded as significant candidate genes (Table 1). \textit{PNPLA3} rs738409 was not extracted by high throughput sequencer. Next, we evaluated 7 SNPs by TaqMan SNP genotyping assay to confirm the variants obtained by high throughput sequencing and then Cohen’s kappa indicated that 4 SNPs; \textit{EXO1} rs1047840, \textit{PTPRD} rs35929428, \textit{IFNAR2} rs2229207, and \textit{FAM3B} rs111988437 were reliable of the diagnosis by the two methods (Table 1).

Finally, we predicted by PROVEAN prediction (http://provean.jcvi.org/index.php)$^{20}$ whether amino acid substitution by these 4 SNPs had an effect on the protein function. Then \textit{Receptor-Type Tyrosine-Protein Phosphatase Delta (PTPRD)} rs35929428 (R995C) was only extracted as a SNP predicting to cause a protein dysfunction.

**Validation of \textit{PTPRD} rs35929428 by TaqMan SNP genotyping assay**

Next in 53 NAFLD patients and 41 HVs as the validation group, we analyzed validation of \textit{PTPRD} rs35929428 by TaqMan SNP genotyping assay. Clinical characteristics in 53 patients are shown in Table 2. Liver biopsy was performed in 29 of 53 patients and 7 patients were diagnosed as liver cirrhosis. As well as the results of
high throughput sequencing, the ratio of PTPRD rs35929428 GA was significantly higher in NAFLD patients than in that of HVs by TaqMan SNP genotyping assay (Fig. 1).

The association of PTPRD rs35929428 with hepatic fibrosis and fat accumulation

Table 2 showed the clinical characteristics of 38 PTPRD rs35929428 GG and 15 PTPRD rs35929428 GA patients. Between the two groups, there were no significant differences in age, sex, blood examinations, the ratio of metabolic syndrome and HCC, and pathological findings in liver biopsy. The ratio of PTPRD rs35929428 GA was significantly increased in patients with higher Fib-4 index than 1.45 (Fig. 2a), suggesting that PTPRD rs35929428 GA may be involved in the pathogenesis of fibrosis because Fig-4 index is deeply associated with liver fibrosis. On the other hand, pathological findings failed to show a relation between the stage of hepatic fibrosis assessed by liver biopsy and PTPRD rs35929428 GG and GA patients (Fig. 2b). Next, we evaluated the relationship between PTPRD rs35929428 and the degree of hepatic fat accumulation. Representative pathological findings were shown in Fig. 2c and 2d. Mild steatosis was seen in the liver in a PTPRD rs35929428 GG patient (Fig. 2c) while severe steatosis was observed in PTPRD rs35929428 GA (Fig. 2d). The analysis of fat
accumulation in 29 patients who received liver biopsy demonstrated that the degree of
fat droplets was significantly higher in *PTPRD* rs35929428 GA patients than in *PTPRD*
rs35929428 GG patients (Fig. 2e), suggesting that *PTPRD* rs35929428 GA may
associate with hepatic lipid accumulation.

The expression of PTPRD in the liver of NAFLD

It was previously reported that PTPases including PTPRD were mainly
expressed in brain, heart, kidney, and placenta.\(^21\) Because little is however known
whether PTPRD is expressed in the liver, we next analyzed the expression of PTPRD
protein in the liver of NAFLD patients by immunohistochemistry in 15 *PTPRD*
rs35929428 GG and 11 *PTPRD* rs35929428 GA patients. Immunohistochemistry
revealed PTPRD protein expression in the cytosol and nucleus of hepatocytes in all
patients (Fig. 3a and 3b), indicating for the first time that PTPRD is expressed in the
hepatocytes. However, the numbers of PTPRD positive cells were not significant in
patients with *PTPRD* rs35929428 GG and *PTPRD* rs35929428GA patients (Fig 3c).

The function of PTPRD R995C encoded by *PTPRD* rs35929428
PTPRD is a transmembrane protein that dephosphorylates STAT3 at tyrosine 705. We evaluated whether PTPRD R995C that is encoded by PTPRD rs35929428 affected to the phosphorylation of STAT3 in hepatocytes. As immunohistochemistry indicated that PTPRD protein expressed in hepatocytes, in vitro study was performed in Huh 7 cells. Firstly, we confirmed that Huh7 cells had PTPRD rs35929428 GG type. IL-6 significantly accelerated the phosphorylation of STAT3 (Tyr 705) in Huh 7 cells transfected with wild type PTPRD, while this acceleration in the phosphorylation of STAT3 (Tyr 705) was not reproduced in cells transfected with PTPRD R995C (Fig. 4a and 4b), suggesting that PTPRD R995C that is encoded by PTPRD rs35929428 is capable of enhancing the ability of dephosphorylation of STAT3, thereby speculating PTPRD R995C may play a role in functions related to STAT3 signaling in the liver.

As in vitro study demonstrated that PTPRD R995C encoded by PTPRD rs35929428 had gain of function of dephosphorylation of STAT3 (Tyr 705) in hepatocytes, next we evaluated the status of pSTAT3 (Tyr 705) in liver tissue of NAFLD patients by immunohistochemistry in 16 PTPRD rs35929428 GG and 9 PTPRD rs35929428 GA patients. Representative immunohistochemistry revealed that phosphorylation of STAT3 (Tyr 705) was strongly positive in nuclei of hepatocytes in patients with PTPRD rs35929428 GG (Fig. 4c) when compared to in those with PTPRD
rs35929428 GA (Fig. 4d). The numbers of pSTAT3 (Tyr 705) positive cells were significantly decreased in patients with *PTPRD* rs35929428 GA in comparison of those with *PTPRD* rs35929428 GG (Fig. 4e), supporting the results of *in vitro* analysis.

**Discussion**

In the present study, we identified *PTPRD* rs35929428 GA as a novel SNP of Japanese NAFLD, which potentially leads to a protein dysfunction. As the prevalence of *PTPRD* rs35929428 GG, GA, and AA has been reported 81.7%, 13.5%, and 4.8%, respectively in Japan (http://www.internationalgenome.org/), it may be reasonable why *PTPRD* rs35929428 AA patients had not been included in our study. There was no association between *PNPLA3* rs738409 and NAFLD patients in the present study. According to previous publications, *PNPLA3* rs738409 has been considered to be strongly associated with NAFLD. Very recently, Kawaguchi et al. have demonstrated that *PNPLA3* rs738409 was strongly associated with Matteoni type 4 NAFLD patients in the analysis of more than five hundreds NAFLD patients in Japan. Because the present study analyzed a relatively small number of patients with Matteoni type 4, we would suggest that *PNPLA3* rs738409 was not be identified as a candidate gene in the present study.
We next examined whether the novel SNP, *PTPRD* rs35929428 GA may be related to the pathophysiology of NAFLD. Fat accumulation detected by liver biopsy specimens revealed that a higher steatosis was observed in the *PTPRD* rs35929428 GA patients when compared with *PTPRD* rs35929428 GG, suggesting that *PTPRD* rs35929428 GA was associated with the lipid accumulation in the liver in NAFLD patients. Furthermore, in our study, the ratio of Fib-4 index > 1.45 for advanced hepatic fibrosis, was significantly higher in *PTPRD* rs35929428 GA, indicating that *PTPRD* rs35929428 GA may also play a role in the process of liver fibrosis in NAFLD. Since hepatic fat accumulation and hepatic fibrosis are considered to be main pathological changes in NASH, *PTPRD* rs35929428 GA might be specifically involved in the development of NASH.

The main function of PTPRD is dephosphorylation of STAT3 (Tyr 705). STAT3 plays a key role in the regulation of hepatic lipid accumulation, inflammation, regeneration, and fibrosis. These findings led us speculate that the STAT3 signaling may mediate the pathophysiologica roles such as steatosis and fibrosis in NAFLD patients with *PTPRD* rs35929428 GA. To address the above speculation, the phosphorylation of STAT3 in cultured hepatic cells, Huh7 was examined. Firstly, we confirmed that Huh7 cells have *PTPRD* rs35929428 GG type and then PTPRD R995C
encoded *PTPRD* rs35929428 GA was transfected. Since STAT3 signaling is activated by various cytokines such as IL-6, we stimulated phosphorylation of STAT3 with IL-6. *In vitro* study demonstrated that cell transfected with PTPRD R995C that is encoded by *PTPRD* rs35929428 GA was not capable of inducing the phosphorylation of STAT3 (Tyr 705) by IL-6, suggesting that PTPRD R995C had a gain of function as regards to dephosphorylation of STAT3 (Tyr 705). In other words, PTPRD R995C may enhance the activity of dephosphorylation of STAT3, followed by inhibiting the phosphorylation of STAT3. The *in vitro* findings may be supported by the results obtained by liver biopsy specimens because STAT3 phosphorylation was deeply suppressed in *PTPRD* rs35929428 GA. It is therefore speculated that the lowered STAT3 phosphorylation in hepatocytes in NAFLD patients with *PTPRD* rs35929428 GA may be involved in the hepatic steatosis and fibrosis, the main pathological characteristics of NAFLD including NASH since STAT3 is deeply involved in the process of hepatic lipid accumulation and fibrosis as described above.24, 25

*PTPRD* rs35929428 GA was associated with higher Fib-4 index. On the other hand, there was no relation between the degree of liver fibrosis assessed by liver biopsy and *PTPRD* genotype. Since paired liver biopsies demonstrated significant sampling variability in patients with NASH,26 a lack of paired sampling might lead no
association. We would raise an additional possibility of a relatively small number of samples in the present study. Based upon these findings, further studies in a large number of the subjects are needed to further clarify whether $PTPRD$ rs35929428 is associated with the liver fibrosis.

In conclusion, the present study revealed the novel SNP $PTPRD$ rs35929428 for Japanese NAFLD. $PTPRD$ rs35929428 was associated with higher Fib-4 index and steatosis. The mechanisms may be explained as followings. $PTPRD$ R995C that was encoded $PTPRD$ rs35929428 attenuated the phosphorylation of STAT3 (Tyr 705) in the liver, thereby enhancing hepatic fibrosis and steatosis and leading to the development of NAFLD including NASH.

**Acknowledgments**

This work was supported, in part, by Health Labour Sciences Research Grant (Research on Hepatitis: 2015) and by Grants-in-Aid for Young Scientists (B) provided by the Ministry of Education, Culture, Sports, Science, and, Technology, in Japan.

**References**

1. Browning JD, Szczepaniak LS, Dobbins R, et al. Prevalence of hepatic steatosis in an
urban population in the United States: impact of ethnicity. *Hepatology* 2004; **40**: 1387–95.

2 Clark JM. The epidemiology of nonalcoholic fatty liver disease in adults. *J Clin Gastroenterol*. 2006 (Suppl 1); **40**: S5–10.

3 Imajo K, Fujita K, Yoneda M, et al. Hyperresponsivity to low-dose endotoxin during progression to nonalcoholic steatohepatitis is regulated by leptin-mediated signaling. *Cell Metab.* 2012; 16: 44–54.

4 Gentile CL, Pagliassotti MJ. The role of fatty acids in the development and progression of nonalcoholic fatty liver disease. *J Nutr Biochem*. 2008; **19**: 567–76.

5 Sawada K, Ohtake T, Hasebe T, et al. Augmented hepatic Toll-like receptors by fatty acids trigger the pro-inflammatory state of non-alcoholic fatty liver disease in mice. *Hepatol Res*. 2014; **44**: 920–34.

6 Szabo G, Velayudham A, Romics L Jr, et al. Modulation of non-alcoholic steatohepatitis by pattern recognition receptors in mice: the role of toll-like receptor 2 and 4. *Alcohol Clin Exp Res*. 2005; **29** (11 Suppl): 140S–5S.

7 Hasebe T, Tanaka H, Sawada K, et al. Bone morphogenetic protein-binding endothelial regulator of liver sinusoidal endothelial cells induces iron overload in a fatty liver mouse model. *J Gastroenterol*. 2017; **52**: 341–51.
8 Anstee QM, Day CP. The Genetics of Nonalcoholic Fatty Liver Disease: spotlight on PNPLA3 and TM6SF2. *Semin Liver Dis*, 2015; **35**: 270–90.

9 Romeo S, Kozlitina J, Xing C, et al. Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease. *Nat Genet*. 2008; **40**: 1461–5.

10 Anstee QM, Day CP. The genetics of NAFLD. *Nat Rev Gastroenterol Hepatol*. 2013; **10**: 645–55.

11 Liu YL, Patman GL, Leathart JB, et al. Carriage of the PNPLA3 rs738409 C >G polymorphism confers an increased risk of non-alcoholic fatty liver disease associated hepatocellular carcinoma. *J Hepatol*. 2014; **61**: 75–81.

12 Kawaguchi T, Sumida Y, Umemura A, et al. Genetic polymorphisms of the human PNPLA3 gene are strongly associated with severity of non-alcoholic fatty liver disease in Japanese. *PLoS One*. 2012; **7**: e38322.

13 Chalasani N, Guo X, Loomba R, et al. Nonalcoholic Steatohepatitis Clinical Research Network. Nonalcoholic Steatohepatitis Clinical Research Network. Genome-wide association study identifies variants associated with histologic features of nonalcoholic Fatty liver disease. *Gastroenterology* 2010; **139**: 1567–76.

14 Watanabe S, Hashimoto E, Ikejima K, et al. Evidence-based clinical practice guidelines for nonalcoholic fatty liver disease/nonalcoholic steatohepatitis. *Hepatol*
Res. 2015; 45: 363–77.

15 Sumida Y, Yoneda M, Hyogo H, et al. Validation of the FIB4 index in a Japanese nonalcoholic fatty liver disease population. *BMC Gastroenterol.* 2012; 12: 2.

16 Fujibayashi S, Sasajima J, Goto T, et al. A high-throughput sequence analysis of Japanese patients revealed 11 candidate genes associated with type 1 autoimmune pancreatitis susceptibility. *BB report* 2016; 6: 76-81.

17 Matteoni CA, Younossi ZM, Gramlich T, et al. Nonalcoholic fatty liver disease: a spectrum of clinical and pathological severity. *Gastroenterology* 1999; 116: 1413–19.

18 Brunt EM, Janney CG, Di Bisceglie AM, et al. Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. *Am J Gastroenterol.* 1999; 94: 2467–74.

19 Landis JR, Koch GG. The measurement of observer agreement for categorical data. *Biometrics* 1977; 33: 159–74.

20 Choi Y, Chan AP. PROVEAN web server: a tool to predict the functional effect of amino acid substitutions and indels. *Bioinformatics* 2015; 31: 2745–7.

21 Pulido R, Serra-Pagès C, Tang M, et al. The LAR/PTP delta/PTP sigma subfamily of transmembrane protein-tyrosine-phosphatases: multiple human LAR, PTP delta, and PTP sigma isoforms are expressed in a tissue-specific manner and associate with the
LAR-interacting protein LIP.1. *Proc Natl Acad Sci U S A* 1995; 92: 11686–90.

22 Veeriah S, Brennan C, Meng S, et al. The tyrosine phosphatase PTPRD is a tumor suppressor that is frequently inactivated and mutated in glioblastoma and other human cancers. *Proc Natl Acad Sci U S A* 2009; 106: 9435–40.

23 Williams CD, Stengel J, Asike MI, et al. Prevalence of nonalcoholic fatty liver disease and nonalcoholic steatohepatitis among a largely middle-aged population utilizing ultrasound and liver biopsy: a prospective study. *Gastroenterology* 2011; 140: 124–31.

24 Ueki K, Kondo T, Tseng YH, et al, Central role of suppressors of cytokine signaling proteins in hepatic steatosis, insulin resistance, and the metabolic syndrome in the mouse. *Proc Natl Acad Sci U S A* 2004; 101: 10422–7.

25 Shigekawa M, Takehara T, Kodama T, et al. N. Involvement of STAT3-regulated hepatic soluble factors in attenuation of stellate cell activity and liver fibrogenesis in mice. *Biochem Biophys Res Commun.* 2011; 406: 614–20.

26 Ratziu V, Charlotte F, Heurtier A, et al. LIDO Study Group. Sampling variability of liver biopsy in nonalcoholic fatty liver disease. *Gastroenterology* 2005; 128: 1898–906.
| Gene   | Chr/Position | SNP ID     | Base substitution | Amino acid substitution | Effect Freq NAFLD | Effect Freq controls | p value | Cohen's Kappa |
|--------|--------------|------------|-------------------|-------------------------|-------------------|----------------------|---------|---------------|
| EXO1   | 0.036 1.00   | rs110447840| G to A             | E589K                   | 18/36 (47.2%)     | 6/27 (22.2%)         | 1.00    | 1.00          |
| PTPRD  | 0.013 1.00   | rs35929428 | G to A             | R996C                   | 14/36 (36.1%)     | 4/27 (11.1%)         | 0.022   | 0.88          |
| IFNAR2 | 0.003 0.88   | rs101082   | G to A             | F58S                    | 18/36 (50.0%)     | 5/27 (18.5%)         | 0.015   | 1.00          |
| IL10RA | 0.003 0.15   | rs2228055  | A to G             | I224V                   | 16/36 (47.2%)     | 6/27 (22.2%)         | 0.036   | 0.63          |
| IFNAR2 | 0.003 0.88   | rs2229207  | G to A             | F58S                    | 18/36 (50.0%)     | 5/27 (18.5%)         | 0.015   | 1.00          |
| IL23R  | 0.006 0.88   | rs1131857  | T to G             | N273H                   | 18/36 (50.0%)     | 5/27 (18.5%)         | 0.015   | 0.63          |
| IFNAR2 | 0.003 0.88   | rs2229207  | G to A             | F58S                    | 18/36 (50.0%)     | 5/27 (18.5%)         | 0.015   | 1.00          |
| IL10RA | 0.003 0.15   | rs2228055  | A to G             | I224V                   | 16/36 (47.2%)     | 6/27 (22.2%)         | 0.036   | 0.63          |
| IFNAR2 | 0.003 0.88   | rs2229207  | G to A             | F58S                    | 18/36 (50.0%)     | 5/27 (18.5%)         | 0.015   | 1.00          |
| IL10RA | 0.003 0.15   | rs2228055  | A to G             | I224V                   | 16/36 (47.2%)     | 6/27 (22.2%)         | 0.036   | 0.63          |
| IFNAR2 | 0.003 0.88   | rs2229207  | G to A             | F58S                    | 18/36 (50.0%)     | 5/27 (18.5%)         | 0.015   | 1.00          |
| IL10RA | 0.003 0.15   | rs2228055  | A to G             | I224V                   | 16/36 (47.2%)     | 6/27 (22.2%)         | 0.036   | 0.63          |
| IFNAR2 | 0.003 0.88   | rs2229207  | G to A             | F58S                    | 18/36 (50.0%)     | 5/27 (18.5%)         | 0.015   | 1.00          |
| IL10RA | 0.003 0.15   | rs2228055  | A to G             | I224V                   | 16/36 (47.2%)     | 6/27 (22.2%)         | 0.036   | 0.63          |
| IFNAR2 | 0.003 0.88   | rs2229207  | G to A             | F58S                    | 18/36 (50.0%)     | 5/27 (18.5%)         | 0.015   | 1.00          |
| IL10RA | 0.003 0.15   | rs2228055  | A to G             | I224V                   | 16/36 (47.2%)     | 6/27 (22.2%)         | 0.036   | 0.63          |
| IFNAR2 | 0.003 0.88   | rs2229207  | G to A             | F58S                    | 18/36 (50.0%)     | 5/27 (18.5%)         | 0.015   | 1.00          |
| IL10RA | 0.003 0.15   | rs2228055  | A to G             | I224V                   | 16/36 (47.2%)     | 6/27 (22.2%)         | 0.036   | 0.63          |
| IFNAR2 | 0.003 0.88   | rs2229207  | G to A             | F58S                    | 18/36 (50.0%)     | 5/27 (18.5%)         | 0.015   | 1.00          |
| IL10RA | 0.003 0.15   | rs2228055  | A to G             | I224V                   | 16/36 (47.2%)     | 6/27 (22.2%)         | 0.036   | 0.63          |
| IFNAR2 | 0.003 0.88   | rs2229207  | G to A             | F58S                    | 18/36 (50.0%)     | 5/27 (18.5%)         | 0.015   | 1.00          |
### Table 2: Clinical features of PTPDR rs35929428 GG and GA in the validation group

|                  | Total | GG | GA | P value |
|------------------|-------|----|----|---------|
| **Number of subjects** | 53    | 38 | 15 |         |
| **Sex (Male/Female)** | 24/29 | 18/20 | 6/9 | 0.76  |
| **Age (Year)** | 60.0 (22-80) | 59.5 (22-79) | 62.0 (34-80) | 0.64 |
| **AST (U/L)** | 52.0 (20-346) | 49.5 (20-262) | 70.0 (31-346) | 0.07 |
| **ALT (U/L)** | 68.0 (18-538) | 62.0 (18-461) | 84.0 (28-538) | 0.29 |
| **Plt (X10\(^4\)/µL)** | 19.2 (6.0-38.0) | 20.4 (6.0-38.0) | 22.0 (6.1-30.4) | 0.44 |
| **T-Chol (mg/dL)** | 190.0 (122-269) | 184.0 (123-269) | 193.5 (123-269) | 0.91 |
| **FBS (mg/dL)** | 108.5 (87-285) | 113.0 (87-285) | 104.0 (90-218) | 0.56 |
| **HbA1c (%)** | 5.9 (5.0-9.0) | 6.3 (5.1-9.0) | 5.7 (5.0-8.6) | 0.20 |
| **Ferritin (ng/mL)** | 235.3 (11.2-2347.9) | 243.3 (11.2-2347.9) | 175.2 (16.3-1586.7) | 0.96 |
| **Alb (g/dL)** | 4.2 (2.9-5.2) | 4.1 (3.2-5.2) | 4.3 (3.0-5.2) | 0.95 |
| **γ-GTP (U/L)** | 75.0 (16.4-468) | 4.2 (2.9-5.2) | 4.3 (2.9-5.2) | 0.5 |
| **DLp (µmol/L)** | 2.7 (0.0-8.6) | 5.7 (0.0-8.6) | 3.7 (0.0-8.6) | 0.54 |
| **HT (+/-)** | 29/24 | 19/18 | 10/5 | 0.54  |
| **DM (+/-)** | 22/31 | 17/21 | 5/10 | 0.54  |

**PTPRD rs35929428**
| Date   | HCC | AST, aspartate aminotransferase | ALT, alanine aminotransferase | ALP, alkaline phosphatase | γ-GTP, γ-glutamyltransferase | DM, Diabetes Mellitus | HT, Hypertension | DLp, Dyslipidemia | T-Chol, Total cholesterol | FBS, Fasting blood sugar | Liver biopsy | HCC (0.26) |
|--------|-----|--------------------------------|------------------------------|--------------------------|----------------------------|------------------------|---------------------|-------------------|-----------------------------|-----------------------------|--------------|------------|
| 8/45   |     |                               |                              |                          |                            |                        |                     |                   |                             |                             |              |            |
| 6/36   |     |                               |                              |                          |                            |                        |                     |                   |                             |                             |              |            |
| 2/13   |     |                               |                              |                          |                            |                        |                     |                   |                             |                             |              |            |
| 0.9    |     |                               |                              |                          |                            |                        |                     |                   |                             |                             |              |            |

Liver biopsy: 1/3/5/3
AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; γ-GTP, γ-glutamyltransferase; DM, Diabetes Mellitus; HT, Hypertension; DLp, Dyslipidemia; T-Chol, Total cholesterol; FBS, Fasting blood sugar; HCC, hepatocellular carcinoma.
Figure Legends

Figure 1 Genetic variants of PTPRD rs35929428 in NAFLD.

The ratio of PTPRD rs35929428 GA was significantly higher in NAFLD patients than in HVs (28.3% vs. 7.3%, p=0.015, OR=5.00, 95%CI: 1.33–18.70). GA: PTPRD rs35929428 GA, GG: PTPRD rs35929428 GG.

Figure 2 Association of PTPRD rs35929428 and hepatic fibrosis and fat accumulation.

(a) The ratio of PTPRD rs35929428 GA was significantly higher in Fib-4 index >= 1.45 than in that < 1.45 (35.9 % vs. 7.1%). (b) The stage of hepatic fibrosis assessed by liver biopsy and PTPRD rs35929428. (c, d) Representative liver biopsy specimens stained with H.E in patient with PTPRD rs35929428 GG and PTPRD rs35929428 GA. (e) The degree of fat droplets was shown in PTPRD rs35929428 GA and PTPRD rs35929428 GG patients (the degree of fat droplets, 0: <5%, 1: <10%, 2: <34%, 3: <67%, 4: >67%). GA: PTPRD rs35929428 GA, GG: PTPRD rs35929428 GG (NS: not significant, *p<0.05).

Figure 3 PTPRD expression in the liver.
(a, b) Immunohistochemical analysis of the liver stained with anti-PTPRD antibody in *PTPRD* rs35929428 GG patients and *PTPRD* rs35929428 GA patients. (c) The numbers of PTPRD positive cells were not significant in patients with *PTPRD* rs35929428 GG and *PTPRD* rs35929428GA patients (GG: n=15, GA: n=11) (NS: not significant).

**Figure 4** Phosphorylation of STAT3 (Tyr 705) in Huh7 treated with IL-6 and the patients with NAFLD.

(a) Representative western blot analysis. (b) Treatment with IL-6 increased the phosphorylation of STAT3 (Tyr 705) in Huh 7 cells transfected with wild type PTPRD, while this increasing of phosphorylation of STAT3 (Tyr 705) was attenuated in Huh 7 cells transfected with PTPRD R995C. Each experiment was performed three times. (c, d) Immunohistochemistry revealed that phosphorylation of STAT3 (Tyr 705) was strongly positive in nuclei of hepatocytes in patients with *PTPRD* rs35929428 GG compared to in those with *PTPRD* rs35929428 GA. (e) The numbers of pSTAT3 positive cells in *PTPRD* rs35929428 GA (GA) and *PTPRD* rs35929428 GG (GG) (GG: n=16, GA: n=9) (NS: not significant, *p*<0.05, **p**<0.01).
NAFLD

HVs

p=0.015, OR: 5.00
Stage of hepatic fibrosis

Degree of fat droplets

GG

GA

*
a

PTPRD

|     | WT | R995C |
|-----|----|-------|
| IL-6| -  | +     |
| pSTAT3 Y705 |     |       |
| Total STAT3 |     |       |
| Actin |     |       |

b

Relative expression of pSTAT3/STAT3

|       | IL-6 | WT | R995C |
|-------|------|----|-------|
|       | -    | +  |       |
|       | -    | +  | NS    |

** p < 0.01
NS


c

GG

[d] 50 µm

d

GA

[e]

pSTAT positive cells (%)

|     | GG  | GA  |
|-----|-----|-----|
| p    | 80  | 20  |

* p < 0.05

GG

GA