The impact of single-nucleotide polymorphisms on liver stiffness and controlled attenuation parameter in patients treated with direct-acting antiviral drugs for hepatitis C infection

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Abstract. Single-nucleotide polymorphisms (SNPs) of patatin-like phospholipase domain-containing 3 (*PNPLA3*), tolloid-like protein 1 (*TLL1*) and interleukin-28 (*IL28*) have been identified as susceptibility factors for liver steatosis, inflammation and fibrosis in patients with hepatitis C virus (HCV) infection. Here, whether these polymorphisms affected predispositions to changes in liver stiffness (LS) and controlled attenuation parameter (CAP) following direct-acting antiviral (DAA) therapy was assessed. The changes in LS and steatosis in 77 HCV-infected patients receiving DAA therapy were compared with *PNPLA3*, *TLL1* and *IL28* genotypes, using CAP, FibroScan and Virtual Touch tissue quantification (VTTQ) before treatment and 12 weeks after the end of the treatment. VTTQ results showed that LS significantly decreased in *PNPLA3* CC (P=0.035), *TLL1* AA (P=0.011) and *IL28B* TT (P=0.005) genotypes; no significant differences were observed in *PNPLA3* CG/GG, *TLL1* AT/TT and *IL28B* TG/GG. FibroScan results showed that LS significantly decreased in *TLL1* AA (P=0.028) and *IL28B* TT (P=0.032), with no significant difference in *PNPLA3* CC. No significant differences were observed in *PNPLA3* CG/GG, *TLL1* AT/TT and *IL28B* TG/GG groups. CAP was significantly increased in *PNPLA3* CG/GG (P=0.039 and P<0.05) and *IL28B* TT (P=0.014); no significant difference was observed in *PNPLA3* CC and all genotypes of *TLL1* and *IL28B* TG/GG. Therefore, these results indicated that SNPs could predict changes in LS and steatosis after DAA therapy.

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Introduction

Research on genome-wide association analyses and the association of single-nucleotide polymorphisms (SNPs) with various diseases have seen great advances (1). The association between the pathophysiology of chronic liver disease and SNPs has also been reported. Patatin-like phospholipase domain-containing 3 (*PNPLA3*) *rs738409* (2-4), tolloid-like protein 1 (*TLL1*) *rs17047200* (5-7) and interleukin-28B (*IL28b*) *rs809917* (8-14) are associated with liver fibrosis, inflammation and steatosis in hepatitis C virus (HCV)-infected patients.

Liver biopsy is considered the gold standard for evaluating liver histology, including steatosis, inflammation and fibrosis; however, it is an invasive procedure that can cause various complications such as pain, fever, and bleeding (15). To address this limitation, FibroScan and Virtual Touch tissue quantification (VTTQ) were developed as modalities for the non-invasive assessment of liver fibrosis (16,17). In addition, FibroScan includes software for assessing hepatic steatosis using controlled attenuation parameter (CAP) (16). These modalities can be used to repeatedly evaluate liver steatosis, inflammation and fibrosis.

Hepatitis C is a disease that causes cirrhosis and hepatocellular carcinoma. It is estimated that 177.5 million individuals worldwide are infected with HCV, which is 2.5% of the world's population (17). HCV infection poses a significant risk of cirrhosis and liver cancer. Originally, interferon-based treatment was used as the treatment for HCV, but the elimination rate of HCV was low, and adverse events frequently appeared (18). Direct-acting antivirals (DAAs) have been used as a treatment for HCV, and a high treatment success rate has been reported (19). Ogasawara *et al* (20) reported the influence of DAA therapy on liver stiffness (LS) and CAP, finding that the treatment of HCV with DAA decreases LS and increases CAP after treatment. Therefore, in this study, the impact of three SNPs (*PNPLA3*, *TLL1* and *IL28B*) on changes in CAP and LS following DAA therapy were examined.

Materials and methods

Patients. A total of 78 seven patients (39 females and 38 males; median age, 68 years; age range, 33-88 years)

who received DAA therapy for chronic HCV infection at Nagasaki University Hospital (Nagasaki, Japan) and were subsequently confirmed as HCV-negative 12 weeks after the end of treatment [sustained virologic response (SVR12)] between September 2014 and December 2017 were enrolled in the study.

The patients' profiles and the results of laboratory data at the start of DAA therapy are summarized in Table I. Written informed consent was obtained from all patients, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki (21). This study was approved by the Ethics Committee of Nagasaki University (approval no. 15012688-3).

Measurement of LS and CAP. LS was measured twice, at the baseline (before DAA therapy) and 12 weeks after the end of the treatment (sustained virologic response, 12 weeks after the end of direct-acting antiviral treatment; SVR12). LS was measured by VTTQ [LS (VTTQ)] using an ACUSON S2000 (Siemens AG) and by transient elastography with the M-probe of FibroScan 502 Touch (Echosens) [LS (FibroScan)]. The CAP was also measured using FibroScan. The patient was examined in a supine position with their right arm raised. The tip of the probe was placed on the skin of the patient between the ribs and the right lobe of the liver. For VTTQ measurement, the region of interest was located 2-4 cm under the capsule in the right lobe to avoid major blood vessels. Measurements were taken five times and the median was used for analysis. The LS results after VTTQ are presented as m/s (22). For FibroScan measurement, the probe was placed on the skin between the ribs and aimed at a location similar to that used for VTTQ. Measurements were taken 10 times, and the median was used for analysis. The LS results after FibroScan are presented as kPa, and CAP results are presented as dB/m (23,24).

SNP genotyping. Genomic DNA was extracted from mononuclear cells in peripheral blood samples of each patient using a FlexiGene DNA kit (Qiagen GmbH). The SNPs in *PNPLA3*, *TLL1* and *IL28B* were genotyped in each sample using TaqMan SNP genotyping assays kit (Thermo Fisher Scientific, Inc.) containing two allele-specific TaqMan MGB probes labeled with different fluorochromes and a PCR primer pair according to the manufacturer's protocol. The following primers were used: PNPLA3 rs738409, AGGCCTTGGTAT GTTCCTGCTTCAT[C/G]CCCTTCTACAGTGGCCTTATC CCTC (cat. no. 4351379); TLL1 rs17047200, TTTTGCCCA CTTATGTCCATTTCAC[A/T]GTTCATTGACATCTATTT CTGAAGG (cat. no. 4351379); IL28B rs8099917, TTTTGT TTTCCTTTCTGTGAGCAAT[G/T]TCACCCAAATTGGAA CCATGCTGTA (cat. no. 4351379). As an example, the data for PNPLA3 are shown in Fig. S1. The protocol was the same as that described in a previous study (25).

Statistical analysis. Data are presented as the median (inter-quartile range). Pre- and post-treatment data were analyzed by a Wilcoxon signed-rank test. $P<0.05$ was considered to indicate a statistically significant difference. Statistical analysis was performed using the StatFlex software (version 6.0; Artech).

Results

Whole group analysis. Fig. 1 shows the changes in LS (VTTQ), LS (FibroScan) and CAP from before treatment and at SVR12. In the whole group, the median LS (VTTQ) values decreased significantly (P=0.006) from 1.33 to 1.21 m/s from the start of the treatment to SVR12. The median LS (FibroScan) values decreased significantly (P=0.017) from 6.10 to 5.80 kPa, and the median CAP values increased significantly (P=0.048) from 210 to 226 dB/m.

The *PNPLA3* genotype frequencies were as follows: CC, 32 patients (42%); CG, 30 patients (39%); and GG, 15 patients (19%). The *TLL1* genotype frequencies were as follows: AA, 57 patients (74%); AT, 20 patients (26%); and TT, no patients (0%). The *IL28B* genotype frequencies were as follows: TT, 57 patients (74%); TG, 18 patients (23%); and GG, two patients (3%). We divided *PNPLA3* into CC and CG/GG groups, *TLL1* into AA and AT(/TT) groups, and *IL28B* into TT and TG/GG groups.

Changes in LS (VTTQ). Fig. 2 shows the changes in the LS (VTTQ) for each SNP. In *PNPLA3*, the median values of CC decreased significantly (P=0.035) from 1.39 to 1.19 m/s. The median values of CG/GG did not change significantly from 1.31 to 1.25 m/s. Even after dividing CG/GG genotypes into CG and GG, no significant difference was found between CG and GG. In *TLL1*, the median values of AA decreased significantly (P=0.011) from 1.31 to 1.19 m/s. The median values of AT did not change significantly from 1.43 to 1.26 m/s. In *IL28B*, the median values of TT decreased significantly (P=0.005) from 1.36 to 1.19 m/s. The median values of TG/GG did not change significantly from 1.31 to 1.36 m/s.

Changes in LS (FibroScan). Fig. 3 shows changes in LS (FibroScan) for each SNP. In *PNPLA3*, the median values of CC decreased from 6.15 kPa before treatment to 5.75 kPa at SVR12, but the decrease was not significant. The median values of CG/GG did not change significantly from 6.10 to 5.80 kPa. Even after dividing CG/GG into CG and GG, no significant difference was found between CG and GG. In *TLL1*, the median values of AA decreased significantly (P=0.028) from 6.10 to 5.75 kPa. The median values of AT did not change significantly from 6.60 to 5.10 kPa. In *IL28B*, the median values of TT decreased significantly (P=0.032) from 6.10 to 5.50 kPa. The median values of TG/GG did not change significantly from 6.70 to 7.40 kPa.

Changes in CAP. Fig. 4 shows the changes in CAP for each SNP. In *PNPLA3*, the median values of CC did not change significantly from 217 to 209 dB/m. The median values of CG/GG increased significantly (P=0.004) from 204 to 233 dB/m. After dividing CG/GG into CG and GG, both CG (P=0.039) and GG ($P<0.05$) increased significantly. In *TLL1*, the median values of AA did not change significantly from 207 to 226 dB/m. The median values of AT did not change significantly from 215 to 225 dB/m. In *IL28B*, the median values of TT increased significantly (P=0.014) from 213 to 226 dB/m. The median values of TG/GG did not change significantly from 206 to 218 dB/m.

Table I. Baseline characteristics of the patients (n=77).

Parameter	Value
Age, years (range)	68 (33-88)
Sex, n	
Males	38
Females	39
Chronic hepatitis, n	53
Liver cirrhosis, n	24
Total bilirubin, mg/dl[a]	0.9 (0.3-2.5)
Albumin, g/dl[a]	3.9 (2.5-4.9)
Low density lipoprotein cholesterol, mg/dl[a]	88.8 (31-158)
Platelet count, $10^4/\mu$l[a]	17.8 (3.9-47.0)
Aspartate aminotransferase, IU/l[a]	49.2 (10-188)
Alanine aminotransferase, IU/l[a]	40.0 (26.5-56.3)
Total cholesterol, mg/dl[a]	166.1 (112-241)
Mac-2 binding protein glycosylation isomer[a]	2.39 (0.37-14.57)
Body mass index, kg/m^2[a]	22.5 (15.6-29.0)
Liver stiffness measured using Virtual Touch Tissue Quantification, m/s[a]	1.57 (0.75-3.77)
Liver stiffness measured using FibroScan, kPa[a]	6.30 (4.40-11.63)
Controlled attenuation parameter, dB/m[a]	213.7 (100-373)
Direct acting antiviral agent, n	14/7/32/9/13/2
Daclatasvir/asunaprevir	14
Sofosbuvir + ribavirin	7
Sofosbuvir/ledipasvir	32
Ombitasvir/paritaprevir/ritonavir	9
Elbasvir/grazoprevir	13
Glecaprevir/pibrentasvir	2
PNPLA3 (rs738409) genotype, n	
CC	32
CG	30
GG	15
TLL1 (rs17047200) genotype, n	
AA	57
AT	20
TT	0
IL28B (rs8099917) genotype, n	
TT	57
TG	18
GG	2

[a]Median (range).

Discussion

In this study, the impact of SNPs on changes in LS and CAP after DAA therapy in patients with HCV infection were determined. LS was evaluated using VTTQ and FibroScan, and CAP was evaluated using FibroScan. LS (VTTQ) and LS (FibroScan) significantly decreased, and CAP significantly increased at SVR12 in all cases. These results are consistent with those of a previous study (20). LS reflects liver fibrosis staging and liver inflammation; therefore, a decrease in LS indicates an improvement in fibrosis and inflammation.

PNPLA3 rs738409 is considered a risk factor for liver steatosis, fibrosis progression, and the development of hepatocellular carcinoma in patients with non-alcoholic fatty liver disease (26,27). Moreover, a previous study reported that *PNPLA3* is a significant risk factor for steatosis, inflammation and fibrosis in patients with hepatitis C (2-4). Furthermore, PNPLA3 is reportedly involved in lipase activity, and a C to G substitution causes an amino acid substitution from isoleucine to methionine at position 148 of the coding sequence (I148M), with the resulting loss of function possibly involved in hepatic steatosis. It is also possible that I148M affects the activation of

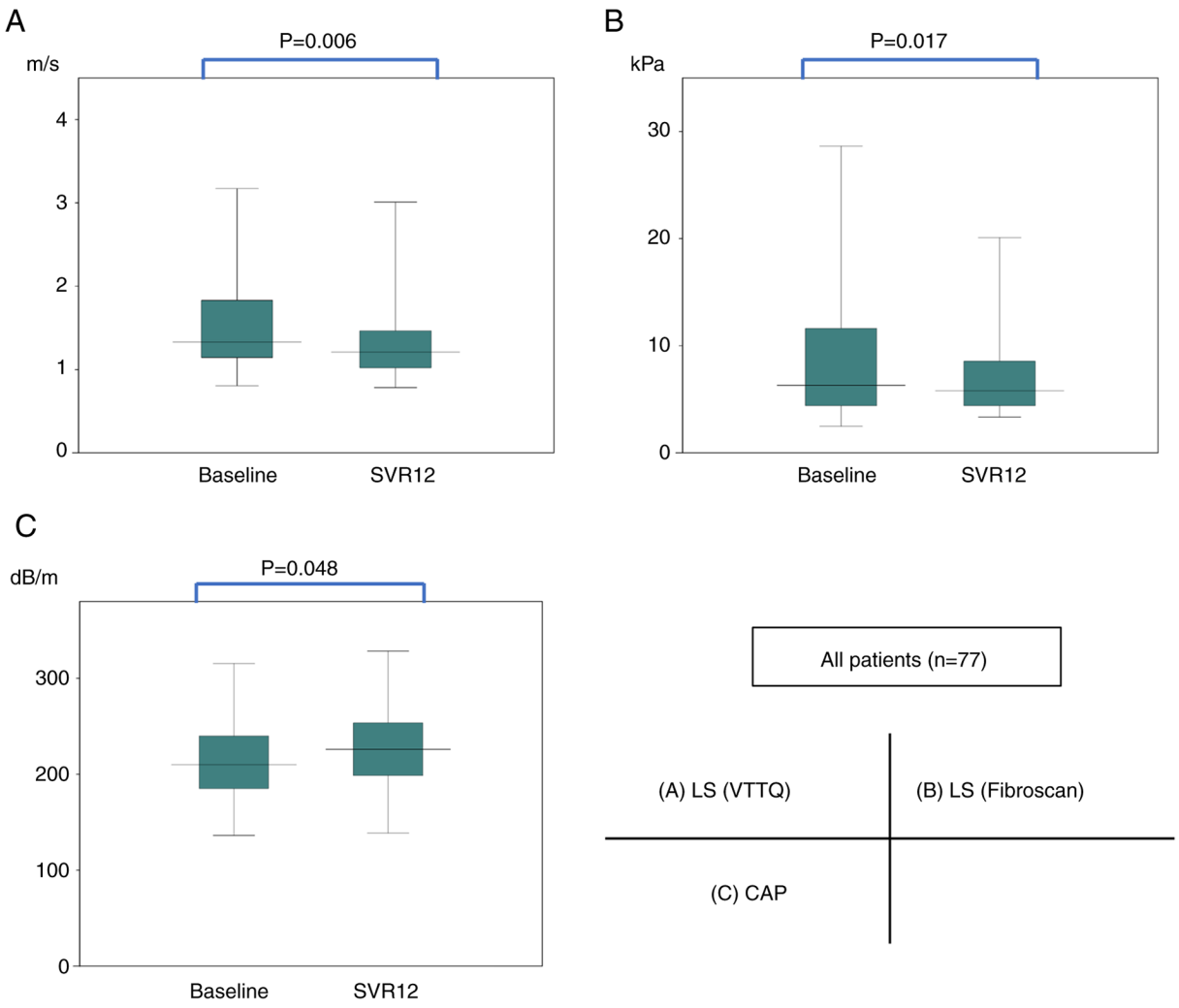

Figure 1. Change in LS and CAP before DAA treatment and at SVR12. Changes in (A) LS (VTTQ), (B) LS (FibroScan) and (C) CAP before treatment and at SVR12 for all patients. The median LS (VTTQ) values decreased significantly (P=0.006) from 1.33 m/s before treatment to 1.21 m/s at SVR12. The median LS (FibroScan) values decreased significantly (P=0.017) from 6.10 to 5.80 kPa. The median CAP values increased significantly (P=0.048) from 210 to 226 dB/m. LS, liver stiffness; VTTQ, virtual touch tissue quantification; HCV, hepatitis C virus; DAA, direct-acting antiviral therapy; CAP, controlled attenuation parameter; SVR12, sustained virologic response, 12 weeks after the end of direct-acting antiviral treatment.

hepatic stellate cells related to fibrosis; however, the associated mechanism has not yet been elucidated (2-4). Previous studies suggested that *TLL1 rs17047200* may contribute to hepatocarcinogenesis through the progression of liver fibrosis and that the AT/TT genotype is a risk factor for the development of HCC after SVR (5-7). Furthermore, *TLL1* may be involved in the development of hepatic fibrosis through extracellular matrix production and TGF-β signal activation (5-7). Another report indicated that SNPs located close to *IL28B* significantly influenced the therapeutic outcome of combination therapy involving pegylated IFN and ribavirin in HCV-infected patients (28). Additionally, liver fibrosis is reportedly exacerbated by the minor (14) and major alleles (8,13) of *IL28B rs80991*, with a previous report suggesting that changes in the *IL28B* allele alter the expression levels of IFN-stimulating genes, including some inflammatory cytokines, and may be involved in fibrosis. Moreover, increased IFN expression reportedly suppresses lipoprotein lipase activity and reduces very-low-density lipoprotein levels through low-density lipoprotein conversion, which may be involved in hepatic steatosis (8,13). These findings suggest the involvement of these SNPs in steatosis, inflammation and fibrosis in patients with chronic hepatitis, and the observed changes in LS and CAP in this study are consistent with previous findings.

Previous studies showed that HCV eradication by IFN and DAA therapy led to downstaging of fibrosis and reduced HCC incidence, particularly in patients who achieved SVR (29-31). However, HCC development can occur even in patients with SVR, and the major risk factors include older age, alcohol intake, pathological factors and diabetes (32-35). Motoyama *et al* (36) reported that stagnation of fibrosis regression is associated with a high risk of HCC development after SVR. Additionally, liver steatosis is reportedly a risk factor for HCC development after SVR (37). Given these findings, elevations in LS and CAP following DAA may represent a risk factor for HCC development. Therefore, patients found to possess these risk alleles need careful follow-ups with their doctors considering the risk of HCC.

In the present study, median LS (VTTQ) and LS (FibroScan) increased after treatment in *IL28B* TG/GG

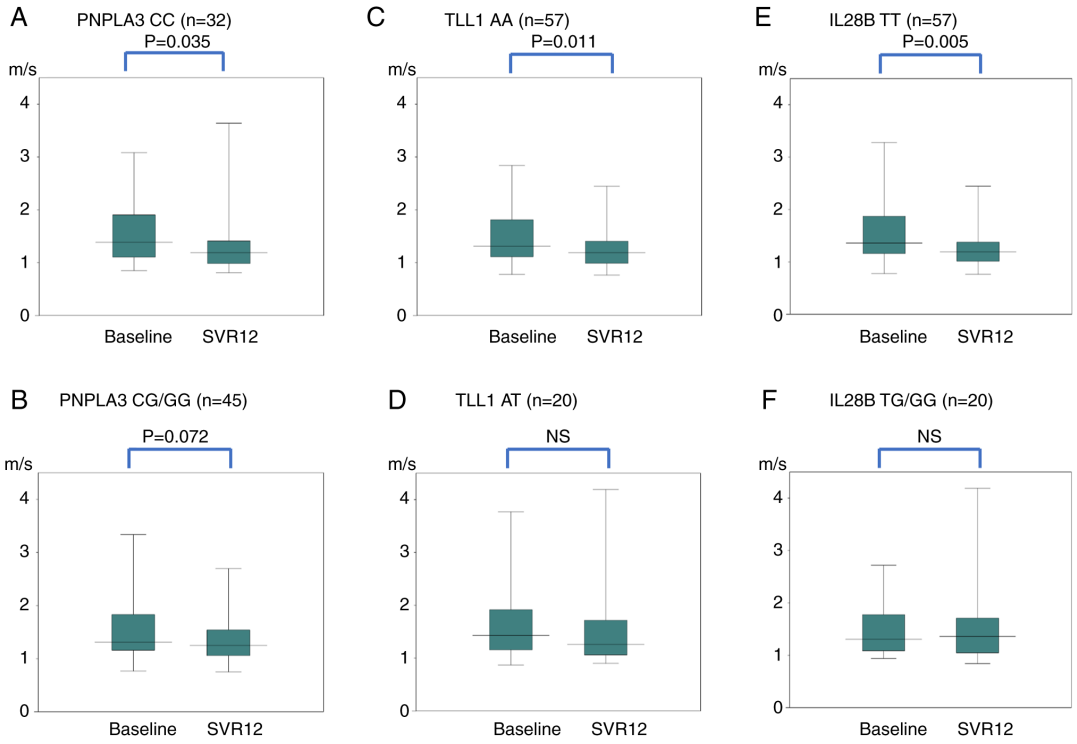

Figure 2. Changes in LS (VTTQ) for each SNP in *PNPLA3*: (A) CC, (B) CG/GG, *TLL1*; (C) AA, (D) AT, *IL28B*; (E) TT and (F) TG/GG genes. In the *PNPLA3* gene, the median values of CC decreased significantly (P=0.035) from 1.39 m/s before treatment to 1.19 m/s at SVR12. The median values of CG/GG did not change significantly; 1.31 to 1.25 m/s. In the *TLL1* gene, the median values of AA decreased significantly (P=0.011) from 1.31 to 1.19 m/s. The median values of AT did change significantly; 1.43 to 1.26 m/s. In the *IL28B* gene, the median values of TT decreased significantly (P=0.005) from 1.36 to 1.19 m/s. The median values of TG/GG did not change significantly; 1.31 to 1.36 m/s. LS, liver stiffness; VTTQ, virtual touch tissue quantification; SNP, single-nucleotide polymorphism; SVR12, sustained virologic response, 12 weeks after the end of direct-acting antiviral treatment.

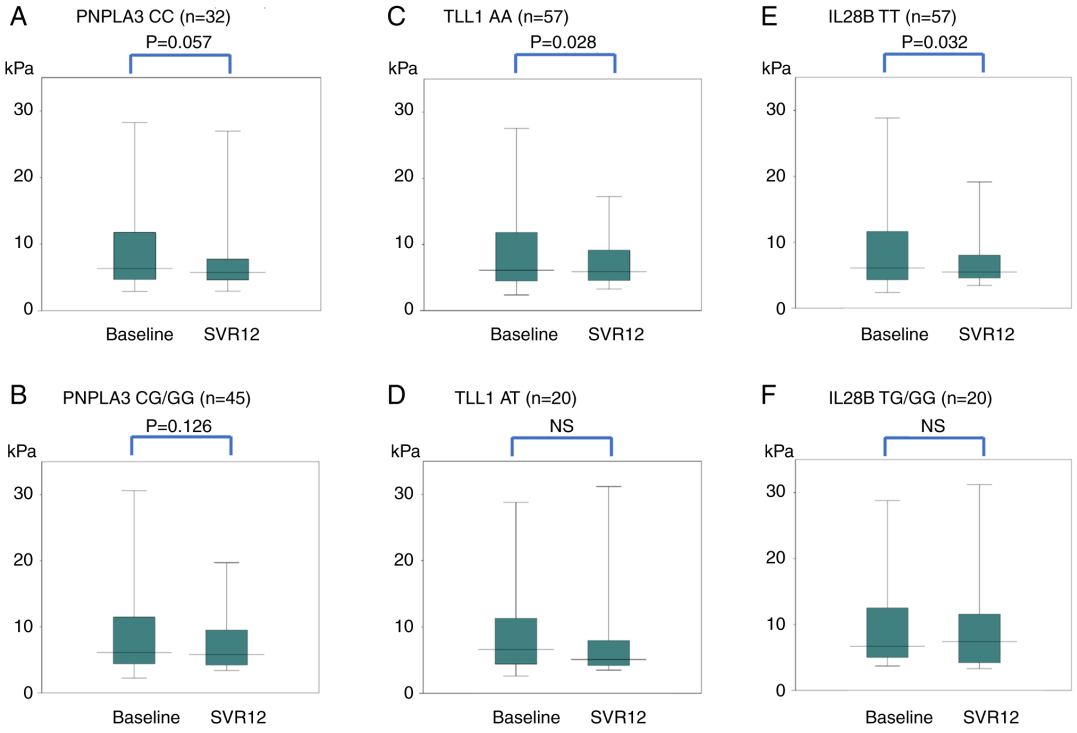

Figure 3. Changes in LS (FibroScan) for each SNP in *PNPLA3*: (A) CC, (B) CG/GG, *TLL1*; (C) AA, (D) AT, *IL28B*; (E) TT, and (F) TG/GG genes. In the *PNPLA3* gene, the median values of CC were 6.15 kPa before DAA treatment and 5.75 kPa at SVR12. There was no significant difference, but there was a decreasing trend (P=0.057). The median values of CG/GG did not change significantly; 6.10 to 5.80 kPa. In the *TLL1* gene, the median values of AA decreased significantly (P=0.028) from 6.10 to 5.75 kPa. The median values of AT did change not significantly; 6.60 to 5.10 kPa. In the *IL28B* gene, the median values of TT decreased significantly (P=0.032) from 6.10 to 5.50 kPa. The median values of TG/GG did not change significantly; 6.70 to 7.40 kPa. LS, liver stiffness; VTTQ, virtual touch tissue quantification; SNP, single-nucleotide polymorphism; SVR12, sustained virologic response, 12 weeks after the end of direct-acting antiviral treatment.

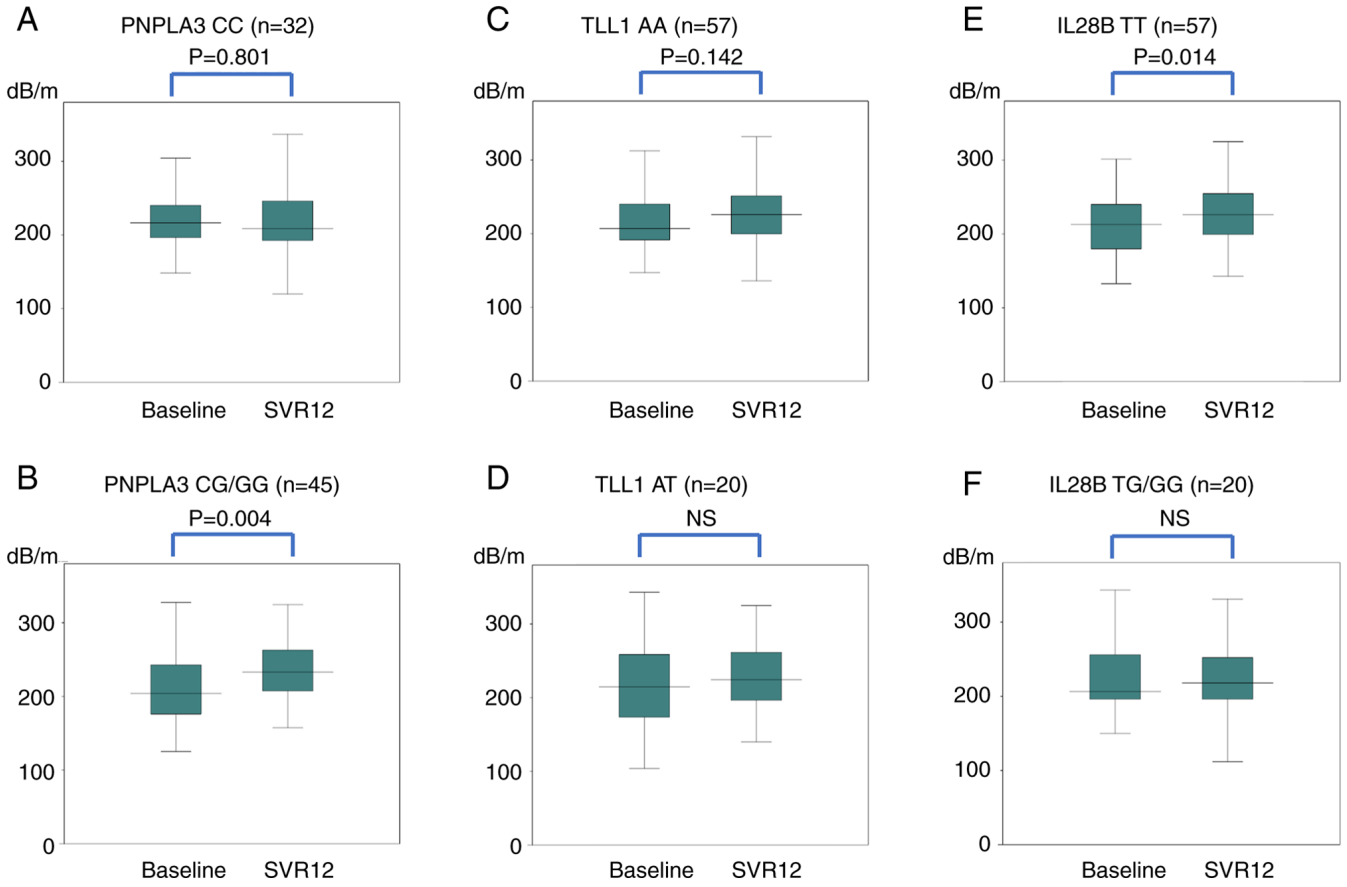

Figure 4. Changes in CAP for each SNP in *PNPLA3*: (A) CC, (B) CG/GG, *TLL1*; (C) AA, (D) AT, *IL28B*; (E) TT and (F) TG/GG genes. In the *PNPLA3* gene, the median values of CC did not change significantly; 217 dB/m before treatment and 209 dB/m at SVR12. The median values of CG/GG increased significantly (P=0.004) from 204 to 233 dB/m. In the *TLL1* gene, the median values of AA did not change significantly; 207/m to 226 dB/m. The median values of AT did not change significantly; 215 to 225 dB/m. In the *IL28B*, the median values of TT increased significantly (P=0.014) from 213 to 226 dB/m. The median values of TG/GG did not change significantly; 206 to 218 dB/m. CAP, controlled attenuation parameter; SNP, single-nucleotide polymorphism; SVR12, sustained virologic response, 12 weeks after the end of direct-acting antiviral treatment.

patients, although no significant difference was observed. After treatment, LS (VTTQ) increased in 26 out of 77 cases, and LS (FibroScan) increased in 29 out of 77 cases. Of the 20 cases of *IL28B* TG/GG, 7 cases exhibited an increase in LS (VTTQ) and 8 cases exhibited an increase in LS (FibroScan). Further analysis is difficult due to the small number of cases, and it is necessary to investigate whether *IL28B* TG/GG exacerbates fibrosis after DAA treatment using a larger cohort. The median decrease in CAP for *PNPLA3* CC showed a similar result; therefore, this should be investigated using a larger cohort further.

In *PNPLA3* CC, the value of LS (VTTQ) decreased significantly after treatment, but there was no significant difference in LS (FibroScan). However, the post-treatment value of LS (FibroScan) apparently showed a downward trend, and therefore, it is necessary to investigate whether this trend may be significant with a larger cohort.

In addition, as LS levels are affected by inflammation, it may be useful to compare the parameters immediately after treatment and 3 months after treatment. However, it was difficult to compare these because the protocol of this survey did not include the measurement of the LS value immediately after the treatment. In future studies, the parameters should be measured immediately after treatment as well.

The present study has certain limitations. The study was conducted in a single center, and the sample size was small. To validate the results observed in this study, a larger number of samples should be investigated in the future. Additionally, this study had a short observation period; therefore, it will be necessary to consider longer observation periods in future studies. Moreover, transient elastography was performed using only the M-probe, which is intended for patients whose distance from the measurement position on the skin to the liver is <25 mm, suggesting a possible disadvantage in that measurement errors are more likely at distances of ≥25 mm. An XL probe can be used for such patients (38).

In conclusion, the results suggested that certain SNPs were associated with the changes in liver fibrosis and steatosis after DAA treatment for HCV infection.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

KM was involved in data curation, formal analysis, investigation and of writing the original draft. HM was involved in conceptualization, methodology and project administration. MF, RS, MH and SM were involved in data curation and formal analysis. KN was involved in data curation and supervision. All authors have read and approved the final manuscript. KM and HM confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Written informed consent was obtained from all patients. This study was approved by the Ethics Committee of Nagasaki University (Nagasaki, Japan; approval no. 15012688-3).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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