with liver stiffness 12.0 kPa or less (Fig. 3). According to the HCC surveillance guidelines, an imaging examination every 6 months is recommended in cases with chronic hepatitis C and once in 3–4 months in cases with liver cirrhosis C.[34] In cases with liver stiffness of more than 12.0 kPa, the guidelines can be considered reasonable. In addition, in cases with liver stiffness of 12.0 kPa or less, it was suggested that the surveillance interval may be prolonged, although further accumulation of such cases was necessary.

In the HBV group, the cut-off value at 8.5 kPa most closely correlated with HCC concurrence (OR, 8.28), and both the cut-off value and OR were lower than those in the HCV group, which indicated that there was a weaker association between fibrosis and HCC in the HBV group than in the HCV group. In the HBV group, it was reported that liver stiffness at 8.0 kPa, a cut-off value lower than that in the HCV group, or higher increased the incidence of HCC development.[15] Subgroup analysis (Fig. 2) revealed that liver stiffness of more than 8.5 kPa was a significant factor irrespective of age and Plt. Unfortunately, we could not analyze the HCC developmental risk in cases with HBV because no case without concurrent HCC initially developed HCC during this limited observation period.

To the best of our knowledge, no report has demonstrated the association between liver stiffness and HCC concurrence in cases with NBNC liver disease, but when liver stiffness at 12.0 kPa was set as the cut-off value, liver stiffness most closely correlated with HCC concurrence and the cut-off value was almost comparable to that in the HCV group. This result demonstrates that fibrosis also plays an important role in HCC development in NBNC though its contribution is weaker than in HCV. Subgroup analysis revealed that HCC concurrence was more frequent in the group with liver stiffness of more than 12.0 kPa among the elderly aged more than 65 years old and cases with low AFP levels as reported previously,[32] demonstrating that the HCC risk was more greatly dependent on fibrosis in the elderly, while it was high irrespective of fibrosis in cases with elevated AFP in the NBNC group. As for etiologies in the NBNC group, most cases were clinically suspected to have fatty liver-associated diseases. Though information on steatosis-related factors was available only from limited cases in this study, high hemoglobin A1c (HbA1c) value (defined as >6.5) was frequent in NBNC cases (25%) compared to HCV (11%) or HBV cases (17%), and this difference reached statistical significance between HCV and NBNC (data not shown). In addition, high HbA1c value and heavy alcohol intake of more than 70 g/day were more significantly identified in HCC cases compared to non-HCC cases in the NBNC group (data not shown). These observations suggested that fatty liver-associated diseases may be one of the main etiologies in the NBNC group. On the other hand, as with the HBV cases, we could not analyze the HCC developmental risk in cases with NBNC because no case developed HCC during this limited observation period.

In conclusion, evaluation of liver fibrosis based on liver stiffness was useful, in particular, in HCV and NBNC liver disease, because HCC development via advancement of liver fibrosis is a major pathway. Accurate evaluation of liver fibrosis would be important to screen the high risk group for HCC development and analyze causal factors for HCC development other than fibrosis.

REFERENCES

1 Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin* 2011; **61** (2): 69–90.
2 El-Serag HB. Hepatocellular carcinoma. *N Engl J Med* 2011; **365** (12): 1118–27.
3 Ikeda K, Saitoh S, Suzuki Y *et al.* Disease progression and hepatocellular carcinogenesis in patients with chronic viral hepatitis: a prospective observation of 2215 patients. *J Hepatol* 1998; **28** (6): 930–8.
4 Inoue A, Tsukuma H, Oshima A *et al.* Effectiveness of interferon therapy for reducing the incidence of hepatocellular carcinoma among patients with type C chronic hepatitis. *J Epidemiol* 2000; **10** (4): 234–40.
5 Takano S, Yokosuka O, Imazeki F, Tagawa M, Omata M. Incidence of hepatocellular carcinoma in chronic hepatitis B and C: a prospective study of 251 patients. *Hepatology* 1995; **21** (3): 650–5.
6 Yoshida H, Shiratori Y, Moriyama M *et al.* Interferon therapy reduces the risk for hepatocellular carcinoma: national surveillance program of cirrhotic and noncirrhotic patients with chronic hepatitis C in Japan. IHIT Study Group. Inhibition of Hepatocarcinogenesis by Interferon Therapy. *Ann Intern Med* 1999; **131** (3): 174–81.
7 Sandrin L, Fourquet B, Hasquenoph JM *et al.* Transient elastography: a new noninvasive method for assessment of hepatic fibrosis. *Ultrasound Med Biol* 2003; **29** (12): 1705–13.
8 Abenavoli L, Beaugrand M. Transient elastography in non-alcoholic fatty liver disease. *Ann Hepatol* 2012; **11** (2): 172–8.
9 Cardoso AC, Carvalho-Filho RJ, Marcellin P. Transient elastography in chronic viral hepatitis: a critical appraisal. *Gut* 2011; **60** (6): 759–64.
10 Castera L. Non-invasive assessment of liver fibrosis in chronic hepatitis C. *Hepatol Int* 2011; **5** (2): 625–34.

11 Yoneda M, Mawatari H, Fujita K *et al.* Noninvasive assessment of liver fibrosis by measurement of stiffness in patients with nonalcoholic fatty liver disease (NAFLD). *Dig Liver Dis* 2008; **40** (5): 371–8.
12 Kim BK, Fung J, Yuen MF, Kim SU. Clinical application of liver stiffness measurement using transient elastography in chronic liver disease from longitudinal perspectives. *World J Gastroenterol* 2013; **19** (12): 1890–900.
13 Akima T, Tamano M, Hiraishi H. Liver stiffness measured by transient elastography is a predictor of hepatocellular carcinoma development in viral hepatitis. *Hepatol Res* 2011; **41** (10): 965–70.
14 Jung KS, Kim SU. Clinical applications of transient elastography. *Clin Mol Hepatol* 2012; **18** (2): 163–73.
15 Jung KS, Kim SU, Ahn SH *et al.* Risk assessment of hepatitis B virus-related hepatocellular carcinoma development using liver stiffness measurement (FibroScan). *Hepatology* 2011; **53** (3): 885–94.
16 Kuo YH, Lu SN, Hung CH *et al.* Liver stiffness measurement in the risk assessment of hepatocellular carcinoma for patients with chronic hepatitis. *Hepatol Int* 2010; **4** (4): 700–6.
17 Masuzaki R, Tateishi R, Yoshida H *et al.* Comparison of liver biopsy and transient elastography based on clinical relevance. *Can J Gastroenterol* 2008; **22** (9): 753–7.
18 Masuzaki R, Tateishi R, Yoshida H *et al.* Prospective risk assessment for hepatocellular carcinoma development in patients with chronic hepatitis C by transient elastography. *Hepatology* 2009; **49** (6): 1954–61.
19 Wang HM, Hung CH, Lu SN *et al.* Liver stiffness measurement as an alternative to fibrotic stage in risk assessment of hepatocellular carcinoma incidence for chronic hepatitis C patients. *Liver Int* 2013; **33** (5): 756–61.
20 Boursier J, Zarski JP, de Ledinghen V *et al.* Determination of reliability criteria for liver stiffness evaluation by transient elastography. *Hepatology* 2013; **57** (3): 1182–91.
21 Castera L. Liver stiffness and hepatocellular carcinoma: liaisons dangereuses? *Hepatology* 2009; **49** (6): 1793–4.
22 Chon YE, Choi EH, Song KJ *et al.* Performance of transient elastography for the staging of liver fibrosis in patients with chronic hepatitis B: a meta-analysis. *PLoS One* 2012; **7** (9): e44930.
23 Lupsor M, Badea R, Stefanescu H *et al.* Analysis of histopathological changes that influence liver stiffness in chronic hepatitis C. Results from a cohort of 324 patients. *J Gastrointestin Liver Dis* 2008; **17** (2): 155–63.
24 Wong VW, Vergniol J, Wong GL *et al.* Diagnosis of fibrosis and cirrhosis using liver stiffness measurement in nonalcoholic fatty liver disease. *Hepatology* 2010; **51** (2): 454–62.
25 Masuzaki R, Tateishi R, Yoshida H *et al.* Risk assessment of hepatocellular carcinoma in chronic hepatitis C patients by transient elastography. *J Clin Gastroenterol* 2008; **42** (7): 839–43.
26 de Oliveria Andrade LJ, D'Oliveira A, Melo RC, De Souza EC, Costa Silva CA, Parana R. Association between hepatitis C and hepatocellular carcinoma. *J Glob Infect Dis* 2009; **1** (1): 33–7.
27 El-Serag HB. Hepatocellular carcinoma and hepatitis C in the United States. *Hepatology* 2002; **36** (5 Suppl 1): S74–83.
28 Asahina Y, Tsuchiya K, Nishimura T *et al.* alpha-fetoprotein levels after interferon therapy and risk of hepatocarcinogenesis in chronic hepatitis C. *Hepatology* 2013; **58** (4): 1253–62.
29 Asahina Y, Tsuchiya K, Tamaki N *et al.* Effect of aging on risk for hepatocellular carcinoma in chronic hepatitis C virus infection. *Hepatology* 2010; **52** (2): 518–27.
30 Lewis S, Roayaie S, Ward SC, Shyknevsky I, Jibara G, Taouli B. Hepatocellular carcinoma in chronic hepatitis C in the absence of advanced fibrosis or cirrhosis. *AJR Am J Roentgenol* 2013; **200** (6): W610–16.
31 Arase Y, Kobayashi M, Suzuki F *et al.* Effect of type 2 diabetes on risk for malignancies includes hepatocellular carcinoma in chronic hepatitis C. *Hepatology* 2013; **57** (3): 964–73.
32 Reddy SK, Steel JL, Chen HW *et al.* Outcomes of curative treatment for hepatocellular cancer in nonalcoholic steatohepatitis versus hepatitis C and alcoholic liver disease. *Hepatology* 2012; **55** (6): 1809–19.
33 Starley BQ, Calcagno CJ, Harrison SA. Nonalcoholic fatty liver disease and hepatocellular carcinoma: a weighty connection. *Hepatology* 2010; **51** (5): 1820–32.
34 Kudo M, Izumi N, Kokudo N *et al.* Management of hepatocellular carcinoma in Japan: consensus-based clinical practice guidelines proposed by the Japan Society of Hepatology (JSH) 2010 updated version. *Dig Dis* 2011; **29** (3): 339–64.

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Original Article

Deep sequencing analysis of variants resistant to the non-structural 5A inhibitor daclatasvir in patients with genotype 1b hepatitis C virus infection

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Aim: Daclatasvir, a non-structural (NS)5A replication complex inhibitor, is a potent and promising direct antiviral agent (DAA) for hepatitis C virus (HCV), being most effective in genotype 1b infection. Although it is known that genotype 1b viruses with Y93H and/or L31M/V/F mutations have strong resistance to daclatasvir, it is not known whether there are some clinical background conditions that favor the occurrence of HCV carrying those NS5A mutations.

Methods: In this study, we carried out deep sequencing analysis of stored sera to determine the presence and significance of daclatasvir-resistant mutants in 110 genotype 1b HCV-infected patients with no previous daclatasvir treatment.

Results: Deep sequencing analysis revealed that the NS5A L31M/V/F and Y93H mutations were present in 13 (11.8%) and 34 (30.9%) of the 110 patients, respectively, and significantly more frequently than in the control plasmid. Simultaneous L31M/V/F and Y93H mutations were detected in four of the 110 patients (3.6%). When the clinical relevance of NS5A resistance was investigated, Y93H was significantly correlated with the IL28B major (TT) genotype of the host ($P = 0.042$).

Conclusion: Y93H was detected frequently by deep sequencing in daclatasvir treatment-naïve patients. Importantly, it seems that the IL28B status of the patients may influence the presence of Y93H mutations, resulting in different treatment responses to daclatasvir.

Key words: deep sequencing, hepatitis C virus, NS5A inhibitor, resistance

INTRODUCTION

RECENTLY, TREATMENT OF hepatitis C virus (HCV) infection has advanced markedly. Specifically, the advent of telaprevir (TPV) and boceprevir (BPV), first-generation protease inhibitors, dramatically increased the sustained virological response (SVR) rate to as high as 60–80% by combination with pegylated (PEG) interferon (IFN)/ribavirin (RBV) therapy.[1] However, high SVR rates following combination therapy have not been seen in null-responders to previous PEG IFN/RBV combination therapy.[2] Under these circumstances, development of more effective drug therapies with less serious adverse effects is anticipated.

Daclatasvir (BMS-790052), a non-structural (NS)5A replication complex inhibitor, is a potent and promising direct antiviral agent (DAA) for HCV. Daclatasvir has anti-HCV activity with broad genotypic coverage, but is most effective for genotype 1b viruses.[3] Moreover, among all NS5A inhibitors, daclatasvir is most advanced in its development for clinical use.[4,5] Drug-resistant mutations have been identified for daclatasvir, and resistance is acquired by Y93H, L31M/V/F or P32L substitutions in NS5A in genotype 1b HCV. In particular, simultaneous substitutions of Y93H and L31M/V/F produce more robust resistance.[6,7]

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In Japan, a clinical phase II trial of 24-week combination therapy of two oral agents, the NS5A inhibitor daclatasvir and NS3 protease inhibitor asunaprevir (BMS-650032), was carried out in 43 patients with genotype 1b HCV infection. The therapy achieved an SVR rate of 90.5% in patients with a null response to PEG IFN/RBV combination therapy and of 63.6% in patients considered ineligible or intolerant for IFN-based therapy.[8,9] The result was that the SVR rate was markedly high, in particular, in patients with a null response to PEG IFN/RBV combination therapy, giving hope to these difficult to treat patients. The study also revealed that the presence of Y93H prior to treatment was significantly associated with non-SVR to the regimen of the two oral agents.[8–11] On the other hand, it remains unknown whether differences in clinical backgrounds, including previous history of IFN therapy and its response, are associated with the presence of Y93H in daclatasvir treatment-naïve genotype 1b patients.

In this study, we carried out deep sequencing analysis using a second generation sequencer to determine the presence of daclatasvir-resistant viruses in genotype 1b HCV patients. By deep sequencing, viral mutants associated with DAA resistance and present as minor populations could be detected.[12–14] Because daclatasvir is considered to be a key DAA for therapy for HCV in the near future, we tried to clarify the possible clinical significance of HCV-resistance mutations, such as Y93H, in the treatment response and their possible association with other viral and host factors.

METHODS

Patients

THE SUBJECTS WERE 110 randomly selected, daclatasvir treatment-naïve patients who were infected with genotype 1b HCV and followed up at the Yamanashi University Hospital. The 110 patients included 59 naïve patients, 30 relapser patients (defined as patients with reappearance of HCV RNA after the completion of previous PEG IFN/RBV combination therapy carried out between 2005 and 2011) and 21 null responder patients (defined as patients without a 2 log drop of HCV RNA at week 12 compared to that at week 0 during previous PEG IFN/RBV combination therapy carried out between 2005 and 2011). These three groups of patients with distinctly different treatment responses to previous therapy (naïve, relapse and null) were included in this study to clarify whether the rate of NS5A mutations varies among different backgrounds of the treatment response. None of the 51 patients who had failed to eradicate the virus during PEG IFN/RBV combination therapy had received antiviral therapy thereafter. In the 110 patients, daclatasvir-resistance mutations were analyzed by deep sequencing of sera collected and stored at the most recent visit to the hospital.

All patients studied fulfilled following criteria: (i) negative for hepatitis B surface antigen; (ii) no other forms of hepatitis, such as primary biliary cirrhosis, autoimmune liver disease or alcoholic liver disease; (iii) free of co-infection with HIV; and (iv) signed consent was obtained for the study protocol that had been approved by Human Ethics Review Committee of Yamanashi University Hospital. The clinical backgrounds of the 110 patients are shown in Table 1.

Direct sequencing

Hepatitis C virus RNA extraction, complementary DNA synthesis, amplification by two-step nested polymerase chain reaction (PCR) from serum samples using primers specific for partial viral regions and direct sequencing were carried out as described previously.[15,16] Generated sequence files were assembled using Vector NTI software (Invitrogen, Tokyo, Japan) and base-calling errors were corrected following inspection of the chromatogram.

This direct sequencing procedure was performed to determine the dominant viral sequences of the core,[17] the IFN sensitivity-determining region (ISDR)[18] and the IFN-ribavirin resistance determining region (IRRDR)[19] from the serum of each patient.

IL28B SNP analysis

Recent reports have disclosed a significant correlation between polymorphisms in the IL28B gene and patients' responses to PEG IFN plus RBV therapy for HCV.[20–22] Human genomic DNA was extracted from peripheral blood using a blood DNA extraction kit (QIAGEN, Tokyo, Japan) according to the manufacturer's protocol. The genotyping of each DNA sample was performed by real-time PCR with a model 7500 sequencer (ABI, Tokyo, Japan) using FAM- and VIC-labeled single nucleotide (nt) polymorphism (SNP) probes for the locus rs8099917 (ABI).

Deep sequencing

Deep sequencing of part of the viral NS5A region was performed for each of the 110 patients. Briefly, RNA was extracted from the stored sera and reverse transcribed to complementary DNA.[23] Then, two-step nested PCR was carried out with primers specific for the NS5A region of the HCV genome. To avoid PCR selection bias, we

Table 1 Patient characteristics classified by their responses to previous PEG IFN/RBV combination therapy

	Naïve $n = 59$	Relapser $n = 30$	Null responder $n = 21$	*P*
Age (years)	62.3 ± 11.5	62.7 ± 9.1	61.2 ± 7.7	0.719
Sex F/M	35/24	16/14	9/12	0.427
AST (IU/L)	35.4 ± 12.6	43.9 ± 53.4	45.3 ± 14.6	0.008
ALT (IU/L)	34.6 ± 18.5	45.3 ± 73.2	51.8 ± 23.5	<0.001
PLT ($\times 10^{-4}/\mu L$)	15.1 ± 5.6	14.3 ± 3.8	13.8 ± 4.8	0.582
Alb (g/dl)	4.2 ± 0.4	4.3 ± 0.3	4.2 ± 0.5	0.334
γ-GT (IU/L)	35.2 ± 37.7	37.6 ± 45.1	67.1 ± 55.2	<0.001
AFP (ng/mL)	5.7 ± 6.3	4.5 ± 3.6	14.7 ± 29.0	<0.001
Core a.a. 70 R	35 (59.3%)	23 (76.7%)	6 (28.6%)	0.003
Core a.a. 91 L	41 (69.5%)	18 (60.0%)	14 (66.7%)	0.672
ISDR 2-	14 (23.7%)	5 (16.7%)	2 (9.5%)	0.340
IRRDR 5-	29 (49.2%)	13 (43.3%)	8 (38.1%)	0.181
IL28B SNP TT	38 (64.4%)	27 (90.0%)	6 (25.6%)	<0.001

γ-GT, γ-glutamyltransferase; a.a., amino acid; AFP, α-fetoprotein; Alb, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; IRRDR, interferon-ribavirin resistance determining region; ISDR, interferon sensitivity-determining region; PEG IFN/RBV, pegylated interferon/ribavirin; PLT, platelets.

searched for the most conserved DNA sequence regions around NS5A by examining sequence information published previously from 43 HCV positive individuals from Japan[16] and designed novel primers for this study (Supplementary Table 1). This PCR procedure amplified 436 viral nt, including the 1st to 432nd nt of the NS5A region. The primers for the second-round PCR had barcodes, 10 nt in length, attached and these differed for each sample, so that the PCR products from each sample were identifiable. After the band densities of the PCR products were quantified using a Pico Green dsDNA Assay Kit (Invitrogen), the concentrations of the samples were adjusted to a common value and pooled samples were prepared.

Libraries were then subjected to emulsion PCR, the enriched DNA beads were loaded onto a picotiter plate and pyrosequencing was carried out with a Roche GS Junior/454 sequencing system using titanium chemistry (Roche, Branford, CT, USA). The Roche Variant Analyzer version 2.5pl (Roche) was used for the analysis.

Statistical analysis

Statistical differences in the parameters, including all available patients' demographic, biochemical, hematological, virological and SNP data in the three groups (naïve, relapser and null responder), classified according to the response to previous PEG IFN/RBV therapy, were determined using the χ^2-test for categorical variables and Kruskal–Wallis test for numerical variables. Statistical differences in the parameters in two groups (Y93H positive, Y93H negative) were determined by Student's *t*-test or Mann–Whitney *U*-test for numerical variables and Fisher's exact test or χ^2-test for categorical variables. Variables that achieved statistical significance ($P < 0.05$) in univariate analysis were entered into multiple logistic regression analysis to identify significant independent factors. We also calculated the odds ratios and 95% confidence intervals. All *P*-values of less than 0.05 by the two-tailed test were considered significant.

RESULTS

Average read numbers obtained by deep sequencing and the background error rate

TO PERFORM DEEP sequencing analysis of the NS5A region from many patients, simultaneous analysis was carried out using the barcode primers and approximately 3826 reads were obtained per sample from each group of patients (naïve, relapser and null responder) (Table 2). Because a previous clinical phase II study had yielded a significantly high SVR rate, especially in the patients with a null response to previous PEG IFN/RBV combination therapy, we classified the patients according to their responses to previous PEG IFN/RBV combination therapy with the assumption that differences in the response to PEG IFN/RBV might influence the daclatasvir response.

The background error rate of pyrosequencing was calculated with a plasmid containing a cloned HCV sequence (pCV-J4L6S)[24] and the read number for the

Table 2 Amplicon read numbers obtained by deep sequencing

	n	Average reads ± SD (range)/sample
Naïve	59	3603.9 ± 1758.4 (655–10 293)
Relapser	30	3980.4 ± 3295.9 (445–14 330)
Null responder	21	4601.6 ± 2385.5 (1187–9579)
Plasmid	7	5448.3 ± 1299.1 (2277–7000)

SD, standard deviation.

plasmid is also shown in Table 2. Though seven runs of the plasmid produced 2277–7000 reads, with an average of 5448 reads, there was no background error at amino acid (a.a.) 31, 32 or 93 in NS5A. Because the background error rate was 0% at each position, the presence of mutations at 0.1% or higher was considered to be significant, based on the 95% confidence interval (0–0.1%) calculated for 0% in 2227 reads. The background error rate coincided almost exactly with the background error rate obtained in our recent study.[23]

Baseline characteristics

The baseline characteristics of the 110 patients are shown in Table 1. The data for viral factors (core a.a. 70, core a.a. 91, NS5A-ISDR and NS5A-IRRDR) in the table were obtained by direct sequencing as described in Methods. As shown in the table, there were significant differences among the three groups in aspartate aminotransferase, alanine aminotransferase, γ-glutamyltransferase, α-fetoprotein, core a.a. 70 and IL28B SNP (rs8099917). Meanwhile, there was no significant difference in background factors of age and sex or factors associated with liver fibrosis such as platelets and albumin.

Detection of NS5A-resistance mutations by deep sequencing

Because previous reports showed that L31M/V/F, P32L and Y93H are resistance mutations in NS5A of genotype 1b HCV, the presence of these mutations was analyzed by deep sequencing. Table 3 shows the rate of NS5A resistance mutations at a.a. 31, 32 and 93. At a.a. 32, no mutation was found in any of the 110 patients. Regarding a.a. 31, resistance mutations (L31M/V/F) were observed in 13 of the 110 patients (11.8%) and, despite no significant difference, tended to occur more frequently in the relapser group and naïve group than in the null group. Meanwhile, the a.a. 93 resistance mutation (Y93H) was observed in 34 of the 110 patients (30.9%) and, despite no significant difference, also tended to occur more frequently in the naïve and relapser groups than in the null group. Simultaneous a.a. 93 and 31 resistance mutations were observed in only four of 110 patients (3.6%) and these four patients all belonged to the naïve group. More detailed deep sequence results for the four patients with simultaneous mutation of L31M/V/F and Y93H are shown in Table 2. Although the substitution rate of L31M/V/F in these patients was low, all isolates with L31M/V/F also featured the Y93H change.

Mutation rates of L31M/V/F and Y93H in each patient

Figure 1 show the mutation rates of L31M/V/F and Y93H in each patient. One bar indicates the resistance mutation rate in one patient, obtained by deep sequencing. It was found that minor viral populations that were not detected by direct sequencing could be detected by deep sequencing.

Table 3 Presence of daclatasvir-resistance amino acid substitutions in daclatasvir treatment-naïve patients, determined by deep sequencing

	Naïve *n* = 59	Relapser *n* = 30	Null responder *n* = 21	Naïve vs relapser *P*	Naïve vs null *P*	Relapser vs Null *P*
L31M/V/F %, median (range)†	2.0 (0.0–99.8)	4.1 (0.0–100.0)	0.2 (0.0–3.4)	0.895	0.295	0.317
Pts with L31M/V/F (%)‡	8 (13.6%)	4 (13.3%)	1 (4.8%)	1.000	0.510	0.612
P32L %, median (range)†	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	1.000	1.000	1.000
Pts with P32L (%)‡	0 (0%)	0 (0%)	0 (0%)	1.000	1.000	1.000
Y93H %, median (range)†	11.7 (0.0–99.1)	7.9 (0.0–100.0)	4.1 (0.0–45.3)	0.824	0.190	0.301
Pts with Y93H (%)‡	21 (35.6%)	10 (33.3%)	3 (14.3%)	1.000	0.112	0.224

†Median proportion per patient (Pts).
‡Number of Pts with the mutant.

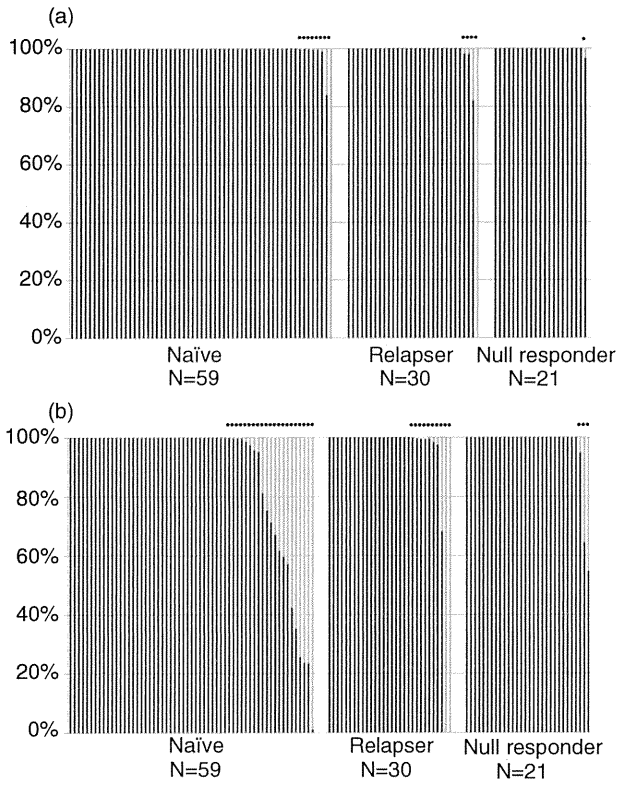

Figure 1 Percentage of mutations in the non-structural (NS)5A region associated with resistance to daclatasvir is presented, classified by the response to previous pegylated interferon/ribavirin (PEG IFN/RBV) therapy (naïve, null responder and relapser). (a) NS5A amino acid (a.a.) 31, (b) NS5A a.a. 93. Each bar indicates the mutation rate in one patient and a dot above a bar shows a patient with a mutation detected by deep sequencing.

In order to compare our deep sequencing data with previous direct sequencing data in terms of the frequency of NS5A mutations, the notion of "cut-offs" was introduced into our deep sequencing data, assuming that direct sequencing could detect minor populations existing above those cut-off levels. When the cut-off level of 50% was defined to detect minor populations by direct sequencing, L31M/V/F mutations and the Y93H mutations were detected in 1.8% (2/110 patients) and 7.3% (8/110) of our patients, respectively, while the values became 1.8% (2/110 patients) and 15.4% (15/110) when 20% was defined as the cut-off level. These results are comparable to the mutation rate determined previously by direct sequencing and that found in the database.[25]

Univariate and multivariate analysis of factors related to the NS5A Y93H mutation

Focusing on the Y93H mutation that is found most frequently in daclatasvir treatment-naïve patients, clinical background factors that would determine efficacy of PEG IFN/RBV combination therapy patients were investigated by univariate analysis of their association with the Y93H substitution (Table 4). Three factors, the IL28B SNP, core a.a. 70 and IRRDR, were found to be correlated with the Y93H substitution with statistical significance in the univariate analysis. In patients with the Y93H mutation, the major type (TT) was frequently observed as the IL28B SNP, while arginine (R) was frequently observed at core a.a. 70 and the number of substitutions in the IRRDR was higher. There was no significant difference in the number of mutations in the ISDR but that number tended to be higher in patients with the Y93H mutation, similar to the IRRDR.

The IL28B SNP, core a.a. 70 and IRRDR, which were correlated significantly with the a.a. 93 mutation by univariate analysis, were subjected to multivariate analysis (Table 4). The IL28B SNP major type (TT) was extracted as an independent significant factor with the odds ratio of 3.67 ($P = 0.042$). The mutation rates of L31M/V/F and Y93H in each patient, classified by the IL28 SNP, are presented in Figure 2. Y93H mutations were found significantly more frequently in IL28B TT patients than that in IL28B non-TT patients.

DISCUSSION

IN THIS STUDY, viral mutations conferring resistance to the NS5A replication complex inhibitor daclatasvir were investigated by deep sequencing in daclatasvir treatment-naïve genotype 1b HCV patients and the mutations, especially Y93H, were detected more frequently than predicted by direct sequencing. Interestingly and importantly, the presence of the Y93H mutation correlated with the IL28B SNP of the host, suggesting the possibility that IL28B major type patients who may show a favorable response to IFN have a greater risk of being infected by daclatasvir-resistant HCV.

Regarding the daclatasvir-resistance mutations L31M/V/F, P32L and Y93H in genotype 1b HCV, it has been reported that a single mutation produces 5–28-fold increased resistance and simultaneous mutations of L31M/V/F and Y93H yield 10 989–21 674-fold increased resistance in genotype 1b HCV infection.[6] Previously, the frequencies of L31M/V/F and Y93H were reported to be 2.7% and 8.2%, respectively, with direct

Table 4 Univariate and multivariate analysis of factors associated with non-structural (NS)5A-Y93H

Variables	No. of patients	NS5A-Y93H substitution		Univariate analysis ($n = 110$)		Multivariate analysis ($n = 110$)	
		Positive ($n = 34$)	Negative ($n = 76$)	Odds ratio (95% CI)	*P*-value	Odds ratio (95% CI)	*P*-value
Age, ≥65 years	48	16 (47.1%)	32 (42.1%)	1.22 (0.54–2.76)	0.629		
Sex male	50	16 (47.1%)	34 (44.7%)	1.10 (0.49–2.47)	0.821		
AST, ≥41 IU/L	38	11 (32.4%)	27 (35.5%)	0.87 (0.37–2.05)	0.746		
ALT, ≥41 IU/L	33	9 (26.5%)	24 (31.6%)	0.78 (0.32–1.92)	0.590		
Platelets, $\leq 12 \times 10^{-4}/mm^3$	35	12 (35.3%)	23 (30.3%)	1.43 (0.61–3.33)	0.601		
Alb, ≤4 g/dL	25	9 (26.5%)	16 (21.1%)	0.69 (0.28–1.70)	0.422		
γ-GT, ≥41 IU/L	30	10 (29.4%)	20 (26.3%)	1.25 (0.51–3.08)	0.628		
AFP, ≥10	16	5 (14.7%)	11 (14.5%)	1.02 (0.32–3.20)	0.974		
IL28B TT	71	29 (85.3%)	42 (55.3%)	4.70 (1.64–13.43)	0.004	3.67 (1.05–12.88)	0.042
Core a.a. 70 R	64	25 (73.5%)	39 (51.3%)	2.64 (1.09–6.38)	0.032	1.19 (0.40–3.55)	0.759
Core a.a. 91 L	73	24 (70.6%)	49 (64.5%)	1.32 (0.55–3.17)	0.531		
ISDR,† ≥2	21	8 (23.5%)	13 (17.1%)	1.49 (0.55–4.02)	0.430		
IRRDR,‡ ≥5	54	23 (67.5%)	32 (42.1%)	2.88 (1.23–6.73)	0.015	2.37 (0.98–5.74)	0.056
NS5AL31 M/V/F positive	11	2 (5.9%)	9 (11.8%)	0.46 (0.10–2.28)	0.345		
History of IFN therapy	59	21 (61.8%)	38 (50.0%)	1.62 (0.71–3.69)	0.255		

†ISDR mutation number.
‡IRRDR mutation number.
γ-GT, γ-glutamyltransferase; a.a., amino acid; AFP, α-fetoprotein; Alb, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CI, confidence interval; IRRDR, interferon-ribavirin resistance determining region; ISDR, interferon sensitivity-determining region; PEG IFN/RBV, pegylated interferon/ribavirin; PLT, platelets.

sequencing in genotype 1b daclatasvir treatment-naïve Japanese patients ($n = 294$) and this was comparable with the frequency (3.8% and 8.3%, respectively) in genotype 1b patients, determined from the European HCV database ($n = 1796$).[6,25] Among the regimens including daclatasvir for genotype 1b HCV infection, until now, only the result of a phase II trial of daclatasvir/asunaprevir therapy for 43 patients has been reported.[8,9] In that study, the pretreatment presence of HCV carrying Y93H was significantly associated with non-SVR to that regimen and, moreover, that viruses carrying mutations in both regions of NS5A (L31M/V/F and Y93H) and of NS3 (D168A/V) emerged in most of the non-SVR patients after virological failure.

In our study, the presence of L31M/V/F and Y93H mutations in daclatasvir treatment-naïve genotype 1b patients was comparable to a previous study which involved direct sequencing, when a cut-off value was introduced to our deep sequencing data, although the prevalence of NS5A mutants changed depending on the cut-off value. However, deep sequencing analysis revealed that NS5A L31M/V/F and Y93H mutations were detectable in 13 (11.8%) and 34 of the 110 (30.9%) patients, respectively, above the background error rate of 0.1% and significantly more frequently than detected by direct sequencing. These results demonstrate that deep sequencing is useful for the detection of viral mutants present as minor variants.

Do HCV populations with Y93H present as minor variants have any association with clinical characteristics? Interestingly, univariate analysis based on the relationship between the presence of the Y93H variant and clinical factors or factors determining treatment efficacy to PEG IFN/RBV combination therapy extracted three significant factors: the IL28B SNP, core a.a. 70 and the IRRDR (Table 4). All these factors were associated with a favorable response to PEG IFN/RBV combination therapy in the group with the Y93H-resistance mutation.[26] Despite that the difference did not reach statistical significance, the number of substitutions in the ISDR also tended to be higher in the group with the Y93H mutation, similar to the IRRDR. It was quite intriguing that multivariate analysis of the presence of Y93H extracted the IL28B major type, the SNP was significantly associated with favorable IFN responses, as an independent factor (Table 4). On the other hand, because it is known that the IL28B SNP is closely linked with core a.a. 70, it is assumed that core 70R should be observed more frequently in the group with Y93H.[16]

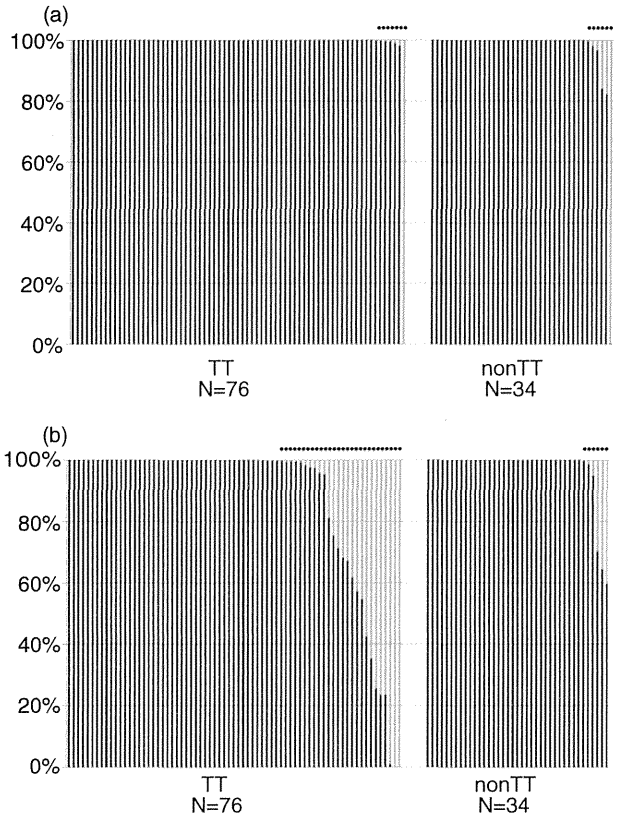

Figure 2 Percentage of mutations at the non-structural (NS)5A region for resistance to daclatasvir is presented, classified by the IL28B SNP (TT or non-TT). (a) NS5A amino acid (a.a.) 31, (b) NS5A a.a. 93. Each bar indicates the mutation rate in one patient and a dot above a bar shows a patient with a mutation detected by deep sequencing.

Then, do NS5A resistant variants with Y93H that are present prior to treatment affect the response to daclatasvir treatment? At present, in genotype 1b infection, daclatasvir is scheduled to be used in combination with other DAA but not with IFN. Considering the correlation between IL28B SNP and Y93H, and the fact that Y93H variants may be sensitive to IFN but resistant to daclatasvir,[27] patients with the IL28B major type may be recommended to receive IFN-based therapy rather than DAA regimens including daclatasvir, because those patients have a greater chance of being infected with daclatasvir-resistant Y93H variants leading to treatment failure. In contrast, the IL28B minor type patients who have poor responses to IFN may be more promising candidates.

The true clinical influence of Y93H on treatment responses remain unknown and further elucidation is mandatory after the approval of daclatasvir for clinical use. In particular, it is important to clarify the cut-off values as to the mixture ratio of Y93H to Y93 wild type in establishing clinical resistance, if the presence of viruses with Y93H before treatment really does affect the response. If so, it is also important to clarify whether the proportion of Y93H variants changes during the clinical course (the natural course or during therapy including IFN) in order to determine the most appropriate timing for introducing daclatasvir. However, it is possible for Y93H variants to disappear after IFN treatment considering that Y93H variants may be sensitive to IFN. The mechanism of the relationship between the IL28B SNP and Y93H also is not clear at present. Considering that wild-type NS5A is known to be associated in its ISDR region with IFN resistance and with the IL28B minor SNP (TG/GG),[28] it is possible that wild-type NS5A Y93 also is associated with IFN resistance and with IL28B minor SNP, although further elucidation is necessary.

We acknowledge that the PCR technique has a risk of producing biased amplicons according to the PCR primer sequences and therefore we designed novel primers in this study by searching for the most conserved sequence regions around NS5A. We speculate that the sequence bias might have been avoided at least to some extent considering the fact that the NS5A mutation rate in this study was quite compatible with that of a previous study and that obtained from the public database.

In conclusion, we detected by deep sequencing the substantial presence of resistance mutations to daclatasvir, Y93H in particular, in daclatasvir treatment-naïve patients and these were not detectable by direct sequencing. We also showed that IL28B major type patients who have favorable responses to IFN may have a higher risk of being infected with Y93H HCV than IL28B minor type patients, suggesting that those patients may have a higher risk of developing daclatasvir resistance, although further studies are needed.

REFERENCES

1 Sarrazin C, Hezode C, Zeuzem S, Pawlotsky JM. Antiviral strategies in hepatitis C virus infection. *J Hepatol* 2012; 56 (Suppl 1): S88–100.
2 Ghany MG, Nelson DR, Strader DB, Thomas DL, Seeff LB. An update on treatment of genotype 1 chronic hepatitis C virus infection: 2011 practice guideline by the American Association for the Study of Liver Diseases. *Hepatology* 2011; 54 (4): 1433–44.
3 Scheel TK, Gottwein JM, Mikkelsen LS, Jensen TB, Bukh J. Recombinant HCV variants with NS5A from genotypes 1-7

have different sensitivities to an NS5A inhibitor but not interferon-alpha. *Gastroenterology* 2011; **140** (3): 1032–42.
4 Lok AS, Gardiner DF, Lawitz E *et al.* Preliminary study of two antiviral agents for hepatitis C genotype 1. *N Engl J Med* 2012; **366** (3): 216–24.
5 Pol S, Ghalib RH, Rustgi VK *et al.* Daclatasvir for previously untreated chronic hepatitis C genotype-1 infection: a randomised, parallel-group, double-blind, placebo-controlled, dose-finding, phase 2a trial. *Lancet Infect Dis* 2012; **12** (9): 671–7.
6 Fridell RA, Qiu D, Wang C, Valera L, Gao M. Resistance analysis of the hepatitis C virus NS5A inhibitor BMS-790052 in an in vitro replicon system. *Antimicrob Agents Chemother* 2010; **54** (9): 3641–50.
7 Fridell RA, Wang C, Sun JH *et al.* Genotypic and phenotypic analysis of variants resistant to hepatitis C virus nonstructural protein 5A replication complex inhibitor BMS-790052 in humans: in vitro and in vivo correlations. *Hepatology* 2011; **54** (6): 1924–35.
8 Karino Y, Toyota J, Ikeda K *et al.* Characterization of virologic escape in hepatitis C virus genotype 1b patients treated with the direct-acting antivirals daclatasvir and asunaprevir. *J Hepatol* 2013; **58**: 646–54.
9 Suzuki Y, Ikeda K, Suzuki F *et al.* Dual oral therapy with daclatasvir and asunaprevir for patients with HCV genotype 1b infection and limited treatment options. *J Hepatol* 2013; **58**: 655–62.
10 Chayama K, Takahashi S, Toyota J *et al.* Dual therapy with the nonstructural protein 5A inhibitor, daclatasvir, and the nonstructural protein 3 protease inhibitor, asunaprevir, in hepatitis C virus genotype 1b-infected null responders. *Hepatology* 2012; **55** (3): 742–8.
11 Londono MC, Lens S, Forns X. Interferon free regimens for the "difficult-to-treat": are we there? *J Hepatol* 2013; **58**: 643–5.
12 Hiraga N, Imamura M, Abe H *et al.* Rapid emergence of telaprevir resistant hepatitis C virus strain from wildtype clone in vivo. *Hepatology* 2011; **54** (3): 781–8.
13 Akuta N, Suzuki F, Seko Y *et al.* Emergence of telaprevir-resistant variants detected by ultra-deep sequencing after triple therapy in patients infected with HCV genotype 1. *J Med Virol* 2013; **85** (6): 1028–36.
14 McPhee F, Hernandez D, Yu F *et al.* Resistance analysis of hepatitis C virus genotype 1 prior treatment null responders receiving daclatasvir and asunaprevir. *Hepatology* 2013; **58** (3): 902–11.
15 Nagayama K, Kurosaki M, Enomoto N *et al.* Time-related changes in full-length hepatitis C virus sequences and hepatitis activity. *Virology* 1999; **263** (1): 244–53.
16 Miura M, Maekawa S, Kadokura M *et al.* Analysis of viral amino acids sequences and the IL28B SNP influencing the development of hepatocellular carcinoma in chronic hepatitis C. *Hepatol Int* 2012; **6** (1): 386–96.
17 Akuta N, Suzuki F, Sezaki H *et al.* Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 1b high viral load and non-virological response to interferon-ribavirin combination therapy. *Intervirology* 2005; **48** (6): 372–80.
18 Enomoto N, Sakuma I, Asahina Y *et al.* Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. *N Engl J Med* 1996; **334** (2): 77–81.
19 El-Shamy A, Nagano-Fujii M, Sasase N, Imoto S, Kim SR, Hotta H. Sequence variation in hepatitis C virus nonstructural protein 5A predicts clinical outcome of pegylated interferon/ribavirin combination therapy. *Hepatology* 2008; **48** (1): 38–47.
20 Ge D, Fellay J, Thompson AJ *et al.* Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature* 2009; **461** (7262): 399–401.
21 Suppiah V, Moldovan M, Ahlenstiel G *et al.* IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. *Nat Genet* 2009; **41** (10): 1100–4.
22 Tanaka Y, Nishida N, Sugiyama M *et al.* Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. *Nat Genet* 2009; **41** (10): 1105–9.
23 Miura M, Maekawa S, Takano S *et al.* Deep sequencing analysis of the association between the quasispecies nature of the hepatitis C virus core region and disease progression. *J Virol* 2013; **87**: 12541–51.
24 Gates AT, Sarisky RT, Gu B. Sequence requirements for the development of a chimeric HCV replicon system. *Virus Res* 2004; **100** (2): 213–22.
25 Suzuki F, Sezaki H, Akuta N *et al.* Prevalence of hepatitis C virus variants resistant to NS3 protease inhibitors or the NS5A inhibitor (BMS-790052) in hepatitis patients with genotype 1b. *J Clin Virol* 2012; **54** (4): 352–4.
26 Maekawa S, Sakamoto M, Miura M *et al.* Comprehensive analysis for viral elements and interleukin-28B polymorphisms in response to pegylated interferon plus ribavirin therapy in hepatitis C virus 1B infection. *Hepatology* 2012; **56** (5): 1611–21.
27 Friborg J, Levine S, Chen C *et al.* Combinations of lambda interferon with direct-acting antiviral agents are highly efficient in suppressing hepatitis C virus replication. *Antimicrob Agents Chemother* 2013; **57** (3): 1312–22.
28 Kurosaki M, Tanaka Y, Nishida N *et al.* Pre-treatment prediction of response to pegylated-interferon plus ribavirin for chronic hepatitis C using genetic polymorphism in IL28B and viral factors. *J Hepatol* 2011; **54** (3): 439–48.

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Original Article

Hepatocellular carcinoma risk assessment using gadoxetic acid-enhanced hepatocyte phase magnetic resonance imaging

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Aim: To investigate whether the patients with hypovascular liver nodules determined on the arterial phase and hypointensity on the hepatocyte phase gadoxetic acid-enhanced magnetic resonance imaging (hypovascular hypointense nodules) are at increased risk of hepatocarcinogenesis, we assessed subsequent typical hepatocellular carcinoma (HCC) development at any sites of the liver with and without such nodules.

Methods: One hundred and twenty-seven patients with chronic hepatitis B or C and without a history of HCC, including 68 with liver cirrhosis, were divided into those with (non-clean liver group, $n = 18$) and without (clean liver group, $n = 109$) hypovascular hypointense nodules. All the patients were followed up for 3 years, and HCC development rates and risk factors were analyzed with the Kaplan–Meier method and the Cox proportional hazard model, respectively.

Results: A total of 17 patients (10 in the non-clean liver group and seven in the clean liver group) developed typical HCC. Cumulative 3-year rates of HCC development were 55.5% in the non-clean liver group and 6.4% in the clean liver group ($P < 0.001$), and those at the different sites from the initial nodules was also higher in the non-clean liver group (22.2%) than the clean liver group (6.4%) ($P = 0.003$). Multivariate analysis identified older age ($P = 0.024$), low platelet counts ($P = 0.017$) and a non-clean liver ($P < 0.001$) as independent risk factors for subsequent HCC development.

Conclusion: Patients with hypovascular hypointense liver nodules are at a higher risk for HCC development at any sites of the liver than those without such nodules.

Key words: gadoxetic acid, hepatocellular carcinoma, hepatocyte phase, magnetic resonance imaging, risk assessment

INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) is one of the most common cancers worldwide and is a major cause of death in patients with chronic viral liver disease. Despite many advances in multidisciplinary treatment, complete curative treatment of early stage HCC remains the only possible therapeutic choice for long-term survival. Therefore, surveillance programs for patients at a high risk for HCC that include imaging-based evaluations are crucial for the detection and treatment of early stage HCC.

The newly introduced magnetic resonance imaging (MRI) contrast agent, gadolinium ethoxybenzyl

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diethylenetriamine pentaacetic acid (gadoxetic acid), has enabled concurrent assessment of tumor vascularity and unique hepatocyte-specific contrast (hepatocyte phase).[1–3] This has led to the frequent identification of hypovascular nodules determined on the arterial phase with hypointensity on the hepatocyte phase (hypovascular hypointense nodules),[4–8] while many of these nodules are difficult to be detected by ultrasonography (US) or computed tomography (CT). Recently, the natural history of hypovascular hypointense nodules themselves were reported in several studies,[9–12] revealing the high risk of subsequent progress to typical HCC from these nodules. However, it is not well known whether patients with such nodules have a higher risk of developing typical HCC at any sites of the liver, including at the different sites from initial nodules, compared to those without such nodules.

If patients with these nodules may have a high risk of developing typical HCC not only at the same sites but also at the different sites from initial nodules, a significant proportion of these nodules are precancerous lesions or early stage HCC as reported,[13–15] and more importantly, the liver with these nodules may reflect a higher potential for hepatocarcinogenesis or the presence of undetectable precursor lesions in other sites of the liver. Conversely, the absence of these nodules potentially identifies the patients at a low risk for subsequent typical HCC development at any sites. The purpose of this study was to assess the risk of subsequent typical HCC development at any sites of the liver with and without hypovascular hypointense nodules on gadoxetic acid-enhanced MRI.

METHODS

Ethical review

THE PROTOCOL OF this retrospective study was approved by the ethics committee of Yamanashi University Hospital, which waived the requirement for written informed consent because the study was a retrospective data analysis, with appropriate consideration given to patient risk, privacy, welfare and rights.

Patients

We recruited 559 consecutive outpatients with chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection who underwent gadoxetic acid-enhanced MRI at Yamanashi University Hospital between January 2008 and December 2010. The exclusion criteria were as follows: (i) presence or history of typical HCC ($n = 420$), because intrahepatic metastasis does not always develop through the usual multistep hepatocarcinogenesis process, skipping the early pathological stage with hypovascularity to an advanced pathological stage even when the size is small;[16,17] (ii) Child–Pugh class C disease ($n = 9$), because the hepatocyte phase findings are not reliable in patients with this condition because of reduced gadoxetic acid uptake in the liver;[18] and (iii) patients who dropped out during the 3-year follow-up period ($n = 3$).

After excluding 432 patients, 127 patients were included in this retrospective cohort study. They were divided into groups with hypovascular nodules determined on the arterial phase and hypointensity on the hepatocyte phase (non-clean liver group; $n = 18$ patients) and without such nodules (clean liver group; $n = 109$ patients) as shown in Figure 1. In this study, we divided cases into two groups according to the presence or absence of these nodules at the baseline, even when such nodules were initially detected during the follow-up period; we assigned these patients to the clean liver group.

Follow up and diagnosis of HCC

All 127 patients were followed up at the liver disease outpatient clinic of our institution with blood tests, including those for tumor markers and diagnostic imaging modality (US, CT or MRI). The development of typical HCC that required treatment as proposed by the American Association for the Study of Liver Diseases (AASLD) guidelines[19] and that was diagnosed according to imaging criteria, showing arterial hypervascularity and venous phase washout, or based on histological examination of liver biopsies from hypovascular nodules that grew to more than 10 mm during follow up. Biopsies were obtained using a 21-G core needle. Two patients each had a liver nodule of more than 10 mm in diameter on initial MRI (12 mm and 13 mm), which were diagnosed on the basis of the biopsy as dysplastic nodules.

The end-point of this study was the development of typical HCC not only from the hypovascular hypointense nodules observed initially but also from other areas without these nodules ("de novo HCC"). Dynamic CT and/or MRI were also performed in cases with hepatic nodules detected by US, liver cirrhosis, a tendency of tumor marker elevation and difficult evaluation of the liver parenchyma by US. All 127 patients were followed up for 3 years after the initial gadoxetic acid-enhanced MRI examination. When imaging

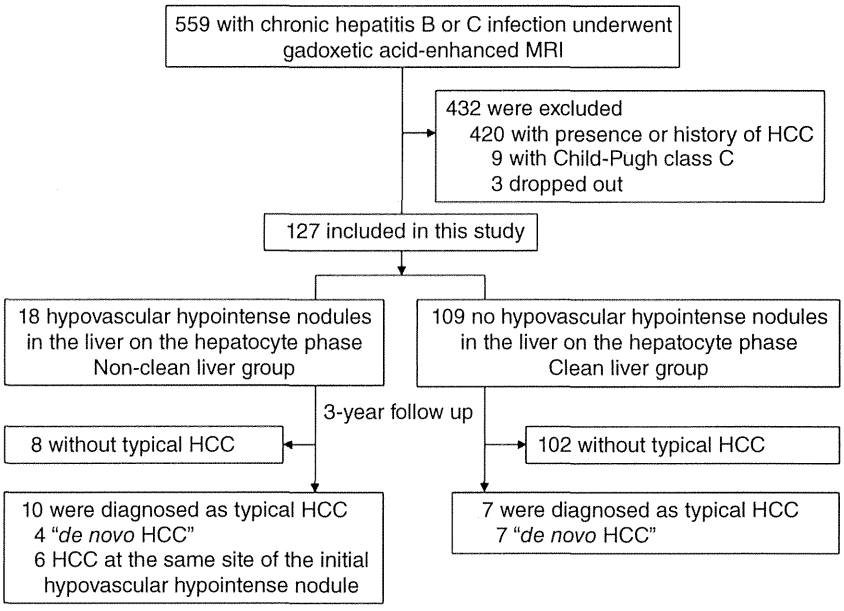


Figure 1 Patient inclusion criteria. "De novo HCC" is a typical hepatocellular carcinoma that developed at sites in which no nodules had been seen on the initial gadoxetic acid-enhanced magnetic resonance imaging (MRI).

modalities led to diagnosis of HCC, recognizing hypervascularization by more than one experienced radiologist and other imaging modalities was regarded as the time of diagnosis of HCC. When needle biopsy was performed to investigate nodules, the time of diagnosis of HCC was when the pathologists and physicians examined pathological tissue and diagnosed as HCC.

MRI

Magnetic resonance imaging was performed using a superconducting magnet that operated at 1.5 Tesla (Sigma EXCITE HD; GE Medical Systems, Milwaukee, WI, USA) and an 8-channel phased-array coil. First, we obtained fast spoiled gradient-echo T_1-weighted images (T1WI) with dual echo acquisition and respiratory-triggered fat-saturated fast spin-echo T_2-weighted images (T2WI). Dynamic fat-suppressed gradient-echo T1WI were obtained using a 3-D acquisition sequence before (precontrast) and 20–30 s, 60 s, 2 min, 5 min, 10 min and 20 min after the administration of gadoxetic acid (Primovist; Bayer Schering Pharma, Berlin, Germany). This contrast agent (0.025 mM/kg bodyweight) was administrated i.v. as a bolus at a rate of 1 mL/s through an i.v. cubital line (20–22 G) that was flushed with 20 mL saline from a power injector. The delay time for the arterial phase scan was adjusted according to a fluoroscopic triggering method.[20] All images were acquired in the transverse plane. Sagittal plane T1WI were also obtained during the hepatocyte phase at 20 min after the injection of the contrast agent.

Statistical analysis

All continuous values are expressed as median (range). Fisher's exact probability test was used for comparisons between categorical variable and the non-parametric Mann-Whitney *U*-test was used to compare differences between continuous variables. Baseline clinical characteristics, including blood test results, were evaluated within 1 month of the initial MRI. We investigated whether or not HCC development was associated with age, sex, fibrosis, etiology (HBV or HCV), platelet count, serum alanine aminotransferase (ALT), γ-glutamyltransferase (γ-GT), α-fetoprotein (AFP), and the presence or absence of hypovascular hypointense nodules.

Cumulative HCC development was estimated according to the Kaplan–Meier method and differences in the curves were tested using the log–rank test. Risk factors for HCC development were determined according to the Cox proportional hazard model. Subgroup analyses with a Cox proportional hazard model were applied to estimation of the hazard ratio (HR) of the non-clean liver group versus clean liver group in the dichotomized subgroups. All statistical analyses were performed using JMP software, version 10 (SAS Institute Japan, Tokyo, Japan). A two-sided *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Characteristics of the patients and nodules

A TOTAL OF 127 patients were enrolled, of whom 26 had chronic HBV infections and 101 had HCV infections, and 68 had virus-associated cirrhosis. No statistically significant differences in the initial clinical characteristics were found between the non-clean liver and clean liver groups (Table 1). Thirty-five hypovascular hypointense nodules were found in 18 patients in the non-clean liver group (1–5 nodules per patient) at baseline (data not shown). Twenty-four of these 35 nodules were detectable only on the hepatocyte phase MRI and were undetectable by US, CT and non-hepatocyte phase MRI. None of the 35 nodules showed high intensity on T2WI. The median nodule diameter was 8 mm (range, 4–13 mm; 33 nodules with ≤10 mm, two nodules with 12 mm and 13 mm).

HCC incidence according to initial MRI findings

Hepatocellular carcinoma was diagnosed in 17 patients, 10 in the non-clean liver group and seven in the clean liver group; 14 of these patients had HCV infection. Thirteen patients were diagnosed according to the AASLD imaging criteria.[19] Four patients were diagnosed pathologically by liver biopsies that were performed, based on enlargement of the nodules of more than 10 mm in diameter during the observation period.

The cumulative 1-, 2- and 3-year HCC incidence rates were 1.5%, 10.2% and 13.4%, respectively. As determined by the Kaplan–Meier method, these rates were 11.1% (95% confidence interval [CI], 0.0–25.6%), 38.8% (95% CI, 16.3–61.4%) and 55.5% (95% CI, 32.6–78.5%) in the non-clean liver group, and 0.0% (95% CI, 0.0–2.3%), 5.5% (95% CI, 0.0–9.8%) and 6.4% (95% CI, 1.8–11.0%) in the clean liver group; the former group showed significantly higher rates of development of typical HCC than the latter ($P < 0.001$) as shown in Figure 2. The median imaging intervals were 3 months (range, 3–6) in the non-clean liver group and 4 months (range, 2–12) in the clean liver group. The imaging interval of the non-clean liver group was shorter than the clean liver group (3 vs 4 months, $P = 0.015$). The median intervals between the initial MRI and HCC diagnosis was 16 months (range, 9–32) in the non-clean liver group and 21 months (range, 16–35) in the clean liver group.

In 11 of 17 patients with HCC development, HCC developed at sites in which no nodules had been seen on the initial gadoxetic acid-enhanced MRI, namely de novo HCC. These HCC were found in four of 18 patients in the non-clean liver group (3-year HCC incidence rates: 22.2%; 95% CI, 4.3–51.0%) and 7 in 109 patients in the clean liver group (3-year HCC incidence rates: 6.4%; 95% CI, 1.8–11.0%). The incidence rates of de novo HCC was significantly higher in the non-clean liver group than the clean liver group ($P = 0.003$, Fig. 3). In the remaining six patients, HCC developed at the same site of the initial nodules exclusively in 18 patients of a non-clean liver group by definition, and those HCC arose among the nodules of 8 mm or more in the initial MRI study.

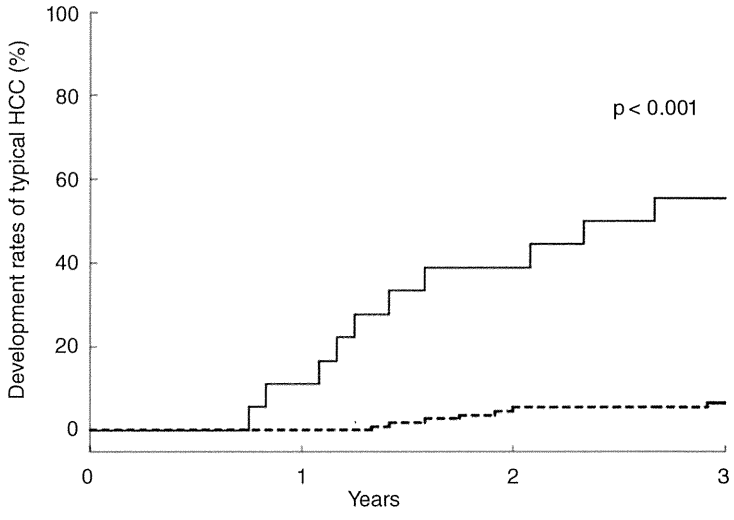
Risk factors for HCC development

Univariate analyses showed that the significant risk factors for HCC development included older age ($P = 0.039$), cirrhosis ($P = 0.009$), a low platelet count ($P = 0.003$), a high AFP concentration ($P = 0.006$) and a non-clean liver ($P < 0.001$). Multivariate analysis with these variables revealed that older age (hazard ratio [HR], 1.08; 95% CI, 1.01–1.16; $P = 0.024$), a low plate-

Table 1 Baseline patient characteristics

Characteristics	Total ($n = 127$)	Non-clean liver ($n = 18$)	Clean liver ($n = 109$)	P
Age, years	65 (30–88)	68 (46–82)	64 (30–88)	0.15
Male/female	68/59	10/8	58/51	1.00
Non-cirrhosis/cirrhosis	59/68	6/12	53/56	0.31
HBV/HCV	26/101	5/13	21/88	0.53
Platelet count ($\times 10^9$/L)	122 (30–410)	102 (46–187)	125 (30–410)	0.07
ALT (IU/L)	32 (7–206)	32 (14–95)	32 (7–206)	0.97
γ-GT (IU/L)	31 (9–305)	31 (13–258)	31 (9–305)	0.68
AFP (ng/mL)	4 (1–582)	8 (2–181)	4 (1–582)	0.19

Continuous data are shown as medians (range).
γ-GT, γ-glutamyltransferase; AFP, α-fetoprotein; ALT, alanine aminotransferase; HBV, hepatitis B virus; HCV, hepatitis C virus.



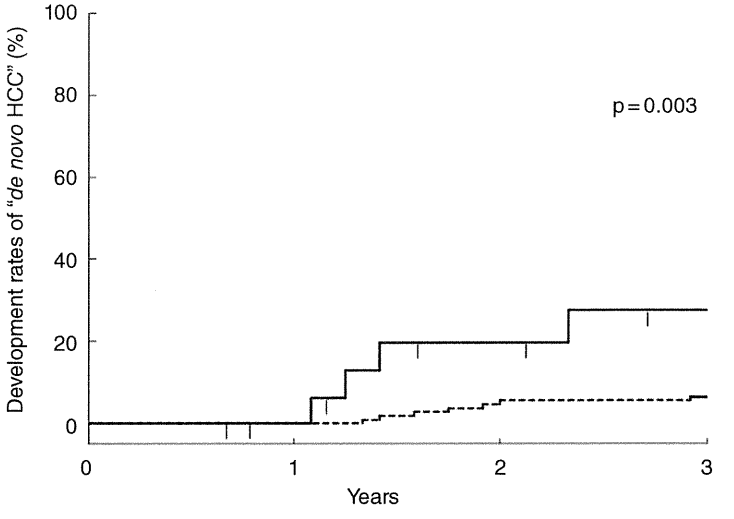
No. of patients at risk

Non-clean liver	18	16	11	8
Clean liver	109	109	103	102

Figure 2 Cumulative incidence rates of typical hepatocellular carcinoma (HCC) development in the non-clean and clean liver groups. ——, non-clean liver group ($n = 18$); ----, clean liver group ($n = 109$).

let count (HR, 1.17; 95% CI, 1.03–1.35; $P = 0.017$) and a non-clean liver (HR, 9.41; 95% CI, 3.47–25.46; $P < 0.001$) were the only independent risk factors for HCC development (Table 2).

We further assessed the effect of a non-clean liver on the risk of HCC development in subgroups of these patients (Fig. 4). We found that belonging to the non-clean liver group was a significant risk factor in patients without HBV. Notably, this designation was particularly valuable for patients who are generally regarded as at low risk for HCC development: those without cirrhosis (HR, 37.23; 95% CI, 3.30–419.71; $P = 0.003$) and those with high platelet counts (HR, 33.42; 95% CI, 6.69–166.94; $P < 0.001$).



No. of patients at risk

Non-clean liver	18	18	15	14
Clean liver	109	109	103	102

Figure 3 Cumulative incidence rates of typical hepatocellular carcinoma (HCC) at sites in which no nodules had been seen on the initial gadoxetic acid-enhanced magnetic resonance imaging, namely, "de novo HCC". ——, non-clean liver group ($n = 18$); ----, clean liver group ($n = 109$).

Table 2 Variables that predict HCC development: univariate and multivariate analyses

Variables	Univariate		Multivariate	
	Hazard ratio (95% CI)	*P*	Hazard ratio (95% CI)	*P*
Male	0.56 (0.29–1.95)	0.755		
Age (per year)	1.06 (1.00–1.12)	0.039	1.08 (1.01–1.16)	0.024
Cirrhosis	14.37 (1.90–108.44)	0.009	3.54 (0.37–33.77)	0.231
HCV (vs HBV)	4.39 (0.58–33.17)	0.151		
Platelet count (per 10^{10}/L)	1.19 (1.06–1.33)	0.003	1.17 (1.03–1.35)	0.017
ALT (per IU/L)	1.00 (0.99–1.02)	0.423		
γ-GT (per IU/L)	1.00 (0.99–1.01)	0.688		
AFP >10 ng/mL	3.98 (1.47–10.77)	0.006	1.47 (0.49–4.33)	0.486
Non-clean liver	12.36 (4.68–32.61)	<0.001	9.41 (3.47–25.46)	<0.001

γ-GT, γ-glutamyltransferase; AFP, α-fetoprotein; ALT, alanine aminotransferase; CI, confidence interval; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus.

DISCUSSION

THIS STUDY REVEALED presence of hypovascular hypointense liver nodules (non-clean liver) on gadoxetic acid-enhanced MRI, is a significant risk factor for subsequent development of typical HCC not only at the same sites but also at the different sites from the initial nodules. The incidence of development of typical HCC in the non-clean liver patients was more than 50% during a 3-year follow-up period, indicating that these higher risk patients should be rigorously investigated for the early detection of HCC during follow up.

In the present study, six of the 18 patients in the non-clean liver group developed typical HCC at the same site of the initial nodules during the subsequent 3 years (11.1%/year). Most of the hypovascular hypointense nodules on gadoxetic acid-enhanced MRI are considered precursor lesions of typical HCC, such as early HCC or high-grade dysplastic nodules, on histological examination,[13–15] while it has been reported that most hypovascular nodules exhibiting high-intensity to isointensity signals in the hepatocyte phase are benign hepatic nodules.[14,15] Recent studies have suggested that a reduction of organic anion-transporting polypeptide 1B3 (OATP 8) transporter expression begins at the earliest stage of hepatocarcinogenesis,[21,22] before changes in vascularity such as decreased portal flow or increased arterial flow. The progression rate of the small

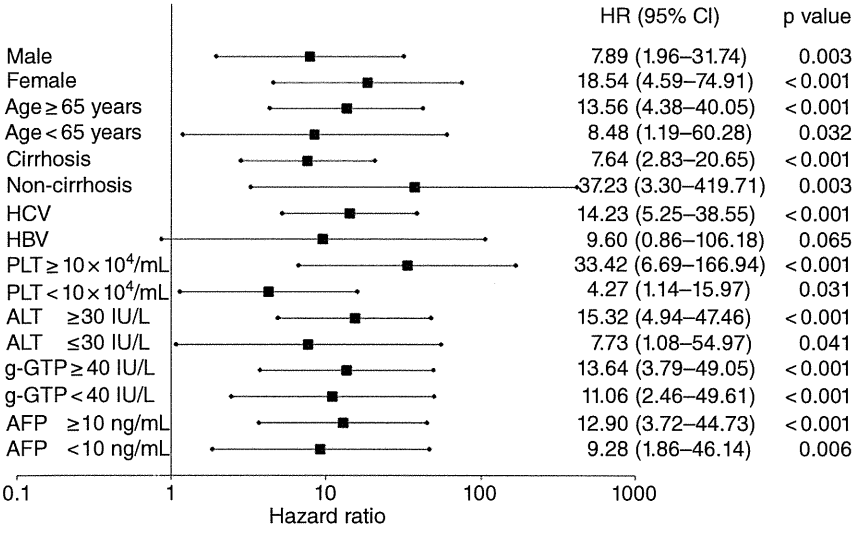


Figure 4 Stratified analyses of the non-clean liver as a risk factor for typical HCC development. AFP, α-fetoprotein; ALT, alanine aminotransferase; CI, confidence interval; g-GTP, γ-glutamyltransferase; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HR, hazard ratio; PLT, platelets.

hypovascular hypointense nodules to typical HCC was reported as 10–17%/year,[9,10] which is comparable to the present study. Typical HCC arose exclusively among the nodules of 8 mm or more, as in previous studies in which the larger hypovascular hypointense nodules were found to be the risk factor for progression to typical HCC in the initial MRI study.[9,10]

Hyperintensity on T2WI[12] or diffusion-weighted images (DWI)[11] also was reported to be useful for prediction of typical HCC progress in hypovascular hypointense nodules. In our patients, none of the nodules in the non-clean liver group showed hyperintensity on T2WI, suggesting that the hepatocyte phase is more sensitive for detecting the early stage of hepatocarcinogenesis.[15] DWI were not evaluated in this study because this usually detects pathologically advanced HCC of larger size or with hypervascularity.[23] Thus, it is reasonable that the hepatocyte phase can effectively recognize the earliest stage of HCC development without T2WI or DWI.

In 11 of 17 patients, typical HCC developed at sites other than the initially detected hypovascular hypointense nodules. As shown in Figure 3, the incidence rates of such HCC in the non-clean liver group was significantly higher than in the clean liver group ($P = 0.003$), indicating that a non-clean liver itself is a risk factor for HCC development, apart from the detectable hypovascular hypointense nodules. In addition, in four patients with nodules even below 8 mm, two developed HCC at different sites from the initial nodules during follow up (data not shown). Taken together, a non-clean liver has the higher potential for hepatocarcinogenesis or for undetectable precursor lesions. The non-clean liver may reflect more advanced genetic or epigenetic changes in the background hepatocytes, however, the detailed biological mechanism is not clear in this study.

Non-clean liver was an independent risk factor for the development of typical HCC, apart from well-documented risk factors (Table 2), such as cirrhosis,[24] ALT,[25] γ-GT,[26] age and AFP.[27] A non-clean liver is a significant risk for HCC development also for those without cirrhosis or with high platelet counts (Fig. 4). This means patients at increased risk of HCC development can be discerned as having a non-clean liver even among low-risk subgroups.

Conversely, patients without such nodules (clean liver group) showed a significantly lower risk of developing typical HCC than those with non-clean livers (0.0% vs 11.1% at 1 year, 6.8% vs 55.5% at 3 years of follow up; $P < 0.001$), suggesting that gadoxetic acid-enhanced MRI could detect precursor lesions sensitively enough to rule out immediate (within 1 year) development of typical HCC. Although seven patients in the clean liver group developed typical HCC only after 1 year, these patients had other risk factors for HCC development, including lower platelet counts, implying more advanced liver cirrhosis or high AFP (data not shown). Such HCC may arise from precursor lesions that cannot be visualized by current imaging techniques.

This study is a retrospective study and has some limitations. We included patients with HBV and HCV together, because gadoxetic acid-enhanced MRI findings or HCC development do not differ between these two groups and HBV or HCV infection is not an independent risk factor for typical HCC development. However, the number of HBV patients was too small ($n = 26$) to statistically confirm the current result when limited to HBV patients only. Prospective studies with larger numbers of patients who have uniform liver disease etiologies and imaging intervals are needed to verify our findings in different settings. Although the imaging interval of the non-clean liver group was shorter than the clean liver group (3 vs 4 months: $P = 0.015$), the median intervals between the initial MRI and HCC diagnosis was 16 months in the non-clean liver group and 21 months in the clean liver group. They are short enough for cumulative detection of HCC development for 3 years and it is assumed that there was little influence on the conclusions.

In conclusion, patients with chronic viral liver disease are at high risk for developing typical HCC at any sites of the liver if they have hypovascular hypointense nodules on gadoxetic acid-enhanced MRI. These patients should be closely followed up for developing typical HCC not only at the same site but also at different sites from the initial nodule.

REFERENCES

1 Ichikawa T, Saito K, Yoshioka N *et al.* Detection and characterization of focal liver lesions: a Japanese phase III, multicenter comparison between gadoxetic acid disodium-enhanced magnetic resonance imaging and contrast-enhanced computed tomography predominantly in patients with hepatocellular carcinoma and chronic liver disease. *Invest Radiol* 2010; 45: 133–41.
2 Halavaara J, Breuer J, Ayuso C *et al.* Liver tumor characterization: comparison between liver-specific gadoxetic acid disodium-enhanced MRI and biphasic CT – a multicenter trial. *J Comput Assist Tomogr* 2006; 30: 345–54.
3 Hamm B, Staks T, Muhler A *et al.* Phase I clinical evaluation of Gd-EOB-DTPA as a hepatobiliary MR contrast

agent: safety, pharmacokinetics, and MR imaging. *Radiology* 1995; **195**: 785–92.
4 Hammerstingl R, Huppertz A, Breuer J *et al.* European EOB-study group. Diagnostic efficacy of gadoxetic acid (Primovist)-enhanced MRI and spiral CT for a therapeutic strategy: comparison with intraoperative and histopathologic findings in focal liver lesions. *Eur Radiol* 2008; **18**: 457–67.
5 Huppertz A, Balzer T, Blakeborough A *et al.* European EOB Study Group. Improved detection of focal liver lesions at MR imaging: multicenter comparison of gadoxetic acid-enhanced MR images with intraoperative findings. *Radiology* 2004; **230** (1): 266–75.
6 Di Martino M, Marin D, Guerrisi A *et al.* Intraindividual comparison of gadoxetate disodium-enhanced MR imaging and 64-section multidetector CT in the detection of hepatocellular carcinoma in patients with cirrhosis. *Radiology* 2010; **256**: 806–16.
7 Inoue T, Kudo M, Komuta M *et al.* Assessment of Gd-EOB-DTPA-enhanced MRI for HCC and dysplastic nodules and comparison of detection sensitivity versus MDCT. *J Gastroenterol* 2012; **47**: 1036–47.
8 Golfieri R, Renzulli M, Lucidi V, Corcioni B, Trevisani F, Bolondi L. Contribution of the hepatobiliary phase of Gd-EOB-DTPA-enhanced MRI to dynamic MRI in the detection of hypovascular small (</= 2 cm) HCC in cirrhosis. *Eur Radiol* 2011; **21**: 1233–42.
9 Kumada T, Toyoda H, Tada T *et al.* Evolution of hypointense hepatocellular nodules observed only in the hepatobiliary phase of gadoxetate disodium-enhanced MRI. *AJR Am J Roentgenol* 2011; **197** (1): 58–63.
10 Motosugi U, Ichikawa T, Sano K *et al.* Outcome of hypovascular hepatic nodules revealing no gadoxetic acid uptake in patients with chronic liver disease. *J Magn Reson Imaging* 2011; **34** (1): 88–94.
11 Kim YK, Lee WJ, Park MJ, Kim SH, Rhim H, Choi D. Hypovascular hypointense nodules on hepatobiliary phase gadoxetic acid-enhanced MR images in patients with cirrhosis: potential of DW imaging in predicting progression to hypervascular HCC. *Radiology* 2012; **265** (1): 104–14.
12 Hyodo T, Murakami T, Imai Y *et al.* Hypovascular nodules in patients with chronic liver disease: risk factors for development of hypervascular hepatocellular carcinoma. *Radiology* 2013; **266**: 480–90.
13 Bartolozzi C, Battaglia V, Bargellini I *et al.* Contrast-enhanced magnetic resonance imaging of 102 nodules in cirrhosis: correlation with histological findings on explanted livers. *Abdom Imaging* 2013; **38**: 290–6.
14 Golfieri R, Grazioli L, Orlando E *et al.* Which is the best MRI marker of malignancy for atypical cirrhotic nodules: hypointensity in hepatobiliary phase alone or combined with other features? Classification after Gd-EOB-DTPA administration. *J Magn Reson Imaging* 2012; **36**: 648–57.
15 Sano K, Ichikawa T, Motosugi U *et al.* Imaging study of early hepatocellular carcinoma: usefulness of gadoxetic acid-enhanced MR imaging. *Radiology* 2011; **261**: 834–44.
16 Motosugi U. Hypovascular hypointense nodules on hepatocyte phase gadoxetic acid-enhanced MR images: too early or too progressed to determine hypervascularity. *Radiology* 2013; **267** (1): 317–8.
17 Asayama Y, Yoshimitsu K, Nishihara Y *et al.* Arterial blood supply of hepatocellular carcinoma and histologic grading: radiologic-pathologic correlation. *AJR Am J Roentgenol* 2008; **190** (1): W28–34.
18 Motosugi U, Ichikawa T, Sou H *et al.* Liver parenchymal enhancement of hepatocyte-phase images in Gd-EOB-DTPA-enhanced MR imaging: which biological markers of the liver function affect the enhancement? *J Magn Reson Imaging* 2009; **30**: 1042–6.
19 Bruix J, Sherman M, American Association for the Study of Liver Diseases. Management of hepatocellular carcinoma: an update. *Hepatology* 2011; **53**: 1020–2.
20 Motosugi U, Ichikawa T, Araki T. Rules, roles, and room for discussion in gadoxetic acid-enhanced magnetic resonance liver imaging: current knowledge and future challenges. *Magnetic Rsonance in Medical Sciences.* 2013; **12**: 161–75.
21 Kitao A, Zen Y, Matsui O *et al.* Hepatocellular carcinoma: signal intensity at gadoxetic acid-enhanced MR imaging – correlation with molecular transporters and histopathologic features. *Radiology* 2010; **256**: 817–26.
22 Narita M, Hatano E, Arizono S *et al.* Expression of OATP1B3 determines uptake of Gd-EOB-DTPA in hepatocellular carcinoma. *J Gastroenterol* 2009; **44**: 793–8.
23 Nasu K, Kuroki Y, Tsukamoto T, Nakajima H, Mori K, Minami M. Diffusion-weighted imaging of surgically resected hepatocellular carcinoma: imaging characteristics and relationship among signal intensity, apparent diffusion coefficient, and histopathologic grade. *American Journal of Roentgenology* 2009; **193**: 438–44.
24 Degos F, Christidis C, Ganne-Carrie N *et al.* Hepatitis C virus related cirrhosis: time to occurrence of hepatocellular carcinoma and death. *Gut* 2000; **47** (1): 131–6.
25 Tarao K, Rino Y, Ohkawa S *et al.* Association between high serum alanine aminotransferase levels and more rapid development and higher rates of incidence of hepatocellular carcinoma in patients with hepatitis C virus-associated cirrhosis. *Cancer* 1999; **86**: 589–95.
26 Ikeda K, Saitoh S, Suzuki Y *et al.* Disease progression and hepatocellular carcinogenesis in patients with chronic viral hepatitis: a prospective observation of 2215 patients. *J Hepatol* 1998; **28**: 930–8.
27 Ikeda K, Saitoh S, Koida I *et al.* A multivariate analysis of risk factors for hepatocellular carcinogenesis: a prospective observation of 795 patients with viral and alcoholic cirrhosis. *Hepatology* 1993; **18** (1): 47–53.

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特集：C型肝炎治療update

C型肝炎治療における宿主因子とウイルス因子

坂本　穣　　榎本信幸

I. 総　　論

C型肝炎治療における宿主因子とウイルス因子

坂本　穣[1,2]　榎本信幸[2]

Host factors and viral factors in hepatitis C treatment

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Abstract

In the interferon-based therapy for hepatitis C, host factors such as age, gender, liver fibrosis and steatosis are important as a therapeutic effect predictor, and viral factors such as HCV genotype, HCV viral load, HCV gene (*IL28B, ITPA*) are also important. In addition in genotype 1b, ISDR/IRRDR and core amino acid substitution are important. Also in the DAA treatment, viral factors are also important at the view of therapeutic effect and difficulty of acquisition of drug resistance mutation. In addition, the goal of treatment of hepatitis C are suppression of liver fibrosis progression and liver cancer and improvement of quality of life due to this (quality of life: QOL) and life prognosis, it is important to understand the host factors and HCV viral factors.

Key words: IL28B, ITPA, ISDR, IRRDR, core amino acid substitution

はじめに

C型肝炎治療の目標はC型肝炎ウイルス(hepatitis C virus: HCV)の排除と，これに基づく持続炎症の鎮静化，そして肝発癌抑止である．現在これを可能とするのはインターフェロン(interferon: IFN)を中心とした治療と，HCVに直接作用するdirect antiviral agents(DAA)である．特にIFN治療は長い歴史があり，単独療法，ポリエチレングリコールを結合させ，作用期間を延長したPEG-IFN，そしてリバビリン(ribavirin: RBV)との併用療法が長く行われてきた．さらにDAAと併用し，我が国では1型のHCVに対し，HCV NS3-4 protease inhibitorのテラプレビル(telaprevir: TVR)，シメプレビル(simeprevir: SMV)，バニプレビル(vaniprevir: VPV)の3剤併用療法が，2型に対してはTVRとの併用が認可されている．一方，DAA製剤のみの治療も実用化され，2014年現在，我が国では1型のHCVに対し，NS3-4 protease inhibitorのアスナプレビル(asunaprevir: ASV)とNS5A阻害剤のダクラタスビル(daclatasvir: DCV)の2剤併用療法が行われ[1]，また海外では既に，NS5B polymerase inhibitorであるソフォスブビル(sofosbuvir: SOF)[2]も用いられている(**表1**)．

1. 宿主因子

これまで，C型肝炎に関する疾患関連遺伝子

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