TABLE IV. Univariate Analysis: Factors Predictive of Sustained Virologic Response

Factors	Sustained virologic response (n = 11)	Non-sustained virologic response (n = 12)	*P*-value
Age (y.o.)	37.9 ± 10.9	39.8 ± 11.3	0.6958
Gender: male/female	10/1	10/2	0.9999
ALT (IU/L)	78.2 ± 50.8	62.6 ± 68.1	0.5435
AST (IU/L)	51.4.4 ± 29.2	48.8 ± 40.4	0.8616
PLT ($\times 10^4$/mm^3)	19.0 ± 5.4	19.3 ± 5.7	0.8870
HCV RNA level (KIU/ml)	1323.1 ± 1077.3	2567.0 ± 2940.8	0.2481
ISDR: wild/mutant	7/4	12/0	0.0373
IL28B:TT/TG	9/1	4/8	0.0115

AST, aspartate aminotransferase; ALT, alanine aminotransferase; PLT, platelet count; HCV, hepatitis C virus; ISDR, interferon sensitivity-determining region; IL28B, interleukin 28B.

the pathogenesis of HCV genotype 1a infection. However, the HCV core region of genotype 1a is well-conserved and no significant mutations were seen in the core region, which is associated with IFN responsiveness. Several reports have also found that the HCV core region, including positions 70 and 91, of HCV genotype 1a is highly conserved [Alestig et al., 2011; Kumthip et al., 2011]. Mutations in the core region of genotype 1a would be rare, so this region might be unsuitable for routine clinical use, unlike in genotype 1b. However, the number of patients in this study was small, and large studies including from other countries are needed to clarify these issues. The ISDR in the NS5A region of HCV genotype 1b is closely associated with response to IFN therapy. ISDR mutations of genotype 1b are well known to be more important in predicting sustained virological response in Japanese patients than European patients [Hofgärtner et al., 1997; Zeuzem et al., 1997; Nakano et al., 1999; Pascu et al., 2004; Hayashi et al., 2011a]. European studies have failed to detect the specific amino acid substitutions in ISDR of genotype 1a associated with IFN responsiveness [Hofgärtner et al., 1997; Zeuzem et al., 1997]. In this study, sustained virological response was achieved in 36.8% of patients with wild-type ISDR and 100% of patients with mutant-type ($P = 0.0373$). The present analysis showed a close relationship between ISDR of genotype 1a and sustained virological response, as in genotype 1b. Recent investigations in Thailand and Iran have failed to identify the usefulness of ISDR for HCV genotype 1a in predicting sustained virological response [Kumthip et al., 2011; Yahoo et al., 2011]. The high virological response rate and low prevalence of patients with mutations in the ISDR do not favor the use of ISDR analysis in predicting IFN responsiveness [Herion and Hoofnagle, 1997; Yokozaki et al., 2011]. Rates of sustained virological response among these studies were much higher than those in the present study (68.4% and 75% vs. 47.8%). The mean number of mutations in patients who achieved sustained virological response in the studies by Kumthip et al. [2011] and Yahoo et al. [2011], and the present group were 1.4, 1.4, and 1.6, respectively. Differences in sustained virological response and the number of mutations to the ISDR might underpin this discrepancy in the evaluation of ISDR. Although the sample size in the present study was small, the results indicate that ISDR represents a strong indicator of progression to sustained virological response for patients with HCV genotype 1a. Amino acid substitutions in the ISDR of genotype 1a thus also play an important role in predicting sustained virological response in Japanese patients compared to patients from other countries. IL28B polymorphisms such as host genetics, as well as mutations in the HCV genome, contribute to IFN treatment outcomes. Rates of sustained virological response in patients in this study with TT and TG were 69.2% and 11.1%, respectively. The TG allele of the IL28B genotype was significantly associated with poor response to IFN therapy ($P = 0.0115$). SNPs of IL28B would regulate the expression of IFN-stimulated genes and affect IFN responsiveness. IL28B and ISDR thus exert independent effects on IFN responsiveness and both host and viral factors impacting IFN responsiveness would improve the prediction of sustained virological response. Several studies have thus reported that both the SNP of IL28B and mutations in the ISDR were associated with sustained virological response in patients with HCV genotype 1b [Akuta et al., 2011; Hayashi et al., 2011b; Kurosaki et al., 2011]. In the present study of HCV genotype 1a, among the 9 patients who had simultaneously the TG allele for IL28B and wild-type ISDR, only 1 achieved sustained virological response (11.1%). The best-sustained virological response was achieved in patients with mutant-type ISDR and the T allele (100%). The combination of SNPs for IL28B and mutations in ISDR may thus predict response to IFN therapy in patients with HCV genotype 1a as well as genotype 1b. Given the small sample size in this investigation, larger cohorts are needed to confirm the present results. Furthermore, infection with genotype 1a in Japanese patients is rare, making large-scale studies difficult to perform.

In conclusion, the prevalence of HCV genotype 1a is rare in Japan and the majority of cases involve patients with hemophilia. The TG genotype of IL28B is associated with poor response, while mutant-type ISDR is associated with good response to combination therapy with pegylated-IFN-α2b and ribavirin in patients with HCV genotype 1a. Combined use of both IL28B and ISDR could improve the prediction of IFN response.

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Favorable association between genetic polymorphisms near the *IL28B* gene and hepatic steatosis: Direct or indirect?

To the Editor,

We read with great interest the article by Tillmann *et al.* [1] that investigated the association between *IL28B* polymorphisms and hepatic steatosis, both of which are associated with a response to combination therapy with peginterferon (PEG-IFN) and ribavirin. It will provide new insight into the role of *IL28B* polymorphisms on the resistance to combination therapy against hepatitis C virus (HCV) infection.

Previous studies have reported the influence of amino acid substitutions at residue 70 of the HCV core region (from arginine to glutamine or histidine) on the resistance to combination therapy with PEG-IFN and ribavirin in patients infected with HCV genotype 1b [2–4]. In addition, prior studies have identified an association between amino acid substitutions at HCV core 70 and hepatic steatosis [5,6]. The percentage of patients with the mutant amino acid at residue 70 of the HCV core region increases with the progression of chronic hepatitis, suggesting that the mutation of the amino acid at residue 70 occurs during the natural course of chronic HCV infection [7]. Several recent studies have reported a lower prevalence of mutant amino acids at HCV core 70 in patients who have the *IL28B* polymorphism that is associated with a favorable response to combination therapy with PEG-IFN and ribavirin (i.e., CC genotype of rs12979860 and TT genotype of rs8099917) than in patients who have an unfavorable genotype [8,9]. These reports suggest that the mutation frequency of the HCV core 70 amino acid may differ according to the genetic polymorphism near the *IL28B* gene.

We analyzed polymorphisms of rs8099917 that corresponded to those of rs12979860, the rate of which is more than 99% of individuals of Japanese ethnicity [10], amino acid substitutions at HCV core 70, and hepatic steatosis based on biopsy specimens, which were obtained just prior to the start of the therapy and evaluated with the same criteria used by Tillmann *et al.*, in our 122 Japanese Mongolian patients infected with HCV genotype 1b. We found higher likelihoods of sustained virologic response in patients with the TT genotype of rs8099917, in patients with arginine at residue 70 of the HCV core region, and in patients without steatosis. We did not find significant association between *IL28B* polymorphisms and hepatic steatosis (absence of steatosis: TT genotype, 66 out of 85 (77.6%) *vs.* TG/GG genotype, 22 out of 37 (59.5%), $p = 0.0658$). We found significant associations between *IL28B* polymorphisms and the amino acid at residue 70 of the HCV core region (patients with arginine at HCV core 70: TT genotype, 71 out of 85 (83.5%) *vs.* TG/GG genotype, 14 out of 37 (37.8%), $p < 0.0001$) and also between the amino acid at HCV core 70 and hepatic steatosis (absence of steatosis: arginine at HCV core 70, 73 of 85 (85.9%) *vs.* glutamine/histidine at HCV core 70, 15 of 37 (40.5%), $p < 0.0001$). These associations may indicate that the polymorphisms near the *IL28B* gene may influence the mutation of the amino acid at residue 70 of the HCV core region, and that the amino acid mutation at HCV core 70 may influence hepatic steatosis over the course of chronic HCV infection.

There are differences between our Japanese Mongolian population and the population studied by Tillmann *et al.* including ethnicity, the rate of correspondence between rs12979860 and rs8099917, the frequency of the mutation at residue 70 of the HCV core region, and the rate of hepatic steatosis. Furthermore, the number of patients infected with HCV genotype 1b in the Tillmann *et al.* cohort is unknown. Despite these facts, it would be interesting if they were to investigate the association of amino acid substitutions at residue 70 of the HCV core both with *IL28B* polymorphisms and with hepatic steatosis.

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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Reply to: "Favorable association between genetic polymorphisms near the *IL28B* gene and hepatic steatosis: Direct or indirect?"

To the Editor:

We thank Drs. Toyoda and Kumada for raising the additional point that HCV amino acid substitutions have also been demonstrated to influence steatosis in the setting of HCV infection. In their study of 122 patients, 85 of whom had a beneficial *IL28B* genotype, Toyoda and Kumada found a trend for steatosis to be associated with *IL28B* polymorphism, as only 22% of the patients with beneficial genotype have steatosis compared to 40% of patients with the less beneficial genotype. Thus, their r, though not significant, is in line with our study, where we likewise found a 25% and 27% higher rate of steatosis in patients with the less beneficial *IL28B* ("non-C/C" for rs12979860 or "non-T/T" for rs8999017) genotype in two different cohorts of 145 and 180 patients, respectively. Similar to our and Toyoda and Kumada's results, Cai *et al.* found an association between the beneficial *IL28B* genotype and lower steatosis frequency [1]. However, a three center study by Trépo *et al.* failed to find a relevant association between *IL28B* genotype and steatosis, according to their statement [2]. This latter paper, however, did not show the data, and therefore it cannot be assessed whether the association was absent or only not significant. Toyoda and Kumada's study showed a similar trend for steatosis with *IL28B*, whereby *IL28B* is associated with different mutations in the core region, the HCV core mutation clearly shows a higher association with steatosis.

In our article, we indicate that response to treatment in relation to steatosis seems unlikely to be explained by *IL28B* alone, and though not specifically mentioned, *IL28B* is likely not solely responsible for the association with steatosis. We had a small cohort of 54 non-genotype 1 patients of whom 19 were genotype 3 and 35 were genotype 2. Despite the fact that steatosis tended to be higher in "non-C/C" patients (4/20 [20%] *vs.* 7/15 [46%] in genotype 2 patients and 4/8 [50%] *vs.* 7/11 [63%] in genotype 3 patients; Table 1) this was not significant. However, the trend was similar across genotypes. Furthermore, we have data on genotype 1a and 1b in 60 and 75 patients from the fibrosis study, respectively. In concordance with the overall results, steatosis was less frequently present in C/C genotype patients with both HCV genotype 1a and 1b (Table 1).

A possible explanation lies in the virus itself as the authors correctly point out with a focus on HCV's core protein. Unfortunately, we do not have the core antigen sequence of our patients. A previous work by Jhaveri *et al.* suggested a role for amino acids 182 and 186 of the core protein, linking steatosis *in vitro* to steatosis [3], but certainly amino acid 70 seems to be especially relevant in genotype 1b infection. There is also some evidence indicating that not all differences can be explained by viral core sequence variation [4].

Table 1. Frequency of steatosis.

		Presence of steatosis	
1A	Non-CC	19/43 (44.2%)	
	CC	2/17 (11.8%)	$p = 0.019$
1B	Non-CC	28/56 (50%)	
	CC	4/19 (21%)	$p = 0.034$
2	Non-CC	7/15 (46%)	
	CC	4/20 (20%)	n.s.
3	Non-CC	7/11 (63%)	
	CC	4/8 (50%)	n.s.

n.s., not significant.

Conflict of interest

The authors declared that they do not have anything to disclose regarding funding of conflict of interest with respect to this manuscript.

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Short Communication

Reference change values for *lens culinaris* agglutinin-reactive α-fetoprotein and des-γ-carboxy prothrombin in patients with chronic hepatitis C

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Abstract

Background: *Lens culinaris* agglutinin-reactive α-fetoprotein (AFP-L3) and des-γ-carboxy prothrombin (DCP) have been routinely used as serological tumor markers of hepatocellular carcinoma (HCC) for surveillance. The aims of this study were: (i) to determine the biological variation of AFP-L3 and DCP in patients with chronic hepatitis C; and (ii) to calculate the reference change values (RCVs) of AFP-L3 and DCP.
Methods: Ten patients with cirrhosis due to hepatitis C virus (HCV) infection and without HCC were enrolled in the study. Serum samples were collected at 14-day intervals, and 10 samples in total were obtained for each patient. AFP-L3 and DCP levels were measured by microchip capillary electrophoresis and liquid-phase binding assay. Intra-individual (CV_I) and inter-individual (CV_G) biological variations and RCVs were estimated from the data generated.
Results: The CV_I was 29.0% for AFP-L3 and 24.6% for DCP, and CV_G was 63.5% for AFP-L3 and 40.4% for DCP. The RCVs for AFP-L3 and DCP were 68.3% and 58.5%, respectively.
Conclusions: Increases in values for AFP-L3 and DCP within 68.3% and 58.5% may be biological variations. Clinician should take these variations into consideration for the management of patients with HCV infection under surveillance of HCC.

Keywords: α-fetoprotein (AFP-L3); biological variation; des-γ-carboxy prothrombin (DCP); hepatocellular carcinoma; reference change value (RCV).

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Hepatocellular carcinoma (HCC) is one of the most prevalent cancers. It is the sixth most common cancer in the world, and the third most common cause of cancer-related death (1). Chronic liver disease caused by hepatitis C viruses (HCV) is an important risk factor for the development of HCC (2). α-Fetoprotein (AFP) (3, 4), the percentage of *lens culinaris* agglutinin-reactive fraction of AFP to total AFP (AFP-L3) (5, 6), and des-γ-carboxy prothrombin (DCP) (7) have been routinely used as serological tumor markers of HCC for surveillance, diagnosis, or the prediction of outcome in clinical practice. Whereas established cut-off with fixed values of AFP, AFP-L3, and DCP are usually used to make clinical decisions, fluctuation of these values are often observed in patients without HCC. Previous studies reported that the reference change value (RCV) for AFP was useful for dynamic monitoring of AFP in patients with liver disease (8). However, clinically minimal significant changes on sequential measurements of AFP-L3 and DCP have never been investigated. The aims of this study were: (i) to determine the biological variation of AFP-L3 and DCP in patients with chronic hepatitis C including cirrhosis; and (ii) to calculate the RCVs of AFP-L3 and DCP as the basis for clinically minimal significant change value.

Ten patients (five males and five females) having cirrhosis with HCV but without HCC were enrolled into the study between April and September 2008. The age of the patients (mean±SD) were 69.6±5.1 years (range, 62–76 years). The platelet counts were 92.2±27.4×1000/mL. All patients were positive for HCV RNA as determined by a commercial assay (HCV COBAS AmpliPrep/COBAS TaqMan System; Roche Molecular Systems, Pleasanton, CA, USA). With respect to liver function, eight patients had Child-Pugh class A and the remaining two patients had Child-Pugh class B liver function. The absence of HCC was confirmed in all patients by ultrasonography and Gd-EOB-DTPA enhanced magnetic resonance imaging that is most sensitive modality to detect hepatic tumors (9) at the time of entry in order to avoid inclusion of patients with occult HCC. In addition, these patients were followed for 2 years after serum sampling for the lack of the development of HCC. Serum samples were collected at 14-day intervals following a standard protocol in order to minimize pre-analytical variations, and 10 samples in total were obtained from each patient. Samples were collected following a standardized protocol in order to minimize

pre-analytical variations. All serum samples were stored frozen at –80°C until measurement. The study protocol was in compliance with the Helsinki Declaration and was approved by the Ethics Committee of Ogaki Municipal Hospital. Written informed consent was obtained from each patient.

AFP-L3%, DCP, and AFP levels were measured by a microchip-based capillary electrophoresis and liquid-phase binding assay on the μTAS Wako i30 autoanalyzer (Wako Pure Chemical Industries, Ltd., Japan) (10). Because we purposed to compare biological variation of AFP-L3, which is expressed as a percentage of total AFP in a current use, with that of AFP-L3 concentration (expressed in units of ng/mL), AFP-L3 concentration (ng/mL) was calculated using the formula total AFP×(AFP-L3/100). Albumin (ALB), total bilirubin (T-Bil), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were measured on a general clinical chemistry analyzer as liver function analytes. ALB was measured by bromcresol purple method (L-Type WAKO ALB-BCP; Wako Pure Chemical Industries, Ltd.), T-Bil was measured by vanadate oxidation method (Total Bilirubin E-HA; Wako Pure Chemical Industries, Ltd.), and AST and ALT were measured by Japan Society Clinical Chemistry transferable method (L-Type AST.J2 and ALT.J2; Wako Pure Chemical Industries, Ltd.). Conversion factors from conventional units to SI units for ALB (g/dL→g/L), T-Bil (mg/dL→μmol/L), AST/ALT (U/L→μkat/L), AFP/AFP-L3 (ng/mL→μg/L) and DCP (mAU/mL→μg/L) are 10, 17.1, 0.017, 1 and 0.012, respectively. Each sample was assayed in duplicates. Measurements were performed by the same analyst. To calculate analytical imprecision (CV_A), controls (AFP: 18.8 ng/mL, AFP-L3: 9.7%, DCP: 99 mAU/mL, ALB: 3.0 g/dL, T-BIL: 0.6 mg/dL, AST: 28 U/L, ALT: 25 U/L) were measured in duplicate with two runs per day over 21 days.

Data was analyzed by calculating intra- (CV_I), and inter-individual (CV_G) biological variations according to Fraser et al. (11, 12). We also calculated the index of individuality (IOI) using an equation of $IOI = CV_I/CV_G$ according to Harris et al (11, 13). When the IOI for a particular test is ≤0.6, conventional population-based reference intervals and cut-off values are of limited value for the classification of some unusual results for a particular individual. When the IOI is ≥1.4, the variation of a particular individual will fit within the assay limits as those of the population and, therefore, population-derived reference value could be applied. The critical differences at 5% significance level (one-tail test) for AFP-L3%, AFP-L3 absolute concentration, DCP, AFP, ALB, T-Bil, AST, and ALT (including analytical variation) were calculated as $2.33\times(CV_I^2 + CV_A^2)^{1/2}$. All statistical analyses were performed with JMP6 statistical software (SAS Institute Japan, Japan).

The median and range of AFP-L3%, AFP-L3 concentration, DCP, and AFP in each of 10 patients are shown in Figure 1. Although all of these values did not show a normal distribution by Shapiro-Wilk test, we did not use logarithmic transformation for these values. It was noted that the median value was below the recommended cut-off of 10% of AFP-L3% and 40 mAU/mL of DCP, respectively. Although the mean values of ALB, T-Bil, and ALT were within the respective reference ranges, the mean value of AST slightly exceeded the reference range (Table 1). Although we observed some cases in which the range was greater in comparison to other cases (ex. patient #2 for AFP and AFP-L3 concentration, patients #8 and #10 for AFP-L3%, and patients #2 and #6 for DCP), these were not outliers (11). Patient #2 showed greater ranges of AFP, AFP-L3 concentration and DCP, but the changes in these values in serial measurement were not spiky, which reportedly indicates the development of HCC in case of AFP (14), and the development of HCC was no

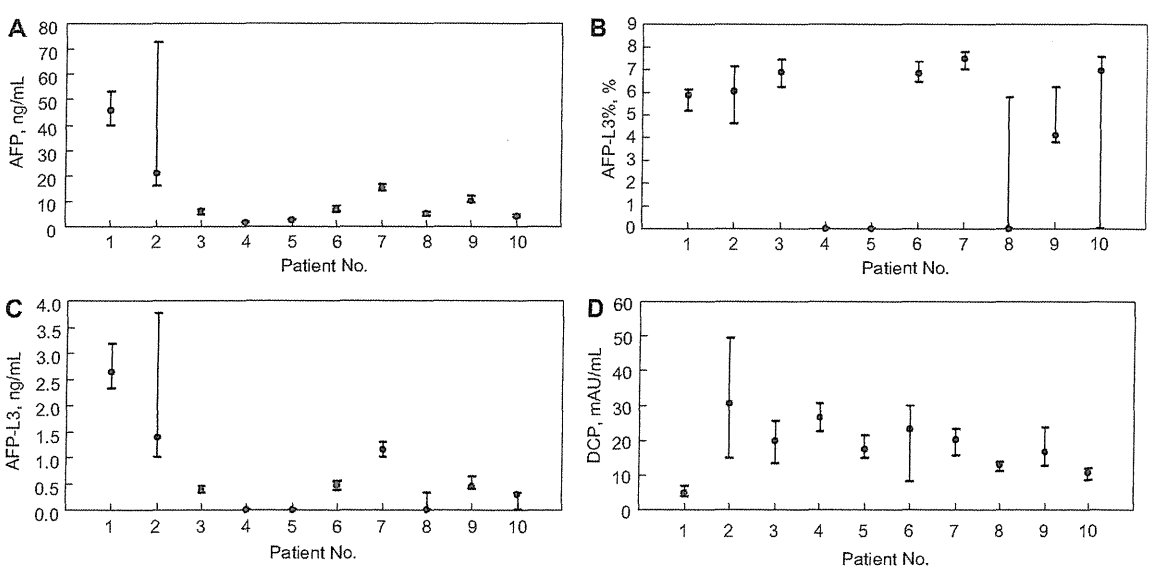

Figure 1 The median and range of AFP (A), AFP-L3% (B), AFP-L3 concentration (C), and DCP (D) for each patient.

Table 1 The mean, range, SD, CVA, CVI, CVG, RCV and IoI for AFP, AFP-L3%. AFP-L3 concentration, DCP albumin, total bilirubin, AST, and ALT.

Marker	Mean	Range	SD	CVA, %	CV_I, %	CV_G, %	RCV, %	IoI
AFP, ng/mL	12.771	71.712	15.3	2.1	52.1	113.1	121.8	0.461
AFP-L3%, %	4.45	8.21	3.0	3.8	29.0	63.5	68.3	0.457
AFP-L3, ng/mL	0.714	4.076	0.9	4.9	42.4	122.0	99.6	0.348
DCP, mAU/mL	18.0	47.2	8.3	4.7	24.6	40.4	58.5	0.609
Albumin, g/dL	3.97	1.95	0.5	0.8	3.7	12.5	8.9	0.300
Total bilirubin, mg/dL	0.81	1.40	0.3	2.4	17.5	32.3	41.2	0.540
AST, U/L	51.4	142.6	22.3	5.5	26.7	36.1	63.5	0.740
ALT, U/L	35.6	106.1	17.8	3.4	36.4	36.3	85.3	1.000

CV_A, analytical imprecision; CV_I, intra-individual biological variation; IoI, index of individuality; CV_G, inter-individual biological variation; RCV, reference change value.

observed under surveillance more than 2 years after study. Results of calculations for CV_A, CV_I, CV_G, RCV, and IOI are summarized in Table 1. The CV_I was 29.0% and CV_G was 63.5% for AFP-L3%, 42.4% and 122.0% for AFP-L3 concentration, 24.6% and 40.4% for DCP, and 52.1% and 113.1% for AFP. The CV_I and CV_G for AFP-L3% were smaller than those of AFP-L3 concentration. This finding indicated that AFP-L3 that was expressed as a percentage of total AFP as a current use is more appropriate than AFP-L3 concentration as a tumor marker for HCC, since the effects of biological variance on clinical decision-making can be reduced when AFP-L3 was expressed as a percentage. The IOI values for AFP-L3%, AFP-L3 concentration, DCP, and AFP were 0.457, 0.348, 0.609, and 0.461, respectively. The value was under 0.6 in case of AFP-L3%, AFP concentration, and AFP, and was far from 1.4 in case of DCP.

Measurement of tumor markers for HCC, AFP, AFP-L3, and DCP, are recommended for patients with high-risk of developing HCC in Japan (15), which plays an important role in HCC surveillance. Fixed cut-off values are used for AFP, AFP-L3, and DCP for clinical decision-making. However, our findings demonstrated that the cut-off values of AFP-L3 and DCP might be of limited clinical utility as an indicator of developing HCC, since the IOI scores were <0.6 in our study; the range of the values for an individual subject spans only a small part of the reference interval for a population. One previous study investigated CV_I, CV_G, and IOI score for AFP (8), with which the data for AFP in the present study was compatible, indicating limited clinical utility of the cut-off value for AFP of 20 ng/mL and 200 ng/mL.

Similar finding are reported for other tumor markers. Carcinoembryonic antigen (CEA) and CA 19-9 also have low IOI scores. It was determined that conventional or population-based reference intervals for CEA and CA19-9 are of limited utilities in interpretation of the assay results, since the IOI for these markers were <0.6 (16, 17). The use of RCVs would improve the clinical utility of these markers. Like these tumor markers, utilization of the RCVs for AFP-L3, DCP, and AFP might improve the accuracy for the detection of HCC under surveillance.

This study includes several limitations. The potential bias may be present in patient selections due to a study performed at a single site, and the number of patients analyzed was small. Larger clinical studies are needed to further evaluate and confirm the RCVs for AFP-L3 and DCP. In addition, all patients analyzed were infected with HCV, and these analyses should be applied for patients with HBV infection or those without hepatitis viral infection.

In conclusion, conventional population-based cut-off values for AFP-L3 and DCP may be of limited value for indicating HCC development in HCV-infected patients at high-risk for HCC under surveillance, due to the effects of significant biological variations of these markers over population based cut-off selection. Analytical and intra-individual component of the biological variations would accounted for the significant changes in sequential marker concentrations, and these should be determined and taken into considerations in interpretation of the assay results. In the management of patients with chronic HCV infection, which is a major cause of HCC development in Japan, clinicians should take these values of biological variation of AFP-L3 and DCP (68.3% and 58.5%) into consideration when evaluating the changes of these biomarkers in serial measurements. Further clinical evaluation is needed to address whether the RCVs for AFP-L3, and DCP would be useful for HCC detection.

Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article. The employment status of F. Kanke and S. Satomura at Wako Pure Chemical Industries Ltd. played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.
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Novel method to measure serum levels of des-gamma-carboxy prothrombin for hepatocellular carcinoma in patients taking warfarin: A preliminary report

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Des-gamma-carboxy prothrombin (DCP) is a useful tumor marker for hepatocellular carcinoma (HCC), but its utility is limited in patients taking vitamin K antagonists. We evaluated the NX-DCP ratio, a newly developed method to measure serum DCP, for its ability to identify DCP elevation induced by HCC in this patient subpopulation. Conventional DCP measurements and the NX-DCP ratio were compared in patients with and without HCC, all of whom were taking the vitamin K antagonist warfarin. We found no differences in conventional DCP measurements between patients with and without HCC due to warfarin treatment. In contrast, the NX-DCP ratio was significantly higher in patients with HCC; the NX-DCP ratio in all patients without HCC was <1.50. When the cut-off was fixed at 1.50, sensitivity and specificity for HCC diagnosis were 60.0% and 100.0%, respectively, which are comparable to those of conventional DCP measurements in patients not taking warfarin. The novel NX-DCP ratio identifies patients on warfarin with elevated DCP due to HCC and is useful as a tumor marker for HCC in this patient subpopulation. (*Cancer Sci*, doi: 10.1111/j.1349-7006.2012.02232.x, 2012)

Prothrombin, or coagulation factor II, is a 71 600 Da protein that consists of three regions: fragment 1; fragment 2; and prothrombin. Fragment 1 consists of 156 amino acids, including 41 amino acids forming an *N*-terminal gamma-glutamic acid (Gla)-containing domain. Prothrombin is first synthesized in the liver as a precursor with 10 glutamic acid (Glu) residues, which are then modified to Gla residues by gamma-glutamylcarboxylase in the presence of vitamin K, O_2, and CO_2 before it is released into the bloodstream.

However, in the absence of vitamin K or in the presence of vitamin K antagonists, gamma-carboxylation is impaired, and prothrombin with the remaining Glu residues, which is inactive with respect to coagulation, is released into the bloodstream.[1] Prothrombin in this form is called des-gamma-carboxy prothrombin (DCP) or protein induced by vitamin K absence/antagonist-II (PIVKA-II). As the number of Glu residues unconverted to Gla varies, DCP is present as a mixture of prothrombin with various numbers of Glu residues, ranging from 1 to 10. In addition, because the Gla residue can bind to calcium, it is known that the 3-D protein structure of DCP will be different in the presence of calcium, and depends on the number of Glu residues.[2]

Des-gamma-carboxy prothrombin is frequently found in the blood of patients with hepatocellular carcinoma (HCC). Because DCP is elevated in many patients with HCC but not in patients with chronic hepatitis or cirrhosis without HCC,[3] it has been routinely used as a tumor marker of HCC in clinical settings.[4–6] However, serum DCP levels are also increased in the absence of HCC when there is a shortage of vitamin K or in the presence of vitamin K antagonists.[7] The value of DCP as a marker of HCC, therefore, is significantly reduced in patients who are taking vitamin K antagonists such as warfarin.

Previous studies reported differences in the number of Glu residues in DCP between patients with HCC and patients taking vitamin K antagonists.[8,9] Conventionally, DCP is measured using a mAb produced by the cell line MU-3 (Picolumi PIVKA-II; EIDIA, Tokyo, Japan), which reportedly reacts predominantly with DCP with 9–10 Glu residues. MU-3 had lower affinity for DCP with one to five Glu residues.[10] However, measuring DCP with this antibody alone can not differentiate between HCC-induced and vitamin K antagonist-associated elevations of DCP, making it difficult to evaluate whether rises in DCP are caused by HCC in patients taking vitamin K antagonists.

In the present study, we attempted to identify HCC-induced DCP in patients with HCC taking the vitamin K antagonist warfarin through the use of two mAbs against DCP, P-11 and P-16 (Sekisui Medical, Tokyo, Japan), which have a reactivity profile different from MU-3. We found clinical utility in DCP as a marker for HCC in patients taking warfarin when measured with the combination of MU-3, P-11, and P-16.

Materials and Methods

Preparation of electrochemiluminescence immunoassay (ECLIA) reagents with P-11 and P-16. Magnetic beads coated with P-16 mAb (Sekisui Medical) were prepared as follows: 1 mL of 30 mg/mL magnetic bead suspension (Dynabeads M-450 Epoxy; Life Technologies, Carlsbad, CA, USA) was placed into a test tube and the magnetic beads were trapped by a magnet to separate the supernatant. After the supernatant was discarded, 1 mL P-16 mAb (0.5 mg/mL in 0.15 mol/L PBS, pH 7.8) was added to the magnetic beads and stirred at 25°C for 18 h. After washing the magnetic beads, 2 mL of 1% BSA in 0.15 mol/L PBS (pH 7.8) were added and stirred at 25°C for 18 h to block the beads. These beads were diluted to 1 mg/mL using the bead dilution reagent (0.05 mol/L Tris buffer (pH 7.5), 0.15 mol/L NaCl, 0.01% Tween 20, 0.1% NaN_3, 10% normal rabbit serum, and 0.1% mouse serum) when in use.

Ruthenium (Ru)-conjugated P-11 mAb was prepared by the following procedure: 68 μL Ru-complex compounds (10 mg Ru (II) Tris (bipyridyl)-NHS ester in 1 mL DMSO) was added

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to 1 mL P-11 mAb (1 mg/mL in 0.15 mol/L PBS, pH 7.8) (Sekisui Medical) for conjugation, and stirred at 25°C for 30 min. Then, 50 μL of 2 mol/L glycine was added to terminate the conjugation reaction, and Ru-conjugated P-11 mAb was isolated by collecting the Ru-bound protein fraction using Sephadex G-25 (previously equilibrated with 10 mmol/L PBS, pH 6.0). The Ru-conjugated P-11 mAb was then diluted to 1 μg/mL using Ru dilution reagent (0.015 mol/L HEPES buffering solution [pH 7.8], 0.15 mol/L NaCl, 0.013 mol/L $CaCl_2$, 0.1% Tween 20, 0.1% NaN_3, 5% normal rabbit serum, and 0.1% mouse serum) when in use.

Measurement of conventional DCP (with MU-3 antibody), NX-DCP (with P-11 and P-16 antibodies), and NX-DCP ratio. Conventional DCP, which is measured using MU-3 antibody and is currently used in clinical settings, was measured with ECLIA using the Picolumi III automated analyzer (EIDIA). NX-DCP was measured by ECLIA. Briefly, 25 μL magnetic beads coated with P-16 mAb (1 mg/mL) and 150 μL Ru-conjugated P-11 mAb (1 μg/mL) were added to samples at 30°C for 9 min to obtain the value of NX-DCP. The NX-DCP ratio was calculated by dividing the value of DCP measured using the conventional Picolumi method by the value of NX-DCP.

Reactivity of MU-3, P-11, and P-16 mAbs based on the time allowed for decarboxylation from prothrombin. We prepared DCP with varying numbers of Glu residues by applying different time intervals for decarboxylation from prothrombin (Enzyme Research Laboratories, Swansea, UK), according to the method of Bajah *et al.*[2] Specifically, 0.78 mL ammonium bicarbonate solution (0.1 mol/L, pH 8.0) was applied to 4.6 mg/mL prothrombin solution overnight for dialysis against an ammonium bicarbonate solution at 4°C. Then 0.1 mol/L EDTA*2Na was applied to the solution after dialysis until a final concentration of 10 mmol/L was achieved, and the solution was allowed to stand at room temperature for 30 min. This solution was dialyzed again against an ammonium bicarbonate solution for 2 h at 4°C then aliquoted into six heat-resistant vials with a screw cap and lyophilized. The vials were then filled with nitrogen gas and heated to 110°C for 0, 30 min, 1, 2, 6, or 23 h to create six different samples. We coated microplates with 100 μL of each sample at 0.1 μg/mL, and tested reactivity of the MU-3, P-11, and P-16 mAbs in the presence of 4 mmol/L calcium chloride. The experiments were repeated three times and the average value was calculated.

Patients. A total of 338 patients were diagnosed with primary, non-recurrent HCC between January 2006 and December 2009 at Ogaki Municipal Hospital (Ogaki, Japan). Of these, 14 patients had been taking warfarin when HCC was diagnosed. Six patients at Osaka Red Cross Hospital (Osaka, Japan) who were diagnosed as having primary HCC during the same period and had been taking warfarin at the time of diagnosis were also enrolled in the study. We analyzed the stored serum samples from these 20 patients, obtained at the time of HCC diagnosis. The diagnosis of HCC was made by histological examination or appropriate imaging characteristics using criteria of the guidelines by the American Association for the Study of Liver Diseases.[11] Tumor stage on imaging findings was assessed according to the TNM classification of the Liver Cancer Study Group of Japan.[12]

Control samples were obtained from 56 patients with chronic liver disease without HCC who were followed up at Ogaki Municipal Hospital. Samples were collected during routine HCC surveillance during the same period. These patients had been taking warfarin when serum samples were collected and provided informed consent for their stored serum samples to be used for research. The diagnosis of chronic liver disease was made with histological examination in 45 patients, including 10 with cirrhosis. The remaining 11 patients were diagnosed with cirrhosis based on imaging findings and biochemical tests. To ensure that controls did not have HCC, these patients were followed for 3 years after serum sampling by ultrasonography, CT, or MRI to ensure that none had developed HCC.

The protocol for the clinical part of this study was approved by the institutional review board of Ogaki Municipal Hospital and carried out in compliance with the Helsinki Declaration. Written informed consent was obtained from all study patients for the use of clinical and laboratory data and stored serum samples.

Statistical analyses. Differences in percentages between groups were analyzed using the χ^2-test. Differences in mean quantitative values were analyzed by the Mann–Whitney *U*-test. Changes in the NX-DCP ratio with increases in HCC stage were analyzed with the Jonckheere–Terpstra test. Data analyses were carried out using JMP statistical software, version 6.0 (Macintosh version; SAS Institute, Cary, NC, USA). All *P*-values were derived from two-tailed tests, with $P < 0.05$ considered to indicate statistical significance.

Results

Reactivity of MU-3, P-11, and P-16 antibodies with DCP based on time allowed for decarboxylation from prothrombin. Figure 1 shows the reactivity of the MU-3, P-11, and P-16 antibodies according to the time allowed for decarboxylation from prothrombin. MU-3 did not react with prothrombin (0 min) and its reactivity increased as the heating time (time allowed for decarboxylation) increased, with maximum reactivity to the sample after 6 h of heating. In contrast, P-11 and P-16 showed maximum reactivity to the 1-h sample and reactivity decreased as the heating time (time allowed for decarboxylation) increased.

Patient characteristics and levels of conventional DCP, NX-DCP, and NX-DCP ratio. Warfarin was used to treat atrial fibrillation in 48 patients, a history of mitral or aortic valve replacement in 13 patients, and a history of cerebral infarction in 15 patients. Table 1 summarizes the characteristics of the patients with and without HCC. There were no differences in patient

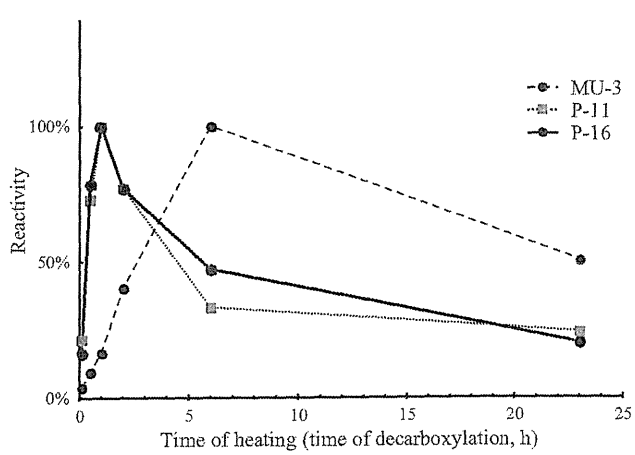

Fig. 1. Reactivity of MU-3, P-11, and P-16 antibodies according to the time allowed for decarboxylation from prothrombin. MU-3 did not react with prothrombin (0 min) and its reactivity increased as the heating time (time allowed for decarboxylation) increased, with maximum reactivity to the sample after 6 h of heating. Both P-11 and P-16 showed maximum reactivity to the 1-h sample and reactivity decreased as the heating time increased.

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Table 1. Background characteristics of study patients with and without hepatocellular carcinoma (HCC) (n = 76)

	Patients with HCC (n = 20)	Patients without HCC (n = 56)	P-value
Mean age ± SD, years (range)	72.4 ± 8.0 (46–83)	70.0 ± 9.8 (46–86)	0.3211
Sex, female/male	6 (30.0)/14 (70.0)	19 (33.9)/37 (66.1)	0.9651
Albumin, g/dL (mean ± SD)	3.82 ± 0.42	3.97 ± 0.51	0.2276
Total bilirubin, mg/dL (mean ± SD)	1.02 ± 0.65	0.82 ± 0.52	0.1288
Platelets ($\times 10^3/\mu L$)	158 ± 75	160 ± 49	0.4754
INR	1.75 ± 0.58	1.76 ± 0.58	0.7816
Mean tumor size ± SD, cm (range)	3.35 ± 1.84 (1.1–8.4)	–	–
Number of tumors, single/multiple	15 (75.0)/5 (25.0)	–	–
Tumor stage, I/II/III†	3 (15.0)/11 (55.0)/6 (30.0)	–	–

†According to the TNM classification of the Liver Cancer Study Group of Japan. Unless otherwise indicated, values in parentheses indicate percentages. INR, International normalized ratio.

age, sex, serum albumin, serum total bilirubin, platelet count, or prothrombin levels.

Figure 2 compares conventional DCP levels, NX-DCP levels, and NX-DCP ratios between patients with and without HCC. No differences were found in conventional DCP levels between patients with and without HCC (median, 2600.5 mAU/mL and range, 1060–96920 mAU/mL in patients with HCC versus median, 20550.5 mAU/mL and range, 1355–71783 mAU/mL in patients without HCC; P = 0.7952). In contrast, NX-DCP levels in patients with HCC (median, 34135.0 mAU/mL; range, 260–67581 mAU/mL) were significantly lower than in patients without HCC (median, 40708.0 mAU/mL; range, 5026–60443 mAU/mL; P = 0.0291). As a result, the NX-DCP ratio was significantly higher in patients with HCC (median, 1.92; range, 0.35–10.32) than in patients without HCC (median, 0.49; range, 0.12–1.33; P < 0.0001).

Sensitivity, specificity, and positive and negative predictive values of NX-DCP ratio for diagnosis of HCC. Figure 3(a) shows the receiver operating characteristic (ROC) curve of the NX-DCP ratio for the diagnosis of HCC. The area under the ROC curve was 0.8928. The highest Youden index was 0.68 when the cut-off was fixed as 0.65 and the highest accuracy was 89.5% when the cut-off was fixed as 1.50, based on the sensitivity and specificity analysis (Fig. 3b). When the cut-off was fixed as 0.65, sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy were 95.0%, 73.2%, 55.9%, 97.6%, and 78.9%, respectively. When the cut-off was fixed as 1.50, sensitivity, specificity, PPV, NPV, and accuracy were 60.0%, 100.0%, 100.0%, 87.5%, and 89.5%, respectively.

Serum alpha-fetoprotein (AFP) and *Lens culinaris* agglutinin-reactive fraction of AFP (AFP-L3) levels in patients with HCC. Serum levels of AFP and AFP-L3 were measured[13] in patients with HCC in the same serum for the measurement of NX-DCP ratio (AFP-L3 was not measured in five patients). The median (range) values were 18.4 ng/mL (0.8–68 470 ng/mL) for AFP and 3.6% (0–45.2%) for AFP-L3. When the cut-off levels of AFP and AFP-L3 were fixed as 20 ng/mL and 5%, respectively, according to previous reports,[14,16] 10 of 20 patients (50.0%) showed elevation of AFP and seven of 15 patients (46.7%) showed elevation of AFP-L3. These two tumor markers were not increased in six of 15 patients (40.0%).

NX-DCP ratio and progression of HCC. Figure 4 shows the NX-DCP ratio in patients according to HCC stage. Despite the small number of patients, there was a gradual increase in the NX-DCP ratio as the stage increased (P = 0.0315).

Discussion

Hepatocellular carcinoma is the sixth most common cancer and the third most common cause of cancer-related death worldwide.[17,18] In Japan, HCC is currently the third most common cause of death from cancer in men and the fifth in women.[19] The incidence of HCC is also increasing in the US.[20,21] Improvements of tumor markers specific for HCC contribute to early detection of HCC. Three markers for HCC are currently used clinically, AFP, AFP-L3, and DCP. The utility of each of these tumor markers for detection and diagnosis of HCC, for evaluation of tumor progression, and for determination of patient prognosis has been reported.[4,22–24] Elevation

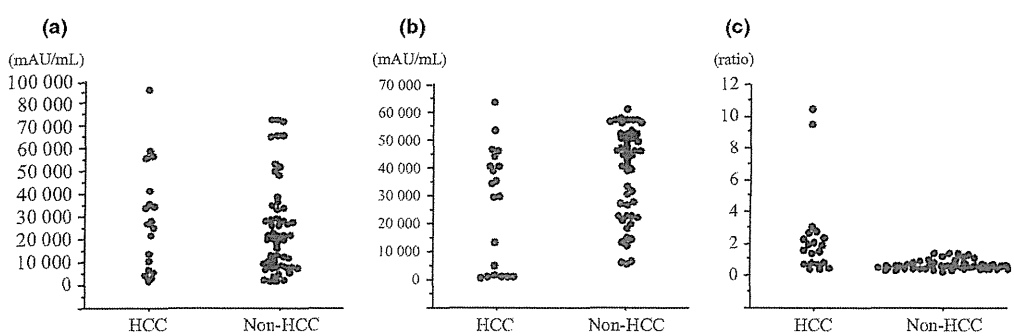

Fig. 2. Serum levels of conventional des-gamma-carboxy prothrombin (DCP), NX-DCP, and the NX-DCP ratio in patients with and without hepatocellular carcinoma (HCC) taking warfarin. (a) Serum levels of conventional DCP. No differences were found between two groups (P = 0.7952). (b) Serum levels of NX-DCP were significantly higher in patients without HCC compared to those with HCC (P = 0.0291). (c) The NX-DCP ratio was significantly higher in patients with HCC than in those without HCC, consequently (P < 0.0001).

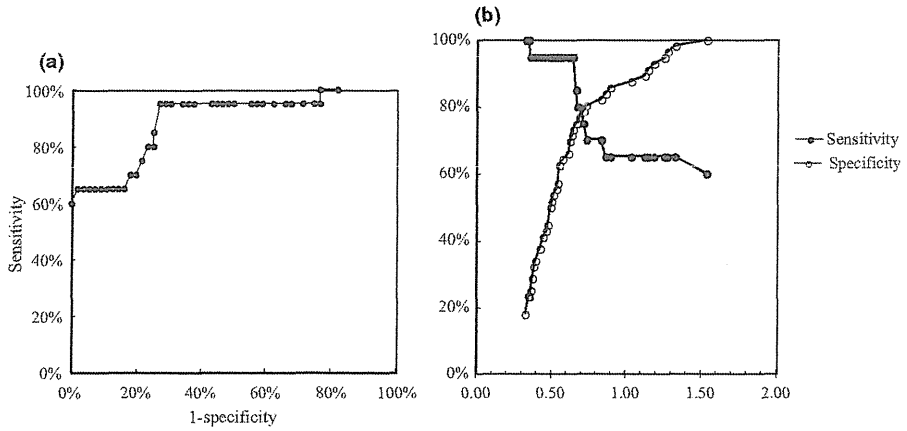

Fig. 3. Receiver operating characteristic (ROC) analysis and the determination of cut-off level of the NX-DCP ratio for the diagnosis of hepatocellular carcinoma. (a) The area under the ROC curve was 0.8928. (b) The highest Youden index was 0.68 when the cut-off was fixed as 0.65 and the highest accuracy was 89.5% when the cut-off was fixed as 1.50.

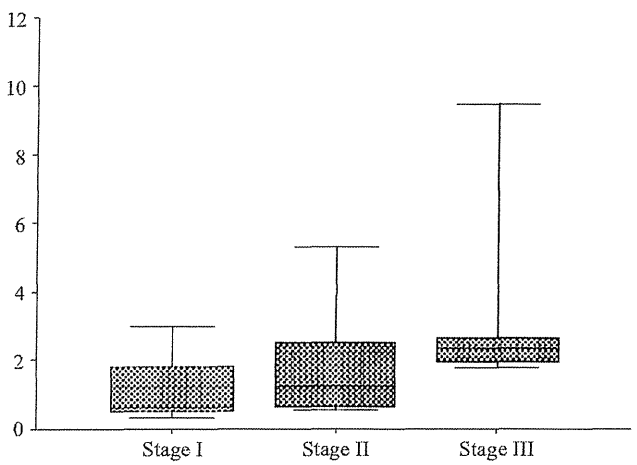

Fig. 4. The NX-DCP ratio according to hepatocellular carcinoma (HCC) stage in 20 patients with HCC taking warfarin (box plot). There was a gradual increase in the NX-DCP ratio as the HCC stage increased (P = 0.0315).

of DCP is often observed in HCC patients without elevation of AFP or AFP-L3, and is useful as a complement to these other two markers in the diagnosis of HCC. In addition, elevation of DCP is reportedly associated with a high rate of portal vein invasion and poor prognosis.[25] Elevation of DCP is also associated with better outcomes when hepatectomy, rather than radiofrequency ablation, is carried out in patients treated with curative intent.[26,27]

However, DCP loses its value as a tumor marker of HCC in patients taking warfarin.[7] Due to the marked decrease in vitamin K level caused by warfarin intake, DCP levels significantly increase in individuals taking warfarin, even in the absence of HCC. Therefore, DCP has no clinical utility as a tumor marker for HCC in this patient subpopulation.

The present study evaluated the reactivity of new antibodies against DCP, antibodies P-11 and P-16, based on the number of Glu residues. The number of Glu residues increases as the time allowed for decarboxylation from prothrombin increases.[2] Our results showed P-11 and P-16 have higher reactivity with DCP with fewer Glu residues than MU-3, the antibody that is conventionally used for the measurement of DCP. The NX-DCP level that is measured by P-11 and P-16 antibodies, therefore, represents predominantly DCP caused by reduced vitamin K availability. Consequently, the elevation of the NX-DCP ratio calculated in the equation: conventional DCP/NX-DCP, reflects more specifically the elevation of DCP by HCC.

There were no differences in the conventional measurements of DCP between patients with and without HCC who are taking warfarin. The NX-DCP ratio was significantly lower in patients without HCC than in patients with HCC. The NX-DCP ratio varied in patients with HCC, as was conventional DCP in patients not taking warfarin, because the production of DCP by HCC is variable. In contrast, in all patients without HCC, the NX-DCP ratio was low despite high conventional DCP levels in the same patients; no patients had an elevated NX-DCP ratio. The results indicate that the NX-DCP ratio could pinpoint the elevation of DCP caused by HCC, thereby restoring the value of DCP as a marker for HCC in patients taking warfarin.

When the cut-off level was fixed at 1.5 on the basis of maximal accuracy, the sensitivity, specificity, PPV, and NPV were comparable to those of conventional DCP in the general population with normal vitamin K levels, as previously reported.[28] The NX-DCP ratio, therefore, seems to be useful as a marker for HCC in patients taking warfarin.

The elevation of other tumor markers for HCC, AFP and AFP-L3, were observed in only half of the patients with HCC taking warfarin. In addition, both AFP and AFP-L3 were negative in 40% of patients with HCC. Des-gamma-carboxy prothrombin is a complimentary marker of AFP/AFP-L3 for HCC. The elevation of DCP without the elevation of AFP and AFP-L3 was observed in 16.1% of patients (cut-off, 20 ng/mL for AFP, 10% for AFP-L3, and 40 mAU/mL for DCP)[29] and 24.8% of patients (cut-off, 400 ng/mL for AFP, 15% for AFP-L3, and 100 mAU/mL for DCP).[30] The measurement of the NX-DCP ratio, therefore, will be important for the detection and diagnosis of HCC even when AFP or AFP-L3 is measured simultaneously.

There are several limitations to this study. The most important limitation was the small number of study patients, especially patients with HCC. The number of patients with HCC taking warfarin is low, so it was difficult to increase the number of study patients. Consequently, it was difficult to evaluate the value of the NX-DCP ratio in indicating progression of

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HCC, including tumor stage progression and portal vein invasion, and in predicting patient outcome. Further studies will be necessary to establish the value of the NX-DCP ratio as a tumor marker for HCC in patients taking warfarin. In addition, the value of the NX-DCP ratio was evaluated only in patients who were taking the vitamin K antagonist warfarin: its value was not evaluated in HCC patients in whom vitamin K is reduced or absent through other mechanisms such as heavy alcohol intake or nutritional deficiency. The value of the NX-DCP ratio as a marker for HCC should be confirmed for these subpopulations in the future.

In conclusion, the novel NX-DCP ratio identified elevation of DCP due to HCC in patients taking the vitamin K antagonist warfarin. Thus, by using this ratio, DCP can be used as a marker for HCC even in patients taking warfarin.

Acknowledgments

We thank Jun Nishimura and Masato Uehara (EIDIA, Tokyo, Japan) for their technical assistance.

Disclosure Statement

The authors have no conflicts of interest to declare.

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Letter to the Editor

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Incidence of hepatocellular carcinoma and response to interferon therapy in HCV-infected patients: effect of factors associated with the therapeutic response and incidence of HCC

To the Editor:

Several previous studies reported a significantly lower incidence of hepatocellular carcinoma (HCC) in hepatitis C virus (HCV)-infected patients who showed sustained virological response (SVR) to or relapsed on antiviral therapy with interferon (IFN) or peginterferon (PEG-IFN), with or without ribavirin compared with no responders (NR; i.e. partial response, viral breakthrough, or null-response) (1, 2). The reduction in HCC incidence was especially marked in patients with SVR. These results have been taken as evidence that antiviral therapy has an effect of suppressing the development of HCC.

Recently reported viral and host factors that are strongly associated with response to anti-HCV therapy (3, 4) may also be associated with the pathogenesis of HCC. Amino acid substitution in the HCV core region, a viral factor reportedly associated with response to PEG-IFN and ribavirin therapy (3), is also associated with the development of HCC (5). Regarding host factors associated with response to anti-HCV therapy (4), genetic polymorphisms near the *IL28B* gene are reportedly associated with hepatic steatosis (6) and interact with amino acid substitutions in the HCV core region (7), both of which are associated with the development of HCC (5, 8).

We analysed the incidence of HCC in 448 patients who completed anti-HCV therapy with IFN or PEG-IFN and in whom the genetic polymorphisms near *IL28B* gene were analysed after the approval of the hospital ethics committee and obtaining written informed consent. We found significant differences in the incidence of HCC between patients with SVR ($n = 247$), relapse ($n = 122$), and NR ($n = 79$) (Fig. 1A, $P < 0.0001$ by Log-rank test). However, the prevalence of patients having TT genotype at rs8099917 near the *IL28B* gene, which is associated with favourable response to anti-HCV therapy, was significantly lower in patients with NR (SVR, 85.8%; relapse, 80.3%; NR, 39.2%; $P < 0.0001$ by Chi-square test). In addition, we found significant differences in the incidence of HCC also according to the genotype of rs8099917 (Fig. 1B, $P = 0.0156$). Although multivariate analysis using Cox proportional hazard model including age, gender, HCV genotype, and the outcome of therapy, but not *IL28B* polymorphisms identified SVR ($P = 0.0083$) and relapse ($P = 0.0493$) as independent factors that were associated with lower incidence of HCC, it failed to detect an independent factor that was associated with the incidence of HCC when *IL28B* polymorphisms were included. These results suggested that the previously reported differences in the incidence of HCC by anti-HCV response may not have been due to the ability of antiviral therapy to suppress HCC, but rather simply they may reflect the ability of such treatment to better identify patients at high-risk for HCC based on response to anti-HCV therapy.

It is not elucidated whether the results in our present analyses were simply due to the small number of

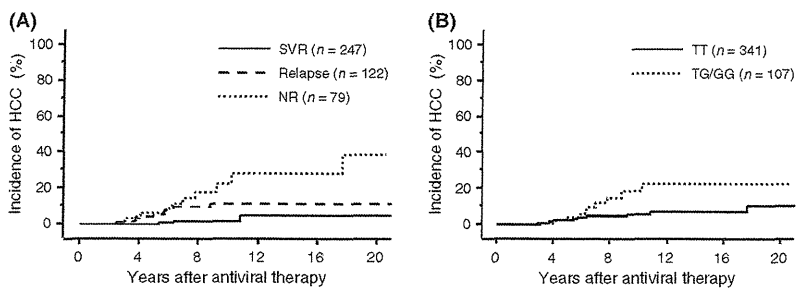

Fig. 1. Cumulative incidence of hepatocellular carcinoma (HCC) after antiviral therapy with interferon or peginterferon. (A) Incidence of HCC is significantly lower in patients with sustained virological response (SVR), those with relapse, and those with no response (NR) that includes partial response, viral breakthrough, or null-response, in that order. (B) Incidence of HCC is significantly lower in patients with TT genotype at rs8099917 near the *IL28B* gene, which is associated with the favourable response to antiviral therapy.

patients analysed or the incidence of HCC after antiviral therapy is similar regardless of response, when they are stratified by host and viral factors. In addition, our present analyses failed to examine the association between amino acid substitutions in the HCV core region and the incidence of HCC due to the small number of patients in whom the information of this substitution was available. Nonetheless, with the emergence of factors that can be independently associated with both the response to antiviral therapy and the development of HCC, the effect of response to antiviral therapy with IFN or PEG-IFN on the incidence of HCC will require re-examination taking *IL28B* polymorphisms and amino acid substitutions in the HCV core region into consideration.

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JOURNAL OF HEPATOLOGY

Week 4 viral response to peginterferon and ribavirin: How should it be used in combination with a baseline predictive factor?

To the Editor:

We read with great interest the article by Marcellin *et al.* [1] evaluating the predictive value of week 4 viral response to peginterferon-alpha 2a and ribavirin combination therapy in patients with hepatitis C virus (HCV) genotype 1. They concluded that patients with a $\geq 3\log_{10}$ drop in HCV RNA at week 4 have a high probability of achieving sustained virologic response (SVR), which is consistent with our previous study [2].

Previous studies reported that the genetic polymorphism near the interleukin 28B (*IL28B*) gene (rs12979860 or rs8099917) is a strong baseline factor associated with the outcome of therapy [3]. However, this variable was not included in the study by Marcellin *et al.*, probably because the actual treatment period in the study predated this finding [4,5]. Given these predictors, i.e. week 4 viral response and a baseline variable, how should they be combined to predict response?

We evaluated the predictive value of week 4 viral response to combination therapy on SVR in 272 patients infected with HCV genotype 1b [6]. Overall, a $\geq 3\log_{10}$ drop in HCV RNA at week 4 was a strong predictor of SVR. SVR was achieved in 77.0% of patients with rapid virologic response (RVR) or a $\geq 3\log_{10}$ drop, whereas only 16.7% of patients with a $<3\log_{10}$ drop achieved SVR ($p<0.0001$). When patients were stratified based on the *IL28B* genetic polymorphism rs8099917, which corresponds to rs12979860 in more than 99% of Japanese ethnicity [7], a $\geq 3\log_{10}$ drop at week 4 was strongly predictive of SVR in patients with the favorable TT rs8099917 genotype (CC rs12979860 genotype). The SVR rate was 79.5% in patients with RVR or a $\geq 3\log_{10}$ drop and 15.6% in patients with a $<3\log_{10}$ drop ($p<0.0001$). In contrast, among patients with an unfavorable TG/GG rs8099917 genotype, no differences were found in the SVR rate between patients with RVR or a $\geq 3\log_{10}$ drop (20.0%) and those with a $<3\log_{10}$ drop (18.3%, $p=0.9265$); the predictive value of week 4 response is low in this subset. In addition, the predictive value of complete early virologic response (EVR) for SVR is lower in patients with the unfavorable TG/GG genotype. The SVR rate was 81.6% in patients with complete EVR and 21.2% in patients without ($p<0.0001$), when patients had the favorable TT rs8099917 genotype. In contrast, the rate of SVR was 25.0% in patients with complete EVR and 18.0% in patients without ($p=0.7279$), when patients had the unfavorable TG/GG genotype. Therefore, it appears to be difficult to identify patients with the unfavorable genotype of the genetic polymorphism near the *IL28B* gene who have a likelihood to achieve SVR by week 4 viral response, although it can identify patients with a high likelihood of achieving SVR in patients with the favorable genotype.

In contrast to our results, a previous large study by Thompson *et al.* [8] reported that patients who attained RVR showed high SVR rate regardless of the genetic polymorphisms near the *IL28B* gene (rs12979860), although they focused on patients with RVR and did not include patients with non-RVR but with a $\geq 3\log_{10}$ drop at week 4. This discrepancy between their study and ours may be partly explained by the difference in the ethnicity of the study population. The study by Thompson *et al.* was based on patients from the IDEAL study including Caucasians, African Americans, and Hispanics, whereas all patients were Japanese Asians in our study. Similarly, the ethnicity was different between the population studied by Marcellin *et al.* and ours. Accordingly, the distribution of rs12979860 or rs8099917 genotypes and the rate of concordance between rs12979860 (analyzed in a study by Thompson *et al.*) and rs8099917 (analyzed in our study) would be different. For example, the rate of favorable homozygote (CC rs12979860 genotype and TT rs8099917 genotype) was largely different: 33.0% in Thompson's study and 76.1% in our study. Moreover, our study involved only patients infected with HCV genotype 1b. These factors should be adjusted when comparing the association between the genetic polymorphisms near the *IL28B* gene and the predictive value of week 4 viral response between studies. Nonetheless, the genetic polymorphism near the *IL28B* gene appears to have a strong impact on the predictive value of early viral response to therapy; the prediction of SVR by week 4 viral response may have to be modified based on this strong baseline predictive factor.

Conflict of interest

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Encephalopathy or hepatic encephalopathy?

To the Editor:

We read with interest the paper by Ginès and co-authors on the management of critically-ill cirrhotic patients [1]. However, we have some concerns on the section on management of hepatic encephalopathy. The authors seem to base their recommendations on a 'statistical' rather than a pathophysiological definition of the syndrome, grouping under the heading 'severe hepatic encephalopathy' a set of different neuropsychiatric symptoms arising in critically-ill cirrhotic patients, to include mental abnormalities relating to sepsis, electrolyte imbalance, and even the side- or desired-effects of drugs such as opioids and benzodiazepines. Within this frame, they state that ammonia levels should not be measured, as they provide no clinical information nor do they relate to clinical outcomes. While we agree with the authors that patients with cirrhosis, especially if critically-ill, may present with more than one metabolic encephalopathy, and these may all contribute and worsen the clinical picture, it seems to us that an effort should be made to differentiate *hepatic* encephalopathy from other forms of metabolic/toxic neuropsychiatric disturbance. For example, we need to be reasonably sure that the encephalopathy we refer to in order to define fulminant hepatic failure is *hepatic* encephalopathy, as we would not want to list for transplant a patient with hepatitis who is confused because of hypoglycaemia, or opioid/benzodiazepine overdose. In this respect, ammonia levels seem useful, as they reflect hepatic failure and portal-systemic shunting [2], they correlate with recognised, quantified indices of hepatic encephalopathy, and they predict the development of hepatic encephalopathy over time [3]. Notably, sepsis, electrolyte imbalance, and psychoactive drugs cause neuropsychiatric abnormalities in critically-ill patients with no liver dysfunction [4]: we would not diagnose these patients with hepatic encephalopathy, we would not expect them to be hyperammonaemic and we would not treat them with ammonia-lowering drugs such as non-absorbable disaccharides/antibiotics. Critically-ill cirrhotic patients are no exception. Should they present with more than one potential cause for neuropsychiatric dysfunction, each cause should be identified and treated according to its pathophysiology. Finally, there seems to be some confusion in Table 2, in relation to the West Haven criteria [5]. These are clinical criteria and they are described, although not in their exact, original form [5], in columns 2 and 3 of the table. However, the table also depicts stages, characterized by parallel alterations in consciousness, cognitive/behavioural features, neurological findings, and electroencephalographic changes. Such correspondence has never been established, which is the reason why Conn and co-workers proposed the use of an index, not unlike the Child–Pugh score, combining the independent scores of five dimensions (mental state based on the West Haven criteria, Trail Making Test A, asterixis, electroencephalographic slowing and arterial ammonia levels) [5]. In addition, the classification of electroencephalographic changes reported in column 5 of the table does not correspond to either the one proposed by Conn *et al.* [5] or to more modern ones [6], most likely in relation to a typo or an alignment problem. An *errata corrige* on the involuntarily misleading information provided in Table 2 of the paper might be necessary.

Conflict of interest

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Original Article

Pegylated interferon monotherapy in patients with chronic hepatitis C with low viremia and its relationship to mutations in the NS5A region and the single nucleotide polymorphism of interleukin-28B

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Aim: Previous studies have suggested that patients with chronic hepatitis C with a low pretreatment hepatitis C virus (HCV) level have a high sustained virological response (SVR) rate, and that there would be a subpopulation of patients in which HCV can be eradicated with pegylated interferon (PEG IFN) alone without a decrease in SVR. However, the efficacy of PEG IFN monotherapy in patients with low HCV RNA levels is unclear. Several studies have reported that interferon sensitivity-determining region (ISDR) and the single-nucleotide polymorphism (SNP) of interleukin-28B (IL-28B) contribute to IFN response, but these relationships are controversial. The aim of this study was to determine whether the SNP of IL-28B (rs8099917) and amino acid substitutions in the ISDR among patients with low HCV levels affect the response to PEG IFN monotherapy.

Methods: One hundred and four patients with low-level HCV infection were studied. Low HCV level was defined as 100 KIU/mL or less.

Results: SVR was achieved in 94 patients (92.2%). HCV levels (≤50 KIU/mL) and ISDR (≥2 mutations) were associated with SVR on univariate analysis. The rates of SVR in the patients with IL-28B genotypes TT, TG and GG were 94.5%, 77.8% and 100%, respectively. The G allele tended to be associated with poor response to IFN therapy ($P = 0.0623$). On multivariate analysis, the ISDR was the factor predictive of SVR ($P = 0.004$).

Conclusion: The ISDR is significantly associated with a good response to PEG IFN monotherapy in patients with low HCV levels.

Key words: hepatitis C virus, interferon sensitivity-determining region, interferon, interleukin-28B, rapid virological response

INTRODUCTION

HEPATITIS C VIRUS (HCV) is a member of the Flaviviridae family and causes chronic hepatitis that can develop into cirrhosis and hepatocellular carcinoma (HCC) that easily progresses to end-stage liver disease.[1] Because 170 000 000 persons are infected with HCV worldwide, HCV infection is a significant global health problem.

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The current recommended therapy for patients with chronic hepatitis C is a combination of pegylated interferon (PEG IFN) and ribavirin and/or telaprevir or boceprevir.[2–6] HCV RNA levels, as well as genotypes, are an important factor associated with sustained virological response (SVR) to IFN therapy.[3,4] Patients with low HCV RNA levels have a high SVR rate, and even standard IFN monotherapy is useful for eradication of HCV in patients with low viral loads.[7–9] Several studies have succeeded in reducing the duration of treatment without risk of relapse.[10,11] Although patients with low HCV RNA have higher response rates to IFN treatment, not all patients achieve SVR. Other factors for improving the prediction of SVR in patients with low HCV RNA levels are needed. The predictive factors for SVR in patients with genotype 1b and high HCV RNA levels have been investigated, and several studies have shown that the single nucleotide polymorphism of interleukin-28B (IL-28B) and amino acid substitutions in the core and NS5A region affect the response to IFN therapy.[12–16] However, the predictive factors for SVR among patients with low HCV RNA levels treated with PEG IFN monotherapy have been unclear.

Hepatitis C virus consists of three structural proteins (core, envelope 1 and envelope 2) and six non-structural proteins (NS2 to NS5). HCV NS5A protein was reported to have a domain associated with IFN response. This domain in the region of HCV genotype 1b is closely associated with response to IFN therapy and is known as the IFN sensitivity-determining region (ISDR).[12,15–21] IFN acts to control replication of the virus by inducing the dsRNA-dependent protein kinase (PKR). The ISDR is located in the PKR-binding domain, is inhibited by PKR *in vitro*,[22] and is useful for prediction in patients with genotypes 2a, 2b and 3a.[23–28] Therefore, ISDR heterogeneity is an important factor that may affect response to IFN in patients with low HCV RNA levels. We hypothesized that ISDR heterogeneity could be predicted in patients with low HCV RNA levels in which HCV can be eradicated with PEG IFN-α alone without a decrease in SVR.

Not only genetic heterogeneity in the HCV genome but also host genetics contribute to IFN treatment outcomes. Therefore, several studies were performed to understand the host factors associated with IFN responsiveness; these showed that IL-28B polymorphisms are strongly associated with response to PEG IFN and ribavirin combination therapy in patients with genotype 1b and high viral load.[13,14,16,29] However, the associations between ISDR and IL-28B and the effects of PEG IFN-α monotherapy in patients with low HCV RNA levels are not well known.

The aim of the present study was to determine whether genomic heterogeneity of the ISDR and the SNP of IL-28B among patients with low HCV RNA levels affects the response to PEG IFN-α-2a monotherapy.

METHODS

A TOTAL OF 295 patients with chronic hepatitis C were treated by PEG IFN-α-2a monotherapy at Nagoya University Hospital and Affiliated Hospitals; 104 patients with low HCV RNA levels were selected for this study. The patients consisted of 62 men and 42 women with a mean age of 55.1 years (range, 19–78). All patients were positive for serum anti-HCV antibody by a commercial enzyme-linked immunosorbent assay (Dinabot, Tokyo, Japan) and for HCV RNA by a commercial polymerase chain reaction (PCR) (Roche Diagnostic Systems, Tokyo, Japan).

A low HCV level was defined as 100 KIU/mL or less, as previously reported.[4,7,9,11] No patient had hepatitis B surface antigen, co-infection with HIV, autoimmune disease or chronic alcohol abuse.

Schedule of IFN therapy

Patients received PEG IFN-α-2a (Pegasys Chugai-Roche, Tokyo, Japan) at a dose of 180 μg injected s.c. once per week for 24 or 48 weeks. The patients were allocated, at the discretion of the physician in charge, to a protocol lasting either 24 or 48 weeks. Laboratory tests and evaluations of adverse events were performed once per week during treatment.

The dose of PEG IFN-α-2a was reduced to 90 μg when clinically significant adverse events or laboratory abnormalities such as neutropenia (<750 cells/mm^3) or thrombocytopenia (<50 000 cells/mm^3) occurred. PEG IFN-α-2a was discontinued when neutropenia of less than 250 cells/mm^3 or a platelet count of less than 25 000 cells/mm^3 was seen.

Hepatitis C virus RNA in serum samples was examined at 4 weeks, at the end of IFN therapy, and at 6 months after the end of treatment (ETR). Serum was stored at −80°C for virological examination at pretreatment.

Patients who were persistently negative for serum HCV RNA and who had a normal serum alanine aminotransferase (ALT) level at 24 weeks after withdrawal of IFN treatment were considered to have SVR. Patients who were HCV negative at the ETR but returned to HCV