that the observed hyperbilirubinemia was not associated with HBV activity. The increase in serum bilirubin level is presumably caused by PIs. Hyperbilirubinemia following PI administration was previously reported [35]. Although it is unclear whether hyperbilirubinemia itself may lead to liver injury, PIs should be used carefully particularly for patients with advanced liver diseases.

Our present study has one major limitation; that is, the effect of alcohol on liver function was not analyzed because the history of alcohol consumption could not be obtained in the majority of the studied patients. Excessive alcohol consumption has been found to be an important risk factor for the development of severe hepatic injury in HIV-infected patients with [3] or without HCV coinfection [5]. Our present study showed that among the 26 patients whose history of alcohol consumption was available, only 2 patients were habitual drinkers. The results suggested that the effect of alcohol on liver function is small in HIV/HBV-coinfected patients in Japan.

In conclusion, ART with anti-HBV drugs may retard the progression of liver diseases and prevent liver-related death in HIV/HBV-coinfected patients. Multiple agents with anti-HBV activity seem essential for the efficacy. PIs should be carefully used particularly for patients with advanced liver diseases.

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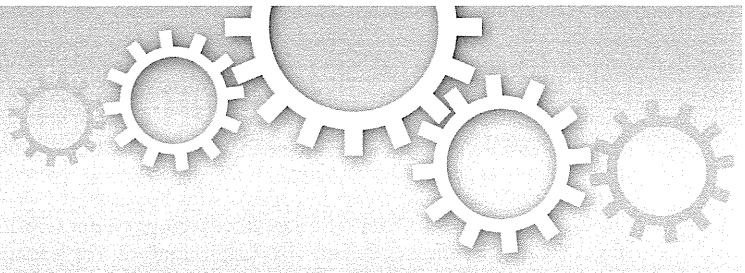

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A serum "sweet-doughnut" protein facilitates fibrosis evaluation and therapy assessment in patients with viral hepatitis

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Although liver fibrosis reflects disease severity in chronic hepatitis patients, there has been no simple and accurate system to evaluate the therapeutic effect based on fibrosis. We developed a glycan-based immunoassay, FastLec-Hepa, to fill this unmet need. FastLec-Hepa automatically detects unique fibrosis-related glyco-alteration in serum hyperglycosylated Mac-2 binding protein within 20 min. The serum FastLec-Hepa counts increased with advancing fibrosis and illustrated significant differences in medians between all fibrosis stages. FastLec-Hepa is sufficiently sensitive and quantitative to evaluate the effects of PEG-interferon-α/ribavirin therapy in a short post-therapeutic interval. The obtained fibrosis progression is equivalent to -0.30 stages/year in patients with sustained virological response, and 0.01 stages/year in relapse/nonresponders. Furthermore, long-term follow-up of the severely affected patients found hepatocellular carcinoma developed in patients after therapy whose FastLec-Hepa counts remained above a designated cutoff value. FastLec-Hepa is the only assay currently available for clinically beneficial therapy evaluation through quantitation of disease severity.

The World Health Organization has estimated that the prevalence of chronic infections with hepatitis B virus (HBV) and hepatitis C virus (HCV) is more than 5% of the world population. The high rate of viral transmission worldwide has also resulted in an explosive increase in incidence of liver cirrhosis (LC), because liver fibrosis caused by the persistent infections with HBV and HCV irreversibly progresses in chronic hepatitis (CH) patients without effective treatment. As the incidence of hepatocellular carcinoma (HCC) increases proportionally to the severity of hepatitis and the presence of LC, it is now clear that about 90% of HCC cases originate from infection with HBV or HCV. It is estimated that more than one million patients worldwide die from liver disease related to HBV or HCV infection each year. Immunomodulatory therapy with PEG-interferon-α and ribavirin is the standard treatment for patients with chronic hepatitis C (CHC)[1]. Recent genome-wide association studies have revealed that variation in the host interleukin-28B gene can predict the outcome of therapies for viral clearance[2–4]. Such pharmacokinetic understanding should allow for more precise treatment protocols and follow-up analyses to optimize the opportunity for patients to achieve sustained virological response (SVR)[5,6]. Linear peptidomimetic HCV and NS3/4A serine protease inhibitors such as telaprevir and boceprevir are new drugs that, in combination with PEG-interferon-α and ribavirin, substantially improve the rates of response among patients with HCV genotype 1 infection[1]. Alternatively, suppression of hepatic decompensation in chronic hepatitis B patients with advanced fibrosis and cirrhosis has been evaluated during long-term treatment with antiviral agents, such as adefovir, lamivudine, entecavir, and tenofovir[7]. For example, cumulative entecavir therapy (for at least 3 years) resulted in substantial histological improvement and regression of fibrosis or cirrhosis[8].

The efficacy of therapy is currently evaluated by frequent monitoring of "viral load" or "liver injury"[5]. From the viewpoint of developing preventive strategies for HCC, the risk of HCC development should also be estimated along with them. For this purpose, liver biopsy is generally considered as the gold standard in which fibrosis is subclassified into 5 stages of severity (F0–4). However, this procedure is invasive and shown to cause a high rate of sampling error (about 15% false-negatives for cirrhosis) in patients with diffuse parenchymal liver diseases.

Furthermore, in a retrospective cohort study[9], the rate of fibrosis progression was estimated at about −0.28 stages/year in patients with SVR and 0.02 stages/year in patients with nonsustained virological response (NVR). This indicates that the biopsy is not suitable for evaluating the effect of therapy after a short interval. The procedure has further disadvantages such as inaccuracy, biopsy-related complications, the need for hospitalization, the time involved, and low cost-effectiveness[10]. Therefore, alternative noninvasive assays are desired and should provide a quantifiable readout of fibrosis progression using a method that is accurate, cost-effective and relatively simple.

To date, several methods have been developed[10] including FibroScan, which measures hepatic fibrosis biomechanically as tissue stiffness based on transient elastography. FibroScan has the advantages of being rapid and technically simple; however, its diagnostic success rate is affected by operator skill. Therefore, it has been suggested that FibroScan, in conjunction with assay of serum fibrosis biomarkers, should improve diagnostic accuracy. FibroTest[11] and FibroMeter[12], believed to be the most reliable indices of fibrosis, have been used in the combination assay aiming to eliminate the need for liver biopsy[13,14]. However, FibroTest and FibroMeter do not complement FibroScan in the development of a rapid "on-site diagnosis" system. This is because each requires both extensive and specialized blood analyses (FibroTest requires α2-macroglobulin, apolipoprotein A1, haptoglobin, γ-glutamyltransferase and total bilirubin whereas FibroMeter requires platelet count, prothrombin index, AST, α2-macroglobulin, hyaluronic acid and urea). In addition, both tests require data on age, and also sex for FibroTest.

Glycans are referred to as the face of cells, which reflect their status such as differentiation stage rather than their state of damage, and therefore they can be great markers for chronic disease. In the case of hepatitis, glycans are considered to reflect more specifically the progression of fibrosis than viral load. In the search for a simple and rapid method that is not markedly affected by tissue inflammation and ALT fluctuation, the possibility of glycomic and glycoproteomic techniques has emerged[15,16], and there are reports of some successful examples applicable for use in the clinical laboratories[17–19]. However, the current glycomic techniques require at least 3 hours of sample preparation for analysis and this has markedly reduced the combination use of glycan-based immunoassays with FibroScan. In this report, we describe for the first time, a rapid and simple glycan-based immunoassay, FastLec-Hepa, that can quantify fibrosis as precisely as FibroTest and also readily evaluate the antifibrotic effects of therapy at the clinical site (**Supplementary Fig. 1**). Moreover, we introduce a novel method for rational selection of the "non-fucose binding type" lectins and provide details of how this concept can be adopted for future development of clinically useful glycodiagnostic tools.

Results

Changes in the *N*-glycosylation of M2BP during progression of liver disease. Based on previous reports[20–23], we adopted the serum 90 K/Mac-2 binding protein (M2BP) as a glycoprotein biomarker for liver fibrosis. M2BP is secreted from many cell types, including hepatocytes (http://www.proteinatlas.org/ENSG00000108679), and it has been shown to modulate many processes, particularly those related to cell adhesion. For example, the interaction of M2BP with matrix fibronectin can modulate adhesion and the high expression of M2BP by tumor cells increases the level in the circulation of affected patients. A prominent feature of native human M2BP is its oligomerization to large ring structures[20], resembling a "sugar-powdered doughnut" which is potentially covered with 70–112 *N*-glycans (**Fig. 1a**). To confirm serum M2BP as a valid marker, we performed a pull-down assay with serum (2 μl each) from five individuals in each of the following groups: HCC, LC, CHC or healthy volunteer with normal liver (HV). Although two bands appeared in all HVs and two CHC patients, M2BPs from patients with relatively severe fibrosis, i.e., LC and HCC, migrated as a single band, the mobility of which was similar to that of the lower band for HVs (**Fig. 1b**). Significant increases in band intensity with excessive smearing of the bands were seen for most HCC patients. A subsequent investigation of 125 HCV patients with stage-determined fibrosis showed alteration in the quality and quantity of M2BP during the progression of fibrosis (**Fig. 1c**) and apparent alteration in the amount of each band (**Fig. 1d and e**), as described in the previous investigations[22,23]. M2BP has been shown to have multibranching and sialylated *N*-glycans. Moreover, it has been suggested that extension of polylactosamine on M2BP controls its binding to galectin-3, a major binding partner *in vivo*. Sialylation and extension of polylactosamine affect the charge and size of M2BP and this results in altered electrophoretic migration. Accordingly, we speculate that the size heterogeneity of M2BP seen on electrophoresis is due to such alterations in glycosylation. In fact, the difference in the band migration was eliminated by Sialidase A treatment, and the smearing of the bands in HCC was reduced by treatment with *N*-Glycosidase F (**Supplementary Fig. 2**). These results indicated that the altered quality of M2BP during progression of liver disease was due to changes in *N*-glycosylation.

Selection of the optimal lectin for direct measurement of disease-related M2BP. To construct a reliable assay (see **Supplementary Fig. 3**), we needed to identify a lectin probe that could most readily discriminate the altered *N*-glycans of M2BP and specifically binds to them in serum without pretreatment. For this purpose, we added a subtraction process to our recently described microarray-based selection strategy[16] (**Supplementary Fig. 4**). In brief, we first obtained a typical glycan profile for serum M2BPs by averaging the glycan profiles of M2BPs immunoprecipitated from 125 HCV patient sera by the antibody-overlay lectin microarray[16,18,24] (**step 1**). In this step, we selected 27 lectins binding to M2BP from a 45-lectin array (**Supplementary Fig. 5a**). Most of them bound not only to M2BP (ca. 10 μg/ml in serum), but also to other abundant serum glycoproteins, whereas some suggested rather specific binding to M2BP. We designated them as high-noise lectins or high signal-to-noise (S/N) lectins, respectively (**Fig. 2a**). We then selected the candidate lectins for the assay by subtracting the high-noise lectins from the M2BP-binding lectins (**step 2**), using a glycan profile of whole serum (**Supplementary Fig. 5**)[25]. Comparing the profiles for M2BP and whole serum (**Fig. 2b**), we quickly identified 6 lectins with a high S/N ratio. Interestingly, all lectins identifying fucose modification, which is the most well-known glyco-alteration in liver disease (*Pisum sativum* agglutinin (PSA), *Lens culinaris* agglutinin (LCA), *Aspergillus oryzae* lectin (AOL), and *Aleuria aurantia* lectin (AAL)), were high-noise lectins (**Fig. 2b**). After subtraction, we used both the Mann–Whitney *U* test as a nonparametric test, and receiver-operating characteristic (ROC) analysis, to characterize the diagnostic accuracy of the candidate lectins at each stage of fibrosis: significant fibrosis (F2/F3/F4), severe fibrosis (F3/F4) and cirrhosis (F4) (**step 3**). As a result, we found that the diagnostic score of *Wisteria floribunda* agglutinin (WFA) was superior to the other 5 lectins at every fibrosis stage (**Fig. 2c and Supplementary Fig. 6**).

"Proof-of-concept" experiment for direct quantitation of the serum WFA-binding M2BP by sandwich immunoassay. We quantitatively analyzed the WFA-binding M2BPs (WFA^+-M2BP) in serum. Sera, pretreated as described in the **Methods**, were firstly subjected to affinity capture with 20 μl slurry of WFA-coated agarose gel. The eluted fraction was immunoprecipitated with a capturing antibody against M2BP and the product was analyzed by Western blot. The intensity of the "smearing-band" signal for WFA^+-M2BP gradually increased in proportion to the severity of liver fibrosis (**Supplementary Fig. 7**), as indicated by the red line shown in

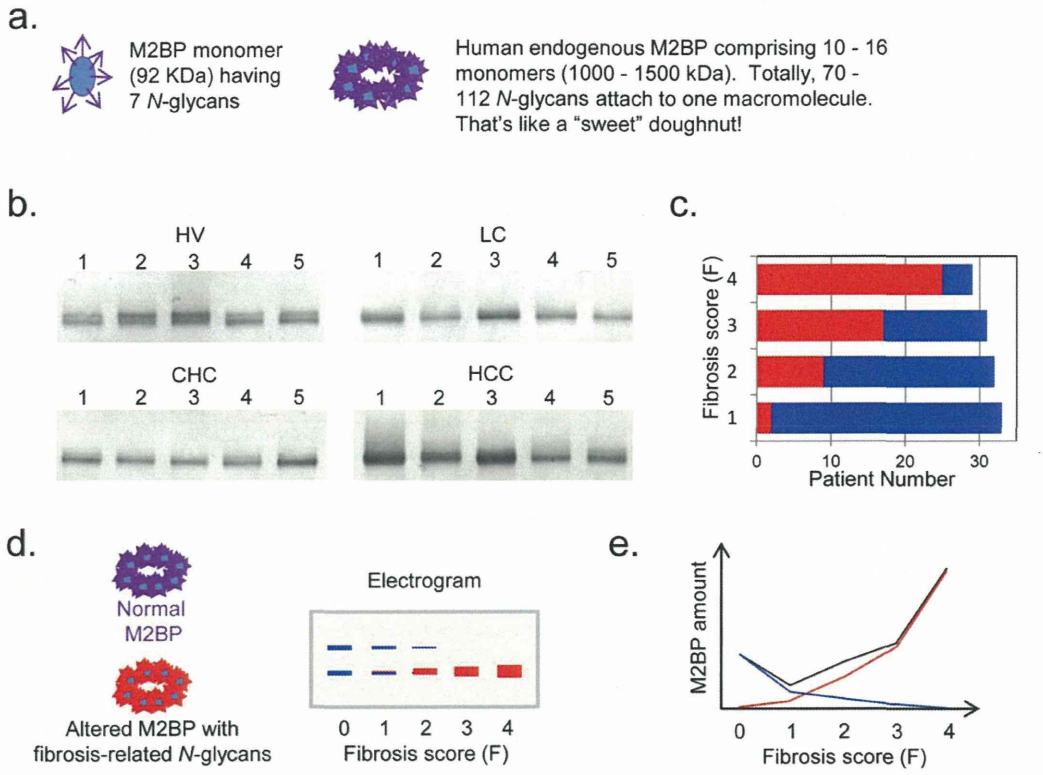
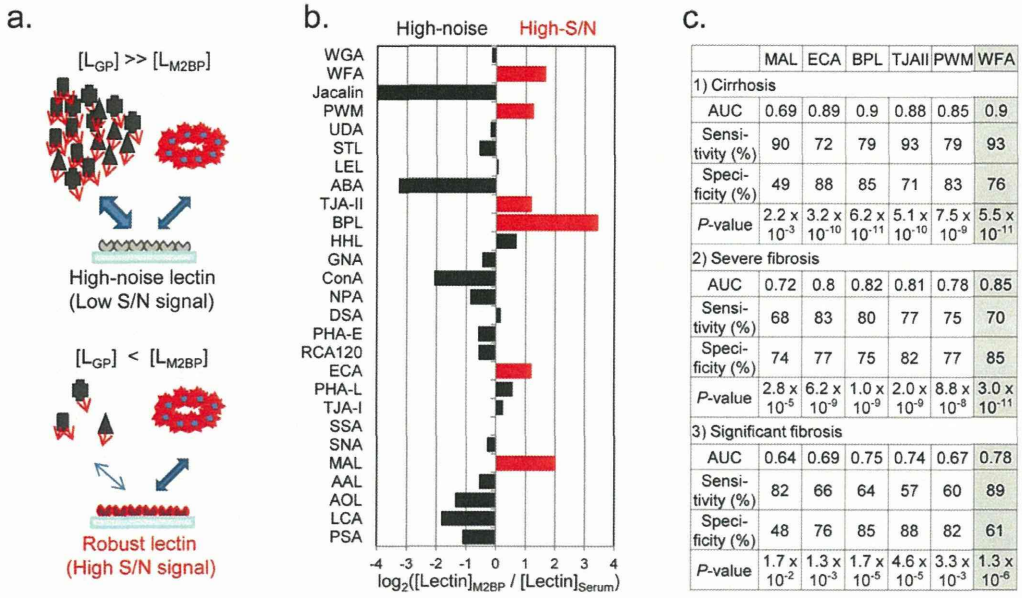


Figure 1 | Changes in the quality and quantity of human serum M2BP with progression of liver fibrosis. (a) The unique shape of human endogenous serum M2BP. The arrowheads and circles represent the *N*-glycan moieties and core protein respectively. (b) Western blot analysis: M2BPs in 2 μl of serum were purified by immunoprecipitation before SDS-PAGE. HV, healthy volunteer; CHC, patient with chronic hepatitis C; LC, HCV-infected patient with liver cirrhosis; and HCC, HCV-infected patient with hepatocellular carcinoma. (c) Number of patients with single (red) or double (blue) band appearance on the blot. The number of bands was determined visually by two independent analysts. The total number of HCV patients who participated in this study was 125 (F0–F1 [$n = 33$], F2 [$n = 32$], F3 [$n = 31$], and F4 [$n = 29$]). (d) Typical changes of serum M2BP band intensities in patients with different fibrosis scores and (e) concentrations based on a previous report on quantitation of serum M2BP by Cheung *et al*[3]. and our present results. The blue bands on the electrogram and blue line on the graph represent M2BPs secreted from normal liver. The red bands and line represent altered M2BP, the concentration of which is suggested to increase with the progression of fibrosis. The black line represents the total concentration of serum M2BP.

Fig. 1e. We next conducted a sandwich immunoassay with WFA and anti-M2BP antibody (see **Supplementary Fig. 3b**). WFA was immobilized on the surface of a 96-well microtiter plate through biotin–streptavidin interaction. We performed the first assay for the WFA-binding activity using recombinant human M2BP (rhM2BP). As a result, a linear regression analysis revealed a linear range of detection from 0.039 to 0.625 μg/ml (**Supplementary Fig. 8a**). Subsequently, we used culture supernatant of a hepatoblastoma cell line HepG2, which expresses WFA^+-M2BP, to illustrate the dose-dependency of the interaction of WFA with M2BP/HepG2. We also showed that heat treatment of the culture supernatant eliminated this binding activity (**Supplementary Fig. 8b**). Finally, we performed a sandwich immunoassay for direct measurement of WFA^+-M2BP in untreated serum samples, and the results correlated well with the quantitative assay using affinity capture and lectin microarray analysis (**Supplementary Fig. 7 and 9**).

FastLec-Hepa: a fully automated sandwich immunoassay for direct quantitation of serum WFA^+-M2BP. We adapted the WFA-antibody immunoassay to the HISCL-2000i bedside clinical chemistry analyzer[18]. We successfully adjusted every reaction condition during the automatic assay by HISCL, which is about a 17-min manipulation. Heat pretreatment of the serum was avoided to ensure both binding avidity and the fast association rate. Repeatability was assessed by performing 10 independent assays of three samples, and the coefficient of variation ranged between 2.1% and 2.5% (data not shown). Sensitivity was determined by triplicate assays of samples generated by 2-fold serial dilution of 50 μg/ml rhM2BP. The linear regression analysis identified a linear range of detection ($R^2 = 1.00$) from 0.025 to 12.5 μg/ml (**Fig. 3a**, a range of 0.025 to 1.6 μg/ml also shown in **Fig. 3b**). The resulting dynamic range was 25-fold that of the manual sandwich immunoassay described above. We next examined whether the HISCL measurements made on serum from HCV patients ($n = 125$) were consistent with lectin microarray analysis, and this comparison resulted in sufficient linearity with coefficient of determination, $R^2 = 0.848$ (**Fig. 3c**). Accordingly, we could perform automatic quantitation of serum WFA^+-M2BP in 180 patients in 1 hour and we have therefore named it FastLec-Hepa.

Validation of FastLec-Hepa. For a validation study, we obtained serum from CH patients at two locations: Nagoya City University Hospital and Hokkaido University Hospital (**Supplementary Fig. 10**). Staging of these patients ($n = 209$) by histological activity index (HAI) was conducted independently by two senior pathologists on ultrasonography-guided liver biopsy samples. F0–F1 was assigned in 82 cases (39.2%), F2 in 52 (24.9%), F3 in 40 (19.1%), and F4 (cirrhosis) in 35 (16.7%). Serum from healthy volunteers (with no history of any hepatitis virus infections) was obtained for analysis from two sites ($n = 48$ from National Institute of Advanced Industrial Science and Technology [AIST]: HV1; $n = 70$ from Nagoya City University: HV2). Their FastLec-Hepa counts



c.

	MAL	ECA	BPL	TJAII	PWM	WFA
1) Cirrhosis						
AUC	0.69	0.89	0.9	0.88	0.85	0.9
Sensitivity (%)	90	72	79	93	79	93
Specificity (%)	49	88	85	71	83	76
P-value	2.2×10^{-3}	3.2×10^{-10}	6.2×10^{-11}	5.1×10^{-10}	7.5×10^{-9}	5.5×10^{-11}
2) Severe fibrosis						
AUC	0.72	0.8	0.82	0.81	0.78	0.85
Sensitivity (%)	68	83	80	77	75	70
Specificity (%)	74	77	75	82	77	85
P-value	2.8×10^{-5}	6.2×10^{-9}	1.0×10^{-9}	2.0×10^{-9}	8.8×10^{-8}	3.0×10^{-11}
3) Significant fibrosis						
AUC	0.64	0.69	0.75	0.74	0.67	0.78
Sensitivity (%)	82	66	64	57	60	89
Specificity (%)	48	76	85	88	82	61
P-value	1.7×10^{-2}	1.3×10^{-3}	1.7×10^{-5}	4.6×10^{-5}	3.3×10^{-3}	1.3×10^{-6}

Figure 2 | Selection of the optimal lectin for the lectin-antibody sandwich immunoassay. (a) The kinetics of lectins binding to serum glycoproteins. The M2BP-binding lectins are divided into two categories: high-noise lectins and high signal-to-noise (S/N) lectins. The high-noise lectins bind to both M2BPs and abundant serum glycoproteins, causing a strong suppression of the M2BP–lectin interaction (see *top panel*). On the other hand, the number of binding targets in serum for the high S/N lectins is negligible, resulting in the specific interaction with the target M2BP (see *lower panel*). (b) Classification of M2BP-binding lectins. The high S/N lectins are those detecting M2BPs with at least twice the signal intensity seen for other serum glycoproteins. The classification strategy is summarized in **Supplementary Fig. 4**. (c) Diagnostic performance of 6 candidate lectins. *P*-values were determined using the nonparametric Mann–Whitney *U* test (Excel 2007, Microsoft).

(**Supplementary Table 1**) are also plotted in a box-whisker diagram in **Supplementary Fig. 11** along with that from a separate group of 1,000 healthy volunteers (HV3). Based on the calibration curve ([FastLec-Hepa counts]/10^6 = 1.027 × [rhM2BP] + 0.006 in **Fig. 3a, b**), the 75th percentiles of HVs of 64,205–107,617 and the 25th percentile of LC of 1,327,596 patients (see also **Supplementary Fig. 11**), we estimate the concentration of WFA$^+$-M2BP to be approximately 0.09 μg/ml in the serum of HV patients and > 1.0 μg/ml in that of LC patients. This means that the linear range shown in **Fig. 3a** is sufficient for accurate quantitation of WFA$^+$-M2BP in all serum samples. The analyses showed a gradual increase with the progression of liver fibrosis, but it did not correlate with the grade of hepatic activity defined by HAI scoring (**Supplementary Fig. 12**).

Next, we made a statistical comparison of FastLec-Hepa with other simple tests for liver fibrosis: the direct fibrosis marker hyaluronic acid (HA), the indirect fibrosis index FIB-4[26] and the glycan-based fibrosis index LecT-Hepa[18,27]. We enrolled 160 patients (F0−F1 = 66, F2 = 41, F3 = 33 and F4 = 20) whose age, platelet count, AST, ALT and HA levels were readily available (**Supplementary Fig. 10** and **Supplementary Tables 1 and 2**). As shown in **Fig. 4a**, the results of all the tests correlated well with the stage of fibrosis ($P < 0.0001$). However, an ROC analysis concluded that FastLec-Hepa detected cirrhosis with the highest diagnostic accuracy (**Fig. 4b** and **Table 1**). Notably, FastLec-Hepa distinguished between F3 and F4 with 90% sensitivity, 85% specificity, and with an AUC of 0.91. These results were superior to LecT-Hepa (sensitivity:

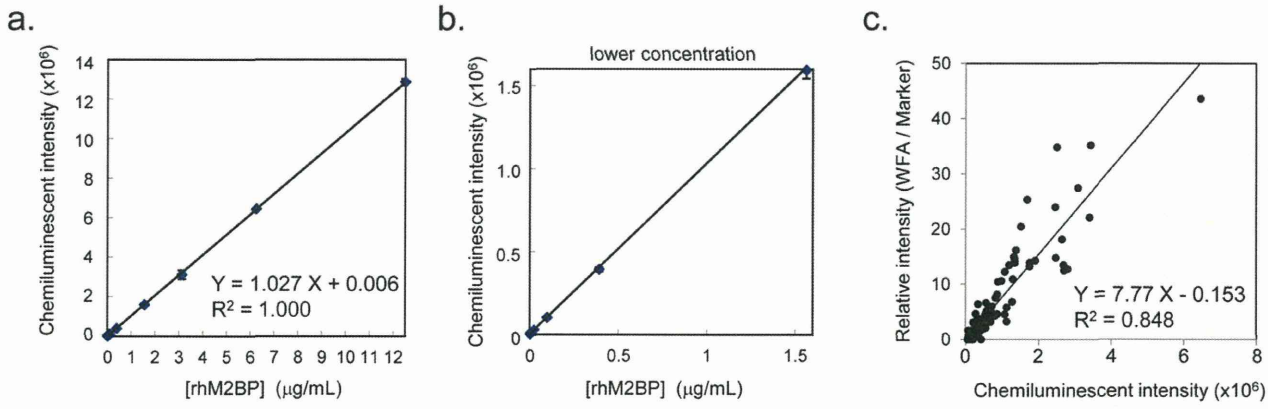


Figure 3 | Description of FastLec-Hepa, a fully automated WFA and anti-M2BP antibody sandwich immunoassay. (a) Standard curve for quantitation of WFA-binding rhM2BP. Plots for the lower concentration of rhM2BP are alternatively highlighted in (b). (c) Scatterplot comparison of WFA$^+$-M2BP data obtained from 125 different serum samples by both HISCL and a manual lectin microarray assay. The best-fit linear comparison with its correlation coefficient was calculated in Excel 2007 (Microsoft).

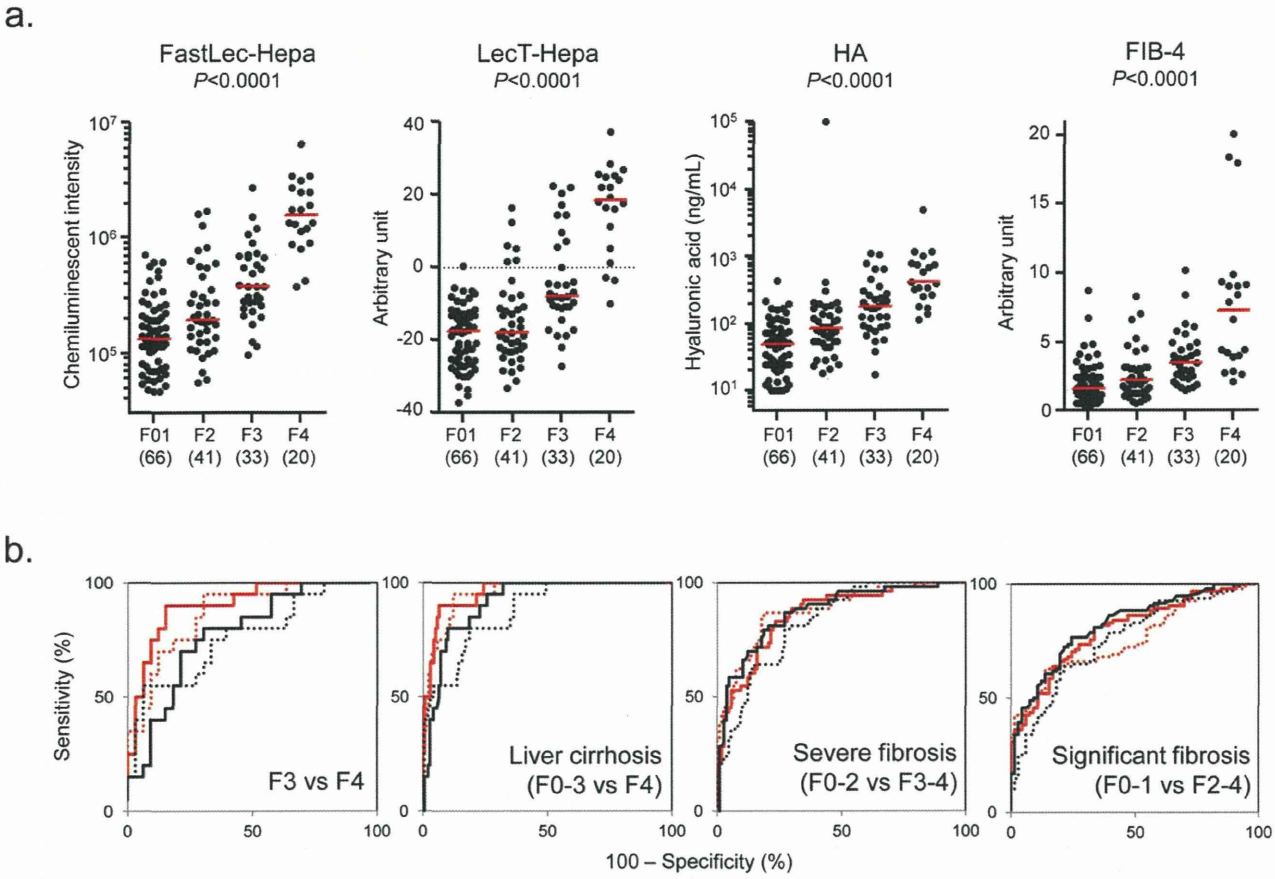


Figure 4 | Comparison of diagnostic performance of FastLec-Hepa, LecT-Hepa, HA, and FIB-4. (a) Scatterplots of the data obtained with FastLec-Hepa, LecT-Hepa, HA, and FIB-4 against the fibrosis score. Red horizontal lines represent the median. Correlation of the data with the progression of fibrosis was evaluated as significant differences in the medians relative to the fibrosis scores ($P < 0.0001$) by a nonparametric method, the Kruskal–Wallis one-way ANOVA. (b) Area under the receiver-operating characteristic (AUC-ROCs) curves of FastLec-Hepa, LecT-Hepa, HA, and FIB-4 for liver cirrhosis (F3 vs F4 or F0–3 vs F4), severe fibrosis (F0–2 vs F3–4), and significant fibrosis (F0–1 vs F2–4). FastLec-Hepa, LecT-Hepa, HA, and FIB-4 are indicated by a red solid line, red dotted line, black solid line, and black dotted line, respectively.

95%, specificity: 70%, and AUC: 0.87), FIB-4 (sensitivity: 55%, specificity: 94%, and AUC: 0.76), and HA (sensitivity: 80%, specificity: 70%, and AUC: 0.78).

Clinical utility of FastLec-Hepa: quantitative monitoring of antiviral therapy. To assess clinical utility, we examined two types of trials—short-interval evaluation and long-term follow-up—both of which are essential for following the patients receiving PEG-interferon-α and ribavirin therapy. For the first trial, we enrolled 41 patients with CHC who had previously undergone 48 weeks of therapy at Hokkaido University Hospital. According to the definition described in the **Methods**, 26 and 15 of them were judged as SVR and NVR/relapse (non-SVR), respectively. For each patient, we performed FastLec-Hepa on serum samples, which were collected just before treatment (Pre) and within a short period (12–22 weeks) after treatment (Post) (**Fig. 5a**). We found a marked decrease from Pre to Post counts ($P = 0.0061$) in SVR patients, but no apparent change for non-SVR patients ($P = 0.9780$) (**Fig. 5b**). Specifically, a median percent decrease of 31% was found for SVR patients (median Pre-count of 161,053 and median Post-count of 110,739), while the level for non-SVR patients was essentially constant. These results show that the assay can evaluate the effect of therapy within a short period after treatment. This is an important advance, because the ALT levels of non-SVR, as well as SVR, are mostly decreased into the range of 10–64 IU/ml during this period (**Fig. 5c**)[5]. In fact, changes in the FastLec-Hepa counts did not correlate with those in the ALT counts (**Supplementary Fig. 13**), thereby invalidating ALT-dependent fibrosis assays, including FIB-4 (**Fig. 5d**).

In support of our finding that the FastLec-Hepa counts correlate excellently with the stage of fibrosis, we found a strong correlation between the histopathological scores and the median of the $\log_{10}$[FastLec-Hepa] counts (**Fig. 5e**). These correlations were approximated to two linear equations: $y = 0.23x + 4.9$ for F0 to F3, and $y = 0.58x + 3.8$ for F3 to F4 histology. This means that FastLec-Hepa can reliably reproduce the assessment of therapeutic effects, which were previously drawn from histopathological scoring[9]. Indeed, the median changes in fibrosis obtained by FastLec-Hepa analysis were about −0.295 stages/year for SVR and 0.010 stages/year for non-SVR (**Fig. 5f**). These data were consistent with the rate of fibrosis progression and regression determined by Shiratori *et al*[9].

For the second trial, we enrolled 6 HCV patients (SVR = 3 and non-SVR = 3) with advanced fibrosis who completed 48 weeks of therapy at Nagoya City University Hospital. Sera were collected before therapy and at 0, 1, 3, and 5 years after the end of therapy (see **Fig. 5g**). FastLec-Hepa counts in SVR patients gradually decreased and reached below the median of F0 patients within 3 years. However, those in non-SVR patients remained above the median for F3 patients during the follow-up period (**Fig. 5h**).

Table 1 | Diagnostic performance of fibrosis markers

n = 160	FIB-4	HA	LecT-Hepa	FastLec-Hepa
a) Significant fibrosis (F0–1 vs F2–4)				
AUC	0.76	0.82	0.76	0.79
(95% CI)	(0.68–0.83)	(0.76–0.89)	(0.69–0.83)	(0.72–0.86)
Diagnostic sensitivity (%)	64	77	63	81
Diagnostic specificity (%)	79	76	86	67
Youden's index (%)	43	52	49	48
b) Severe fibrosis (F0–2 vs F3–4)				
AUC	0.83	0.87	0.88	0.84
(95% CI)	(0.76–0.89)	(0.81–0.93)	(0.82–0.93)	(0.77–0.91)
Diagnostic sensitivity (%)	81	81	87	83
Diagnostic specificity (%)	71	79	81	77
Youden's index (%)	52	61	68	60
c) Liver cirrhosis (F0–3 vs F4)				
AUC	0.88	0.91	0.95	0.96
(95% CI)	(0.80–0.95)	(0.86–0.96)	(0.92–0.99)	(0.93–0.99)
Diagnostic sensitivity (%)	80	80	95	90
Diagnostic specificity (%)	81	90	88	94
Youden's index (%)	61	70	83	84
d) Liver cirrhosis (F3 vs F4)				
AUC	0.76	0.78	0.87	0.91
(95% CI)	(0.63–0.90)	(0.65–0.90)	(0.77–0.97)	(0.82–0.99)
Diagnostic sensitivity (%)	55	80	95	90
Diagnostic specificity (%)	94	70	70	85
Youden's index (%)	49	50	65	75

Interestingly, HCC had developed in two non-SVR patients whose FastLec-Hepa counts remained above the median of F4 patients throughout. Other fibrosis indices, such as FIB-4 and biochemical parameters (ALT and AST), did not distinguish between SVR and non-SVR or appear to predict this occurrence (**Fig. 5i-k**).

Discussion

We have described the development and use of a fully automated, glycan-based immunoassay termed FastLec-Hepa, for the evaluation of liver fibrosis. A high degree of reliability in the quantitative aspects of this method should establish it as a clinically significant test,

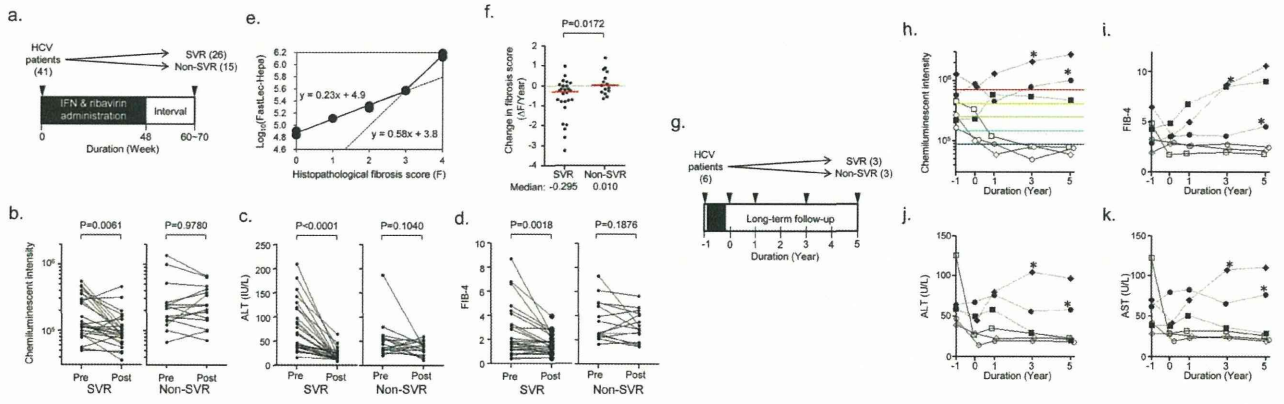


Figure 5 | Evaluation of the curative effect of interferon therapy by FastLec-Hepa. (a) Validation of FastLec-Hepa in short-interval evaluation. The numbers in parentheses represent the number of patients participated in this experiment. Arrowheads indicate the timing of blood collection. At week 0, blood was collected immediately before the treatment. Black box indicates the period of PEG-interferon-α and ribavirin therapy. Changes in the FastLec-Hepa counts (b), ALT (c), and the FIB-4 index (d) in patients with sustained virologic response (SVR) and relapse/nonresponders (non-SVR) during interferon therapy. The *P*-value was determined by a nonparametric method, the Wilcoxon matched pairs signed-rank test. (e) Dot-plot representation of the histopathological fibrosis score and the medians of FastLec-Hepa counts. Best-fit linear curves were calculated in Excel 2007 (Microsoft) allowing conversion of the FastLec-Hepa counts into fibrosis score. (f) Yearly changes in the converted fibrosis score. Changes for patients with SVR and non-SVR are indicated in the dot plots. Red horizontal lines represent the median. The *P*-value was determined by the Mann–Whitney *U* test. (g) Validation of FastLec-Hepa in long-term follow-up. The numbers in parentheses represent the number of patients participated in this experiment. Arrowheads indicate the timing of blood collection. At year -1 and 0, the blood was collected immediately before and after the treatment, respectively. Black box indicates the period of PEG-interferon-α and ribavirin therapy. Yearly changes of FastLec-Hepa counts (h), FIB-4 index (i), ALT (j), and AST (k) in individual patients after therapy. The five colored lines in (h) represent the median values obtained for each fibrosis stage (red, F4; orange, F3; green, F2; cyan, F1; blue, F0). Closed and opened symbols indicate the data obtained from non-SVR and SVR patients, respectively. * indicates the period when the development of HCC was found.

particularly for revealing and managing patients at a high risk of progression to liver complications such as HCC and related life-threatening events. The most striking advantage of FastLec-Hepa is not only its simplicity but also its capacity to provide fibrosis read-outs that are not influenced by fluctuations in the ALT value or inflammation, both of which can cause falsely high estimates in most of the other fibrosis tests available[10]. In fact, our study has illustrated robust capacity of FastLec-Hepa to evaluate the effects of antiviral therapy and subsequent disease progression in both the short and long term.

Many retrospective and prospective studies have demonstrated that achieving SVR through the PEG-interferon-α/ribavirin treatment significantly reduces liver-related morbidity and mortality (i.e., hepatic decompensation, HCC, and liver-related death)[28–30]. As this combination therapy is effective in only about 50% of patients with HCV genotype 1, new agents[1] and targets[31] for antiviral treatments of HCV have been developed to achieve SVR more effectively after the therapy. Long-term follow-ups often show that the risk of disease progression is significantly high in patients with non-SVR after PEG-interferon-α/ribavirin treatment. Furthermore, the development of HCC in patients with SVR remains at a significant cumulative rate (2%)[28,30,32,33]. For these reasons, a new data-mining model using individual factors (age, platelet count, serum albumin and AST) was developed recently to identify patients at a high risk of HCC development[34]. This is, however, a statistical procedure for estimating the chance of disease progression, and there is not a direct evaluation of fibrosis. In the present report, we performed a long-term retrospective study with serially collected sera from SVR and non-SVR patients, in which we showed the potential use of FastLec-Hepa for improved prognostic accuracy. Indeed, recent advances in the development of antifibrotic agents lead us to expect the therapeutic elimination of health risks associated with HCC and decompensation[35]. Moreover, we expect that FastLec-Hepa will be proved for its usefulness in rapid evaluation of progression and regression of fibrosis in clinical trials of newly developed antifibrotic agents. Hence, FastLec-Hepa should be very useful for fibrosis stage screening and evaluation of disease progression in untreated individuals or patients under or after treatment, as well as evaluation of the most recently developed drugs.

It is important to note that FastLec-Hepa has many merits, including speed (possibly 1,000 assays per day) and full automation for measurement of a serological glycobiomarker: these attributes will enable retrospective studies with valuable serum specimens that have been collected previously. In addition, our recently developed calibrator for FastLec-Hepa will improve traceability and enable simultaneous assay and data storage in multiple diagnostic facilities. The data obtained with diluted serum samples demonstrated a high level of assay reproducibility and a very favorable linear detection range (**Supplementary Fig. 14**). Furthermore, we found an excellent agreement between assay values for serum and plasma prepared simultaneously from the same patient. Presently, we have about 10,000 sera and plasma available with detailed clinical notes collected in more than 10 facilities in Japan, and a series of retrospective studies is under way. We will shortly conclude licensing of our system for clinical implementation, based largely on the trials of the present study. In contrast to this, the majority of recent noninvasive techniques are currently shifting to physical measurements such as FibroScan, acoustic radiation force impulse[36] and real-time strain elastography[37]. Any on-site assay of large numbers of blood samples should provide a diagnostic value comparable to that of FibroTest, and a direct comparison in the same patient group will be necessary to evaluate this. We note here that according to a recent statistical validation method[38], predicted AUC of the diagnostic value of FibroTest for detection of advanced fibrosis in our sample set (DANA score = 1.81) was approximately 0.77, which was comparable to the AUC of FastLec-Hepa we obtained (0.79).

FastLec-Hepa has adopted a new paradigm for clinical diagnosis, "glyco-diagnosis", which is based on the quantity and quality of protein glycosylation patterns that well indicate disease progression. To detect such changes in glycosylation by conventional methods (e.g., mass spectrometry, liquid chromatography, or capillary electrophoresis), it is absolutely necessary to liberate the glycans of interest from their protein linkages[15,17,39]. It is possible to employ an alternative technology, which is based on a lectin–antibody sandwich immunodetection system for intact glycoproteins bearing disease-specific glyco-alterations. Such assays have been used to detect changes in fucosylation of *N*-linked glycans, which are associated with liver disease. However, in the present study, fucose-binding lectins were classified as "high noise" (**Fig. 2b**), and thus an enrichment of the target protein was the essential process in the assay. Lectin-overlay detection is performed typically after on-plate enrichment of the target glycoproteins by an immobilized antibody. In such cases, detection relies on a low avidity (high dissociation rate) between the captured glycoprotein and the overlaid lectin probe (see *right* of **Supplementary Fig. 3b**). These kinetic considerations essentially eliminate the use of an automated bedside clinical chemistry analyzer. Even though a fucose-binding lectin was immobilized on the beads (see *left* of **Supplementary Fig. 3b**), it still remains a problem for reliable quantitation by autoanalyzer. Our previous system LecT-Hepa[16,18,19,26], which detects the level of fucosylated α1-acid glycoprotein, requires enrichment of the protein prior to the assay.

In the present study, we have developed a strategy to overcome these problems in glyco-diagnosis associated with clinical implementation, and realized a rapid "on-site diagnosis" system (17 min, within the minimum time required for single assay by HISCL), based on analysis of a glycomarker (**Supplementary Fig. 1**). The strategy for selecting the most robust lectin led us to WFA, and away from the use of fucose-binding lectins, for the direct measurement system (**Fig. 2**). The diagnostic utility of M2BP, a protein resembling "sweet-doughnut"[20], brought a favorable density and orientation of the disease-related glycan on the homomultimer. These characteristic structures resulted in a major increase in the avidity of M2BP for the plated WFA. The resulting glycan–lectin interaction, which is remarkably strong and specific, made it possible to develop the rapid and highly sensitive assay (see *left* of **Supplementary Fig. 3b**). We believe that this unique strategy will revolutionize the use of glyco-diagnosis in clinical medicine and potentially provide a framework for the development of a new generation of biomarker assays.

Methods

Patient samples, biochemical parameters and indices. Patients with chronic hepatitis were enrolled at Nagoya City University Hospital and Hokkaido University Hospital. Healthy volunteers as the controls were randomly selected in Nagoya City University Hospital (70 individuals) and AIST (48 individuals). The institutional ethics committees at Nagoya City University Hospital, Hokkaido University Hospital, and AIST approved this study, and informed consent for the use of their clinical specimens was obtained from all participants before the collection. In addition, we used 1,000 serum samples from virus-negative Caucasians as the normal population, which were purchased from Complex Antibodies Inc. (Fort Lauderdale, FL) and collected under IRB-approved collection protocols. Fibrosis was graded in the patients according to the histological activity index (HAI) using biopsy or surgical specimens. Biopsy specimens were classified as follows: F0, no fibrosis; F1, portal fibrosis without septa; F2, few septa; F3, numerous septa without cirrhosis; and F4, cirrhosis. The three diagnostic targets in this study were defined as significant fibrosis: F2+F3+F4; severe fibrosis: F3+F4; and cirrhosis: F4. Hepatic inflammation was also assessed according to the HAI, as follows: A0, no activity; A1, mild activity; A2, moderate activity; and A3, severe activity. Cirrhosis was confirmed by ultrasonography (coarse liver architecture, nodular liver surface, and blunt liver edges), evidence of hypersplenism (splenomegaly on ultrasonography) and/or a platelet count of $< 100{,}000/mm^3$. Virological responses during PEG-interferon-α and ribavirin therapy were defined as follows[5]: SVR, absence of HCV RNA from serum 24 weeks following discontinuation of therapy; nonresponder, failure to clear HCV RNA from serum after 24 weeks of therapy; relapse, reappearance of HCV RNA in serum after therapy was discontinued. For all patients, age and sex were recorded and serum levels of the following were analyzed: aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyltransferase (GGT), total bilirubin,

albumin, cholinesterase, total cholesterol, platelet count (PLT), hyaluronic acid (HA). The FIB-4 index was calculated as follows: [age (years) × AST (U/L)]/[platelets (10^9/L) × ALT (U/L)$^{1/2}$][26]. Fibrosis-specific glyco-alteration of α1-acid glycoprotein was determined by lectin–antibody sandwich immunoassays with a combination of three lectins (*Datura stramonium* agglutinin (DSA), *Maackia amurensis* leukoagglutinin (MAL), and *Aspergillus oryzae* lectin (AOL))[16]. All assays used an automated chemiluminescence enzyme immunoassay system (HISCL-2000i; Sysmex Co., Kobe, Japan)[18].

Enrichment of M2BP from serum. An automated protein purification system (ED-01; GP BioSciences Ltd., Yokohama, Japan) was used to immunoprecipitate M2BP from serum specimens. In brief, sera (2 μl) were diluted 10-fold with PBS/0.2%(w/v) SDS, heated at 95°C for 20 min, mixed with 10 μl of Triton X-100 in TBS (TBSTx) and injected into a 96-well SUMILON microtiter plate (Sumitomo Bakelite Co., Ltd., Tokyo, Japan). The plate and working reagents, including biotinylated anti-M2BP antibody (10 ng/μl), streptavidin-coated magnetic beads, washing buffer (1% TBSTx) and elution buffer (TBS containing 0.2% SDS), were loaded into the system. This generated 110 μl of purified M2BPs per serum sample (96 samples in 3.5 h).

Western blot analysis. Anti-human M2BP polyclonal antibody was purchased from R&D Systems, Inc. (Minneapolis, MN) and biotinylated with Biotin Labeling Kit – NH_2 (Dojindo Laboratories, Kumamoto, Japan). Purified serum M2BPs were electrophoresed under reducing conditions on 5–20% polyacrylamide gels (DRC, Tokyo, Japan) and transferred to PVDF membranes. After treatment with Block Ace® (DS Pharma Biomedical Co., Ltd., Osaka, Japan), the membranes were incubated with biotinylated anti-M2BP polyclonal antibody, and then with alkaline phosphatase-conjugated streptavidin (1/5000 diluted with TBST; ProZyme, Inc., San Leandro, CA). The membranes were incubated with Western Blue stabilized substrate for alkaline phosphatase (Promega, Madison, WI).

Lectin microarray analysis. Enriched M2BPs were analyzed with an antibody-overlay lectin microarray[24]. Purified protein (14 μl) was diluted to 60 μl with PBS containing 1%(v/v) Triton X-100 (PBSTx); this was applied to a LecChip™ (GP BioSciences Ltd.), which included three spots of 45 lectins in each of seven reaction wells. After incubation for 12 h at 20°C, 2 μl of human serum IgG (10 mg/ml) was added to the reaction solution on each chip and incubated for 30 min. The reaction solution was then discarded, and the chip was washed three times with PBSTx. Subsequently, 60 μl (200 ng) of biotinylated anti-human M2BP in PBSTx was applied to the chip, and incubated for 1 h. After three washes with PBSTx, 60 μl (400 ng) of a Cy3-labeled streptavidin (GE Healthcare, Buckinghamshire, UK) solution in PBSTx was added and incubated for 30 min. The chip was rinsed with PBSTx, scanned with an evanescent-field fluorescence scanner (GlycoStation™ Reader1200; GP BioSciences Ltd.) and analyzed with the Array Pro Analyzer software package, version 4.5 (Media Cybernetics, Inc., Bethesda, MD). The chip was scanned with the gain set to register a maximum net intensity < 40,000 for the most intense spots. The net intensity value for each spot was calculated by subtracting the background value from the signal intensity value. The relative intensity of lectin-positive samples was determined from the ratio of their fluorescence to the fluorescence of the internal-standard lectin, DSA.

Quantitation of *Wisteria floribunda* agglutinin (WFA)-binding M2BP. Serum was pretreated as described above under enrichment of M2BP from serum. Pretreated samples (50 μl) were diluted with an equal volume of starting buffer (0.1%(w/v) SDS in PBSTx), added to the WFA-coated agarose in a microtube (20 μl slurry; Vector Lab., Burlingame, UK), and incubated at 4°C for 5 h with gentle shaking. After centrifugation of the reaction solution at 2000 × *g* for 10 min, the supernatant was removed to a new microtube. The precipitate was suspended in 50 μl of the starting buffer, recentrifuged and this second supernatant combined with the first (designated as path-through fraction T). The precipitate was then washed with 200 μl of the starting buffer and the bound glycoproteins were eluted with 60 μl of 200 mM galactosamine/0.02%(w/v) SDS in PBS (designated as elution fraction E). M2BP was immunoprecipitated from fractions T and E and examined by electrophoresis under reducing conditions on 5–20% gradient SDS–polyacrylamide gels.

WFA-antibody sandwich ELISA. Flat-bottomed 96-well streptavidin-precoated microtiter plates (Nunc, Int., Tokyo, Japan) were treated with biotinylated WFA (Vector, 250 ng/well) for 1 h at room temperature. The plates were incubated with the diluted serum samples (50 μl) in PBS containing 0.1%(v/v) Tween20 (PBS-t) for 2 h at room temperature and then with 50 ng/well of the anti-human M2BP polyclonal antibody, in PBS-t for 2 h at room temperature. The plates were washed extensively and then incubated with 50 μl of horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc., Philadelphia, PA) at 1:10,000 in PBS-t for 1 h at room temperature. The substrate 3,3′,5,5′-tetramethylbenzidine (Thermo Fisher Scientific, Fremont, CA) solution (100 μl) was added to each well. The enzyme reaction was stopped by adding 100 μl of 1 M sulfuric acid, and the optical density measured at 450 nm.

WFA-antibody sandwich immunoassay by HISCL. The fibrosis-specific form of glycosylated M2BP was measured based on a sandwich immunoassay approach. Glycosylated M2BP was captured by WFA immobilized on magnetic beads, and the bound product was assayed with an anti-human M2BP monoclonal antibody linked to alkaline phosphatase (ALP-αM2BP). Two reagent packs (M2BP-WFA detection pack and a chemiluminescence substrate pack) were loaded in the HISCL. The detection pack comprised three reagents: a reaction buffer solution (R1), a WFA-coated magnetic beads solution (R2) and an ALP-αM2BP solution (R3). The chemiluminescence substrate reagent pack contained a CDP-Star substrate solution (R4) and a stopping solution (R5). Typically, serum (10 μl) was diluted to 60 μl with R1 and then mixed with R2 (30 μl). After the binding reaction, R3 (100 μl) was added to the reaction solution. The resultant conjugates were magnetically separated from unbound components, and mixed well with R4 (50 μl) and R5 (100 μl) before reading of the fluorescence. The chemiluminescent intensity was acquired within a period of 17 min in the operation described above. The reaction chamber was kept at 42°C throughout.

Statistics. Statistical analyses and graph preparation used Dr. SPSS II Windows software (SPSS Co., Tokyo, Japan), GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA), and Windows Excel 2007. This facilitated selection of the optimal lectin for analysis of fibrosis and a comparison of the diagnostic value of other serological fibrosis markers and indices. Because the data distribution for each parameter was non-Gaussian, the *P*-values were determined by nonparametric tests, such as the Mann–Whitney *U* test and Wilcoxon signed-rank test. Correlations with liver fibrosis were estimated as the significance of differences among the staging groups (F0–1, F2, F3, and F4) determined by Kruskal–Wallis nonparametric one-way analysis of variance. To assess classification efficiencies for detecting significant fibrosis, severe fibrosis and cirrhosis, the receiver-operating characteristic (ROC) curve analysis was also carried out to determine the area under the curve (AUC) values. Cutoff values obtained from Youden's index were used to classify patients. Diagnostic accuracy was expressed in terms of specificity, sensitivity and AUC.

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Author contributions:

A.K. conceived and designed the study, performed most of the biochemical experiments, analyzed data and wrote the paper with comments from Y.T., M.M. and H.N.; Y.I. conceived and designed the study, performed the sample pre-treatment for the assay, and analyzed data; Y.T., K.I., M.M., and S.H collected clinical samples, designed the validation study, and analyzed data; A.M. and S.S. performed the biochemical experiments including lectin microarray analysis and analyzed data; M.S. and M.K. performed staging of biopsy specimens by histological activity index (HAI); H.N. conceived and designed the study, and supervised all aspects of the work; and all authors discussed the results and implications, and commented on the paper.

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Association between S21 substitution in the core protein of hepatitis B virus and fulminant hepatitis

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ABSTRACT

Background: The viral factors of hepatitis B virus (HBV), such as genotypes and mutations, were reported to affect the development of fulminant hepatitis B (FHB), but the mechanism is still unclear.
Objectives: To investigate HBV mutations associated with FHB, especially in the subgenotype B1/Bj HBV (HBV/B1), which are known to cause FHB frequently in Japan.
Study design: A total of 96 serum samples from acute self-limited hepatitis B (AHB) patients and 13 samples from FHB patients were used for full-genome/partial sequencing. A total of 107 chronic infection patients with HBV were also examined for the distribution of mutants.
Results: In the analysis of full-genome sequences of HBV/B1 (FHB, $n = 11$; non-FHB, $n = 35$) including those from the databases, mutations at nt 1961 [T1961V (not T)] and nt 1962 [C1962D (not C)], which change S21 in the core protein, were found more frequently in FHB than in non-FHB (100% vs. 20%, 55% vs. 3%, respectively). When our FHB and AHB samples were compared, T1961V and C1962D were significantly more frequent in FHB than in AHB, both in the overall analysis (46% vs. 6%, 39% vs. 3%, respectively) and in HBV/B1 (100% vs. 29%, 100% vs. 14%, respectively). A newly developed PCR system detecting T1961V showed that HBV/B1 and low viral load were independent factors for the mutation among chronic infection patients.
Conclusions: T1961V/C1962D mutations were found frequently in FHB, especially in HBV/B1. The resulting S21 substitution in the core protein may play important roles in the development of FHB.

1. Background

Hepatitis B virus (HBV) infection is one of the most common viral diseases affecting humans. HBV causes a spectrum of liver diseases such as acute hepatitis, chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma.[1] Patients with acute HBV infection sometimes develop fulminant hepatitis B (FHB). The survival rate of FHB patients is still low[2] and they require careful management including liver transplantation.[3]

HBV contains a small (3.2 kb), circular, partially double-stranded DNA genome.[1] Based on the genomic variability noted among HBV isolates, HBV sequences have been classified into at least 8 genotypes (A–H), and recently, additional 2 genotypes (I and J) were proposed tentatively.[4,5] The genotypes/subgenotypes of HBV have been known to affect the disease outcome.[6–8] As for FHB, the subgenotype B1/Bj HBV (HBV/B1) was reported to be more often associated than subgenotypes B2/Ba, genotype A, and genotype

Abbreviations: AHB, acute self-limited hepatitis B; CTL, cytotoxic T lymphocyte; FHB, fulminant hepatitis B; HBV, hepatitis B virus; HBV/B1, subgenotype B1/Bj HBV; HBV/C2, subgenotype C2/Ce HBV; SSP, sequence-specific primer.

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C.[6,9] Although HBV genotypes are classified by a sequence divergence of more than 8% in the entire genome,[10] little is known about which region or nucleotide substitutions of the genome are responsible for the disease outcome differences among genotypes.

Several mutations in the precore region such as G1896A,[6,9,11–15] which makes a stop codon and abrogates HBeAg, and those in the core promoter such as double mutations of A1762T/G1764A[6,9,16] were reported to be associated with FHB. Also, T1753V [A, C or G (not T)],[14] T1754V,[14] G1899A,[9] and A2339G[9] were found in FHB patients more frequently than in AHB patients. Some of them were described as having the ability to enhance the replication capacity of HBV in vitro.[6,17–19] Recently, we reported that insertions/deletions in the precore region, which cause a frameshift of the precore protein and abrogates HBeAg, was found frequently in FHB patients with HBV/B1 and enhanced the replication capacity of HBV in vitro.[15] However, some conflicting results have been described[20,21] and it is considered that there are still unknown mechanisms in regard to the pathogenesis of FHB.

2. Objectives

Here we studied HBV mutations that were associated with FHB by analyzing full-genome sequences. Next, additional patients with acute and chronic HBV infection were analyzed for clarifying prevalence of mutations.

3. Study design

3.1. Serum samples

A total of 109 serum samples from patients with acute HBV infection including 13 FHB patients and a total of 107 serum samples from patients with chronic HBV infection were obtained from March 1998 to August 2011 in Tohoku University Hospital and other 5 hospitals in the same area. The diagnosis of acute infection was made based on the detection of high-titered immunoglobulin M anti-hepatitis core without any history of prior liver diseases. There were no acute infection patients who retained HBsAg for more than 6 months. The diagnosis of fulminant hepatitis was made based on a slightly modified definition in Inuyama Symposium (Aichi, Japan, 1981) of the original definition[22]: coma grade II or higher, and a prothrombin time of less than 40% developing within 8 weeks after the onset.

3.2. Amplification of HBV sequences and determination of genotypes

To determine the full-genome sequences of HBV, serum samples from 10 of 13 FHB patients were used. Because the sample volume of the remaining 3 samples was relatively small, they were used only for partial sequencing. Additionally, a serum sample from HBV carrier who developed FHB was studied only for full-genome comparison. The same samples that were used for full-genome sequencing in this study had been used also in a previous report to determine only partial sequences.[15] Amplification of the entire HBV genome was performed by methods essentially similar to those described previously.[23] To amplify the 548-nt sequence in the core promoter/precore/core region [nt 1719–2266 (excluding primer sequences), the nucleotide numbers are in accordance with an HBV/B1 isolate of 3215 nt (D00329)], the DNA solution was subjected to nested PCR. The first round PCR was carried out with primers B053 (Table S1) and B055, and the second round was carried out with primers B054 and B052. The amplification products were sequenced on both strands directly using the BigDye Terminator v3.1 Cycle Sequencing kit on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). The AHB/FHB samples used for partial sequencing had never used in the previous study.

For patients with chronic HBV infection, an HBsAg subtype EIA kit (Institute of Immunology, Tokyo, Japan) was used to determine the genotypes of HBV. Only genotype B samples were subjected to direct sequencing for subgenotyping.

3.3. PCR with sequence-specific primers detecting the mutation at nt 1961

To detect mutations at nt 1961 (T1961V) rapidly without the sequencing analysis, a PCR system with sequence-specific primers (SSP) was newly developed in this study. Because the sequences around nt 1961 were relatively conserved, two opposite primers, B050 and B051, whose 3′ ends are located at nt 1961 could be designed. B050 can anneal only with T1961V mutants to make 349 bp products. B051 can detect sequences without T1961V and make 208 bp products. The DNA solution was subjected to PCR with primers 8 pmol of B049, B051, and B052, and 64 pmol of B050, and AmpliTaq Gold PCR Master Mix (Applied Biosystems) in 20 μl reaction. The PCR was carried out for 40 cycles (95 °C for 15 s, 60 °C for 15 s, 72 °C for 60 s). If both 208 bp and 349 bp products were amplified, the HBV was considered to have the mix type mutations at nt 1961.

3.4. Statistical analysis

Statistical analyses were performed using Fisher's exact probability test or the chi-square test for comparison of proportions between two groups and Mann–Whitney *U* test for comparison of continuous variables between two groups. Multivariate analyses were performed with a logistic regression model using SPSS Statistics 19 (IBM, Armonk, NY).

4. Results

4.1. Full-genome comparative analysis of HBV

Of 10 full-genome sequences from FHB patients, 4 sequences were revealed to belong to HBV/B1 and 5 sequences were subgenotype C2/Ce (HBV/C2), and one sequence was subgenotype B2/Ba. A sequence from an HBV carrier who developed FHB (BFJT2009-1) was HBV/B1. A phylogenetic tree was constructed based on the full-genome sequences of genotype A-J HBV (Fig. S1) and the sequence data have been assigned to the GenBank/EMBL/DDBJ with the accession numbers AB642091–AB642101.

In August 2011, a total of 41 full-genome sequences of HBV/B1 could be retrieved from Hepatitis Virus Database[24] and GenBank/EMBL/DDBJ. Of them, 6 were obtained from FHB patients, and 35 were others including chronically infected patients. First, a consensus sequence of HBV/B1 was deduced from the 35 non-FHB strains. Then the numbers of nucleotide mutations, which were different from the consensus sequence, in all of the 3215 nt of the HBV/B1 genome were compared between FHB ($n = 11$, including 5 sequences determined in this study) and non-FHB. When the *p*-values of mutations were calculated in each nucleotide, only nt 1961 and 1962 had lower *p*-values than 1.0×10^{-3} (Fig. 1A). The full-genome sequences of HBV/C2 were also compared between FHB ($n = 15$, including 5 sequences determined in this study) and non-FHB ($n = 35$) in the same way (Fig. 1B). However, such a low *p*-value was not found in the HBV/C2 strains. Table S2 shows the nucleotides that had lower *p*-values than 1.0×10^{-2}. The partial nucleotide sequences of HBV/B1 around nt 1961/1962 are shown in Fig. 1C. All of the 11 FHB strains had the mutation at nt 1961 and 6 strains had the mutation at nt 1962. Although the mutations at nt

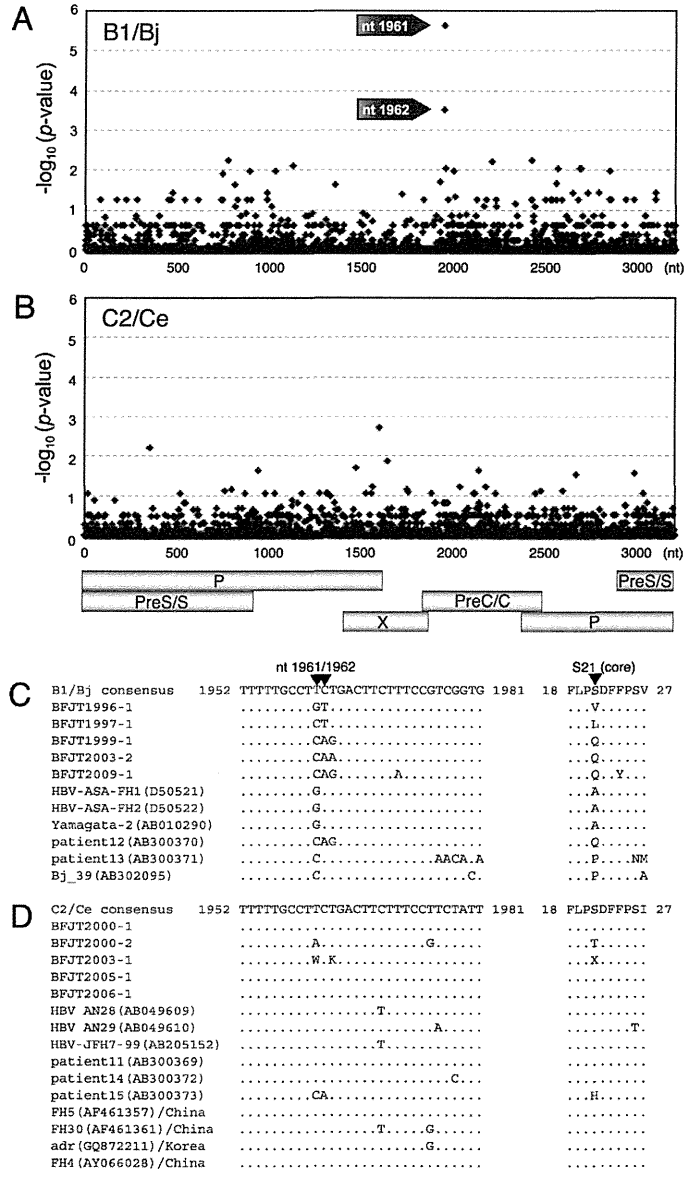

Fig. 1. Comparison of HBV full-genome sequences between FHB and non-FHB. (A) Comparison of HBV/B1 between FHB ($n=11$) and non-FHB ($n=35$). The frequencies of mutations at each nucleotide were analyzed, and the reciprocal numbers of p values were plotted. (B) Comparison of HBV/C2 between FHB ($n=15$) and non-FHB ($n=35$). (C) Partial nucleotide sequences of HBV/B1 around nt 1961/1962 and deduced amino acid sequences of FHB strains. The consensus sequences deduced from non-FHB sequences are indicated at the top. (D) Sequences of HBV/C2. W (nucleotide), T or A; X (amino acid), S or T.

1961/1962 were minor in HBV/C2 (Fig. 1D), 3/15 (20%) of FHB and 2/35 (6%) of non-FHB had the mutations. These mutations could change the amino acid of S21 in the core protein (Fig. 1C and D), which is located in an HLA-A2 restricted cytotoxic T lymphocyte (CTL) epitope of HBcAg18–27.[25]

4.2. Distribution of S21 substitution among patients with HBV acute infection

Next we compared partial sequences containing nt 1961/1962 using our acute self-limited hepatitis B (AHB, $n=96$) and FHB ($n=13$) samples. The clinical characteristics of FHB/AHB patients are shown in Table 1. Consistent with previous reports,[6,9] the HBeAg positive rate was significantly lower in FHB patients. As reported previously from Japan, HBV/C2 was most prevalent

Table 1
Clinical characteristics of patients with FHB and AHB in this study.

	FHB ($n=13$)[a]	AHB ($n=96$)[a]	p-Value
Age	45.2 ± 16.0	32.5 ± 13.1	0.004
Male	11 (84.6)	59 (61.5)	0.088
HBeAg positive	1 (7.7)	61 (67.8)	<0.0005
Peak T. Bil (mg/dl)[b]	15.1 ± 8.8	9.0 ± 9.0	0.096
Peak AST (IU/l)[b]	4663 ± 3786	1451 ± 1686	0.074
Peak ALT (IU/l)[b]	4841 ± 3850	2350 ± 1735	0.300
Lowest PT (%)[b]	19.7 ± 11.6	73.7 ± 23.8	<0.0005
HBV DNA (log copies/ml)[c]	6.7 ± 2.2	6.7 ± 1.9	0.954
HBV subgenotype			
A2/Ae	0 (0)	19 (19.8)	0.070
B1/Bj	4 (30.8)	14 (14.6)	0.141
B2/Ba	2 (15.4)	5 (5.2)	0.196
B7	0 (0)	1 (1.0)	0.238
C1/Cs	0 (0)	3 (3.1)	0.681
C2/Ce	7 (53.8)	54 (56.3)	0.680
Therapy			
Liver transplantation	3 (23.1)	0 (0)	0.001
Nucleoside analog[d]	4 (38.5)	9 (9.4)	0.048
Deceaced	6 (46.2)	0 (0)	<0.0001

T. Bil, total bilirubin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; PT, prothrombin time.

[a] Values are expressed as the mean ± standard deviation or numbers of patients (%).

[b] Data of 28 patients could not be obtained.

[c] Data of 8 patients could not be obtained.

[d] Lamivudine or entecavir was used.

(61/109, 56%). HBV/B1 was found more frequently (18/109, 17%) than the reports of all Japan (7%)[26] and was reported to be a characteristic of northeast Japan.[7,15]

Besides the mutations at nt 1961 and 1962, several mutations that were reported to be associated with FHB[6,9,11–14,16] were compared. The frameshift insertions/deletions in the precore region that we reported recently[15] were also included. In the overall analysis, the mutations of G1896A, T1961V, and C1962D [A, G or T (not C)] were found much more frequently in FHB patients than in AHB patients (62% vs. 8%, $p=3.0\times10^{-5}$; 46% vs. 6%, $p=5.4\times10^{-4}$; 39% vs. 3%, $p=5.1\times10^{-4}$, respectively) (Fig. 2A). The mutations of A1762T/T1764A and the precore frameshift were significantly more frequent in patients with FHB (48% vs. 16%, $p=0.018$; 23% vs. 1%, $p=0.0051$), as well. Notably, when HBV/B1 isolates were compared, the frequencies of only T1961V, C1962D, and the precore frameshift were significantly different (100% vs. 29%, $p=0.023$; 100% vs. 14%, $p=0.0049$; 75% vs. 7% $p=0.019$) (Fig. 2B). In the analysis of HBV/C2 isolates, G1896A (71% vs. 7%, $p=3.9\times10^{-4}$), A1762T/G1764A (71% vs. 26%, $p=0.026$), and T1753V (43% vs. 9%, $p=0.042$) were significantly more frequent in FHB patients (Fig. 2C), different from HBV/B1.

Additionally, we analyzed the T1961V frequency in combination with the major mutations G1896A and A1762T/G1764A. In the overall analysis, the frequency of isolates without these mutations was significantly higher in AHB than FHB (77% vs. 8%, $p=1.8\times10^{-6}$) (Fig. 2D). The combinations of A1762T/G1764A and G1896A, and all three mutations were found more frequently in FHB patients than in AHB patients (31% vs. 3%, $p=0.0036$; 15% vs. 1%, $p=0.037$, respectively). When analyzed separately 2 subgenotypes, the distribution of combination pattern in FHB patients was quite different (Fig. 2E and F). Whereas T1961V was present alone (50%) or with G1896A (50%) in HBV/B1, all HBV/C2 isolates with T1961V had both A1762T/G1764A and G1896A. Because the all HBV/B1 isolates only with T1961V had the precore frameshift, which abrogates HBeAg like as G1896A,[15] T1961V seemed to be related to HBeAg-defective strains.

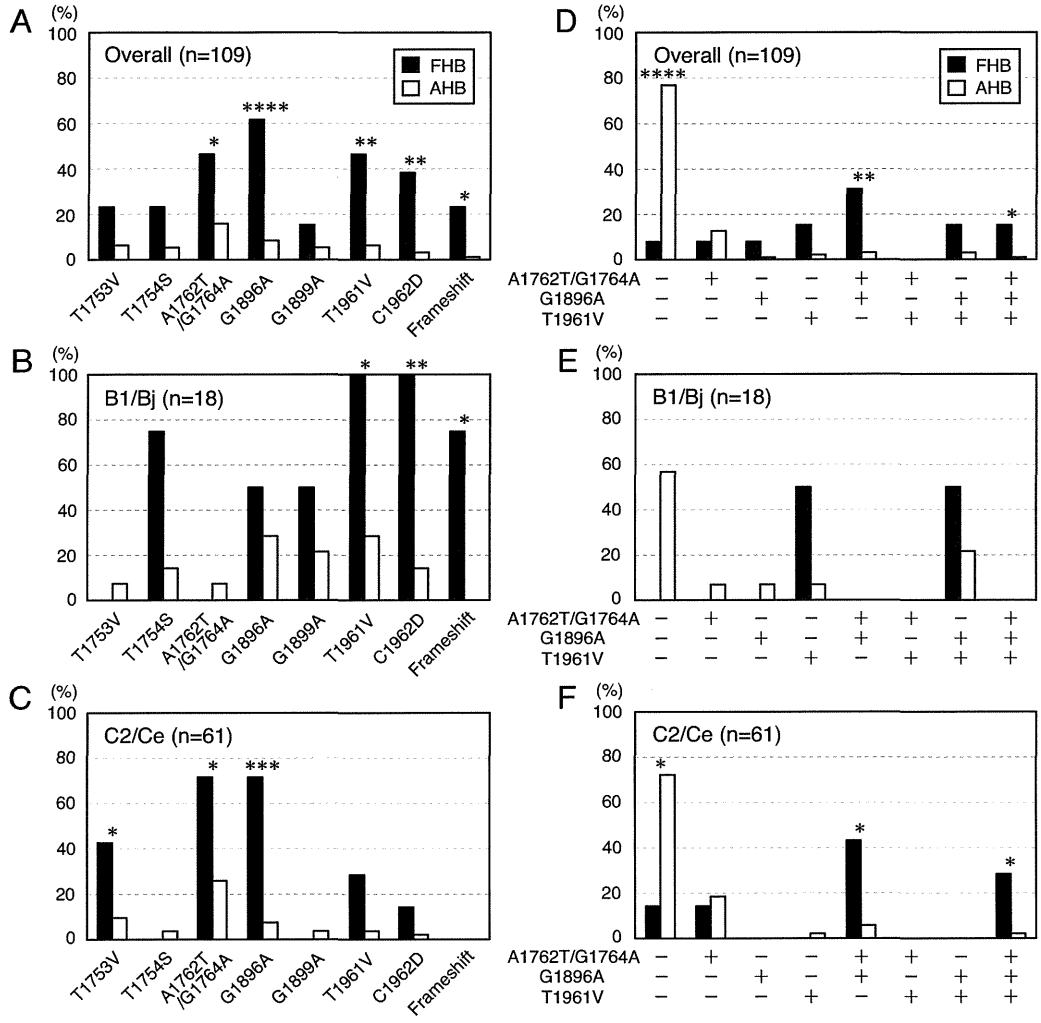

Fig. 2. Comparison of mutations between FHB and AHB in the core promoter/precore/core region. Besides T1961V and C1962D, several mutations that had been reported to be associated with FHB were also included. (A) Comparison of the overall HBV sequences between FHB ($n=13$) and AHB ($n=96$). (B) Comparison of HBV/B1 between FHB ($n=4$) and AHB ($n=14$). (C) Comparison of HBV/C2 between FHB ($n=7$) and AHB ($n=54$). D = A, G or T (not C); S = C or G; V = A, C or G (not T); *$p<0.05$; **$p<0.005$; ***$p<0.0005$; ****$p<0.00005$.

4.3. Distribution of T1961V in chronically HBV-infected patients

A PCR system with SSP was established in this study to detect T1961V more easily (Fig. 3). Using the PCR system, the frequency of T1961V was evaluated using 107 chronically HBV-infected patients [mean age, 50.6 ± 13.8; male, 73 (68.2%); HBeAg positive, 49 (45.8%); mean ALT (IU/l), 142.9 ± 177.7; mean HBV DNA (log copies/ml), 6.4 ± 1.7; liver cirrhosis, 27 (25.2%); hepatocellular carcinoma, 20 (18.7%)]. Of them, 103 (96%) could be determined to be a wild type or a mutant at nt 1961. The remaining 4 patients showed only 512 bp products. Sequencing analysis showed that HBV of the 4 patients had minor mutations within the B051-annealing site and no mutation at nt 1961. In total, 18/107 (17%) patients had T1961V mutants including the mixed type (Table 2). Of note, the frequency of T1961V including the mixed type was significantly higher in HBeAg-negative patients than in HBeAg-positive patients (29% vs. 2%, $p=1.7\times10^{-4}$). Patients with low HBV DNA levels (<6.6 log copies/ml) in the serum had T1961V mutants more frequently than those with high levels (31% vs. 4%, $p=1.8\times10^{-4}$). Although patients treated with nucleos(t)ide analogs had T1961V mutants frequently, it may be a result from the fact that HBeAg-negative patients with low HBV DNA are rarely treated with nucleos(t)ide analogs. When the HBV genotypes were compared, the T1961V mutants were detected more frequently in HBV/B1 than in the others (41% vs. 11%, $p=0.002$). HLA-A2 positivity, which was detected as described previously,[27,28] was not associated with the mutation significantly. A multivariate analysis showed that the HBV/B1 (odds ratio 7.635, $p=0.006$) and HBV DNA levels (odds ratio 0.548, $p=0.009$) were independent factors for the T1961V mutation.

5. Discussion

In this study, we found first that the S21 substitution in the core protein due to T1961V (±C1962D) was associated with FHB, especially in HBV/B1. Because the first stage analysis of HBV full-genome sequences included HBV strains from chronically infected patients in the databases, mutations that were common in chronic infection such as G1896A[29] and A1762T/G1764A[30] were not detected. In the analysis, only mutations at nt 1961 and 1962 in HBV/B1 were picked up with lower p-values than 1.0×10^{-3}, and we could confirm that these mutations were more frequent in FHB patients in the second stage analysis of our acutely infected patients. Therefore, the rapid examination of T1961V by PCR with SSP in this study was considered to be useful to predict the development of FHB in acutely infected patients.

Table 2
Distribution of the mutation at nt 1961 among HBV chronically infected patients detected by PCR with SSP.

	n	Wild type (%)	Mixed type (%)	Mutant (%)	*p*-Value[a]
Overall	107	89(83.2)	6(5.6)	12(11.2)	
Age (years)					0.245
<53	56	49(87.5)	1(1.8)	6(10.7)	
≥53	51	40(78.4)	5(9.8)	6(11.8)	
HBeAg					<0.0005
Positive	49	48(98.0)	0(0)	1(2.0)	
Negative	58	41(70.7)	6(10.3)	11(19.0)	
HBV DNA (log copies/ml)					<0.0005
<6.6	52	36(69.2)	6(11.5)	10(19.2)	
≥6.6	55	53(96.4)	0(0)	2(3.6)	
Liver cirrhosis					0.496
Present	27	22(81.5)	3(11.1)	2(7.4)	
Absent	80	67(83.8)	3(3.8)	10(12.5)	
Hepatocellular carcinoma					0.083
Present	20	14(70.0)	4(20.0)	2(10.0)	
Absent	87	75(86.2)	2(2.3)	10(11.5)	
Treatment					0.049
Nucleos(t)ide analog[b]	24	23(95.8)	0(0)	1(4.2)	
Naïve	83	66(79.5)	6(7.2)	11(13.3)	
Genotypes					
A	4	3(75.0)	1(25.0)	0(0)	
B	25	16(64.0)	4(16.0)	5(20.0)	0.006[c]
B1/Bj	22	13(59.1)	4(18.2)	5(22.7)	0.002[c]
B2/Ba	3	3(100)	0(0)	0(0)	
C	78	70(89.7)	1(1.3)	7(9.0)	
HLA-A2					0.056
Positive	32	30(93.8)	0(0)	2(6.3)	
Negative	36	29(80.6)	2(5.6)	5(13.9)	

[a] Wild type vs. mixed type and mutant.
[b] Twenty patients were treated with lamivudine, and 4 patients were treated with combination of lamivudine and adefovir dipivoxil. They were treated for more than 3 months.
[c] Genotype B or subgenotype B1/Bj vs. others.

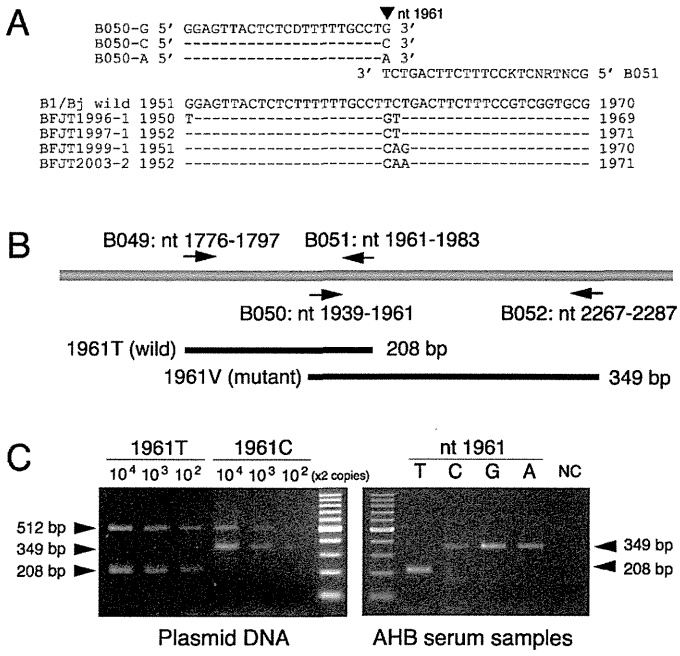

Fig. 3. Schema of the PCR with SSP established to detect the S21 mutation. (A) Primer sequences used in the PCR and corresponding sequences of the HBV/B1 wild type and 4 HBV/B1 FHB strains. Because the S21 mutants always had T1961V in our analysis, SSP was designed to anneal with either T1961V mutant or wild type. (B) Schema of the positions of primers used in the PCR, and the size of the resulting PCR products. T1961V mutants were distinguished by the difference of amplified products (wild type, 208 bp; mutant, 349 bp). (C) Patterns of the electrophoresis of the PCR products. The left panel shows the products amplified from the plasmid DNA with 1961T and 1961C of 2.0×10^4 to 2.0×10^2 copies, and the right panel shows the products amplified from DNA of AHB patient sera whose partial HBV sequences including nt 1961 were determined. The 512 bp products amplified by primers of B049 and B052 were not necessarily detected.

nt 1961–1963 of HBV encodes S21 in the core protein, which is located within an HLA-A2 restricted CTL epitope of HBcAg18–27,[25] and T1961V ± C1962D changes S21 to various amino acids. Interestingly, this mutant epitope had been suggested to act as T-cell receptor antagonist leading to inhibition of the CTL response to the wild type CTL epitope,[31] and the S21 substitution was considered to be a CTL escape mutation.[32] However, of our 3 FHB patients whose HLA-A types were known, 2 patients with S21 substitution did not have HLA-A2 (data not shown). Therefore the pathogenesis might not be necessarily due to the immune escape. Also, in patients with chronic infection, there were several HLA-A2-negative patients with T1961V mutants. The association between S21 substitution and HLA types has to be clarified further both in acutely infected patients and in chronically infected patients.

Because this study was limited by the small number of FHB patients and the cross-sectional nature, causal relationship could not be established well. While it is possible that S21 substitution predisposes acute infection patients to FHB, immune pressure during active hepatitis can also induce mutations. The latter possibility is less likely because of the short duration from infection, but further mechanistic study is needed to address the issue.

Through HBeAg seroconversion, which is an important event in chronic HBV infection, the HBV specific CTL repertoire develops, and mutations in the core gene increase.[33] It was reported that the S21 substitution was more frequent in HBeAg-negative chronic hepatitis B patients with low levels of HBV DNA than in those with high levels of HBV DNA,[32] which is concordant with our results. A study from New Zealand reported that, in the core protein, S21 was one of the amino acids under significant positive selection pressure.[34] These data suggested that the S21 substitution favors the persistence of HBV in the late stage of the natural course of HBV chronic infection, despite the developed immunity.

In conclusion, the S21 substitution in the core protein due to T1961V ± C1962D was found more frequently in FHB patients,

especially those with HBV/B1, than in AHB patients or chronically infected patients. This finding may give an insight into the mechanism of FHB and may be useful to predict the development of FHB in acute HBV infection.

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Competing interests

None.

Ethical approval

Not required.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jcv.2012.06.011.

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Original Article

Combination of hepatitis B viral antigens and DNA for prediction of relapse after discontinuation of nucleos(t)ide analogs in patients with chronic hepatitis B

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Aim: The factors associated with hepatitis recurrence after discontinuation of nucleos(t)ide analogs (NAs) in patients with chronic hepatitis B were analyzed to predict the risk of relapse more accurately.

Methods: A total of 126 patients who discontinued NA therapy were recruited retrospectively. The clinical conditions of a successful discontinuation were set as alanine aminotransferase (ALT) below 30 IU/L and serum hepatitis B virus (HBV) DNA below 4.0 log copies/mL.

Results: Relapse of hepatitis B were judged to occur when maximal serum ALT became higher than 79 IU/L or when maximal serum HBV DNA surpassed 5.7 log copies/mL following NA discontinuation since these values corresponded with mean values of ALT (30 IU/L) and HBV DNA (4.0 log copies/mL), respectively. At least 90% of patients with either detectable hepatitis B e antigen or serum HBV DNA higher than 3.0 log copies/mL at the time of NA discontinuation relapsed within one year. In the remaining patients, higher levels of both hepatitis B surface and core-related antigens at the time of discontinuation, as well as a shorter course of NA treatment, were significantly associated with relapse by multivariate analysis.

Conclusions: It appears that negative results for hepatitis B e antigen and serum HBV DNA lower than 3.0 log copies/mL are essential for successful NA discontinuation, which may be attained by a longer treatment period. Levels of hepatitis B surface and core-related antigens are also significant factors independently associated with relapse of hepatitis.

Key words: discontinuation, hepatitis B core-related antigen, hepatitis B surface antigen, nucleos(t)ide analogs, relapse of hepatitis

INTRODUCTION

HEPATITIS B VIRUS (HBV) infection is a major health concern that has an estimated 350 to 400 million carriers worldwide. Chronic infection with HBV can cause chronic hepatitis, and may eventually develop into liver cirrhosis and hepatocellular carcinoma.[1–3] Over the last decade, major advances in the treatment of chronic hepatitis B have been made with nucleos(t)ide

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analogs (NAs) such as lamivudine (LVD), adefovir dipivoxil (ADV), and entecavir (ETV).[4] NAs are orally administered and are associated with low rates of adverse effects. Treatment with NAs shows strong suppression of HBV replication and consequently rapid improvement of elevated ALT levels. Furthermore, these drugs have been reported to lower the risk of complicating cirrhosis and hepatocellular carcinoma,[5–7] and so NAs are becoming widely used to treat patients with chronic hepatitis B. On the other hand, NAs carry the risk of developing drug-resistance;[8] drug-resistant viruses emerging during treatment may be associated with hepatitis flare-ups. Hepatitis B patients are also required to undergo prolonged treatment with NAs because early discontinuance often leads to relapse of hepatitis and ensuing hepatic failure following rises in alanine aminotransferase (ALT) level.[9,10]

Serum HBV DNA is normally used to monitor the antiviral effect of NAs. HBV DNA decreases rapidly and becomes undetectable in the majority of patients who are treated with NAs,[11–13] but relapse after discontinuation is not rare.[14–17] Since it is also true that favorable virological and biochemical responses to NAs may continue indefinitely in some patients,[9,15] reliable markers that can predict relapse of hepatitis after NA discontinuation are needed. Such markers would benefit not only patients who are considering discontinuation of NA treatment, but also clinicians, hospitals, and the medical economy.

In the present study, we assessed several factors associated with relapse of hepatitis after discontinuation of NAs in patients with chronic hepatitis B, including hepatitis B viral antigens, which have been reported as new and promising markers for monitoring the effect of antiviral agents, such as interferon and NAs.

METHODS

Patients

A TOTAL OF 126 patients with chronic hepatitis B who underwent and completed NA treatment between 2000 and 2010 were enrolled in this study. Patients were recruited retrospectively from 11 hospitals across Japan (Toranomon Hospital, Hokkaido University Hospital, Nagoya City University Hospital, Shinshu University Hospital, Hiroshima University Hospital, National Hospital Organization Nagasaki Medical Center, Chiba University Hospital, The Hospital of Hyogo College of Medicine, Japanese Red Cross Nagoya Daini Hospital, and Tokyo Women's Medical University Hospital, Sapporo Kosei General Hospital) and met the following conditions: (i) serum ALT higher than 30 IU/L and serum HBV DNA higher than 4.0 log copies/mL were observed at least twice within the 6 months prior to administration of NAs; (ii) stored serum samples at initiation and discontinuation of NAs were available for measurements of viral markers; (iii) clinical outcomes were followed for at least 6 months after the discontinuation of NAs; and (iv) tests for hepatitis C and human immunodeficiency virus antibodies were negative. Hepatitis B surface antigen (HBsAg) was confirmed to be positive on at least two occasions at least 6 months apart in all patients before treatment. Patients complicated with hepatocellular carcinoma or signs of hepatic failure at treatment discontinuation were excluded from the study. Our cohort consisted of 83 men and 43 women with a median age of 46 (range, 19 to 79) years when NA administration was discontinued. Hepatitis B e antigen (HBeAg) was positive in 64 patients (51%) at the initiation of treatment and in 24 patients (19%) at its discontinuation. HBV genotype was A in two (2%) patients, B in five (4%), C in 102 (81%), and undetermined in 17 (13%). Thirty-five of the 126 patients in this study were younger than 35 years old. Although not recommended as the first line treatment for this group by Japanese guidelines,[18] NA treatment was commenced since chronic active hepatitis had been persisting in all cases irrespective of their HBeAg status (26 positive and nine negative) at the initiation of treatment.

The decision to discontinue NAs was made by individual physicians using similar, but not uniform, conditions. Four patients who halted NAs for financial reasons were included. No patient underwent interferon treatment during or after NA treatment. The decision to recommence NA administration was also made by individual physicians, essentially when relapse of hepatitis became obvious. With few exceptions, patients were seen at least once a month during the first year after discontinuation of NAs, and at least once every several months afterwards. Stored serum samples were kept frozen at −20°C or below until assayed. This study was approved by the Ethics Committees of all participating institutions.

Hepatitis B viral markers

Serological markers for HBV, including HBsAg, HBeAg, and antibody to HBe (anti-HBe) were tested using commercially available enzyme immunoassay kits (Abbott Japan Co., Ltd, Tokyo, Japan; Fujirebio Inc., Tokyo, Japan; and/or Sysmex Co., Kobe, Japan) at each hospital. Quantitative measurement of HBsAg[19] was done using a chemiluminescence enzyme immunoassay

(CLEIA)-based HISCL HBsAg assay manufactured by Sysmex Corporation (Kobe, Japan). The assay had a quantitative range of −1.5 to 3.3 log IU/mL. End titer was determined by diluting samples with normal human serum when initial results exceeded the upper limit of the assay range.

Serum concentration of HBV DNA was determined using an Amplicor HBV monitor kit (Roche, Tokyo, Japan),[20] which had a quantitative range of 2.6 to 7.6 log copies/mL. Serum HBV DNA was also determined using a COBAS TaqMan HBV kit (Roche, Tokyo, Japan)[21] with a quantitative range of 2.1 to 9.0 log copies/mL in 43 patients whose serum samples were available at the time of NA discontinuation. According to the manufacturer's instructions, detection of a positive signal below the quantitative range was described as a positive signal, and no signal detection was described as a negative signal. Six HBV genotypes (A–F) were evaluated according to the restriction patterns of DNA fragments from the method reported by Mizokami *et al.*[22]

Serum hepatitis B core-related antigen (HBcrAg) levels were measured using a CLEIA HBcrAg assay kit with a fully automated Lumipulse System analyzer (Fujirebio Inc., Tokyo, Japan) as described previously.[23,24] Briefly, 150 μL of serum was incubated with pretreatment solution and then added to a ferrite microparticle suspension in an assay cartridge. Ferrite particles were coated with a monoclonal antibody mixture against denatured HBcAg, HBeAg, and the 22 kDa precore protein. After incubation and washing, further incubation was carried out with alkaline phosphatase conjugated with two kinds of monoclonal antibodies against denatured HBcAg, HBeAg, and the 22 kDa precore protein. Following washing, a substrate solution was added to the test cartridge and then incubated. The relative chemiluminescence intensity was measured, and HBcrAg concentration was calculated by a standard curve generated using recombinant pro-HBeAg. The immunoreactivity of pro-HBeAg at 10 fg/mL was defined as 1 U/mL. We expressed HBcrAg in terms of log U/mL, with a quantitative range set at 3.0 to 6.8 log U/mL.

Statistical analyses

A linear regression model was used to examine for associations between mean and maximal values of both ALT and HBV DNA. Correlations between variables were calculated using the Spearman's rank correction correlation coefficient test. Each cut-off value was decided using receiver operating characteristic curve (ROC) analysis and results were evaluated by measuring the area under the curve (AUC). The Fisher's exact and Pearson's χ^2 tests were adopted to test for differences between subgroups of patients. To compare continuous data, the Mann–Whitney *U*-test was used. The Kaplan–Meier method was used to estimate rates of non-relapse observations, and the log-rank test was used to test hypotheses concerning differences in non-relapse observations between selected groups. Multivariate analyses were performed using the Cox regression model. Variables associated with a P-value < 0.2 in univariate analyses were included in a stepwise Cox regression analysis to identify independent factors associated with relapse of hepatitis after discontinuation of NAs. All tests were performed using the IBM SPSS Statistics Desktop for Japan ver. 19.0 (IBM Japan Inc., Tokyo, Japan). *P*-values of less than 0.05 were considered to be statistically significant.

RESULTS

Definition of hepatitis relapse after discontinuation of NAs

THE CLINICAL CONDITIONS of a successful discontinuation of NAs were set at serum HBV DNA below 4.0 log copies/mL and ALT below 30 IU/L according to the Japanese guidelines for the treatment of hepatitis B.[18] However, these criteria could not be directly applied to our cohort as post-therapy fluctuations in ALT and HBV DNA were difficult to evaluate consistently. In total, 26 (76%) of 34 patients with successful discontinuation of NAs showed transient abnormal levels of ALT and/or HBV DNA, especially during the early phase after cessation. We therefore used mean and maximal values of these markers to evaluate relapse of hepatitis B in this study; mean values were used to evaluate relapse of hepatitis as a whole, and maximal values were used to dynamically assess relapse during the follow-up period after NA discontinuation. Both ALT and HBV DNA were measured 11.0 times per year on average during the first year and 4.1 times per year on average thereafter.

The mean values of HBV DNA were significantly ($P < 0.001$) correlated with maximal values with a correlation coefficient of 0.853. Similarly, the mean values of ALT were significantly ($P < 0.001$) correlated with maximal values with a correlation coefficient of 0.940 (Fig. 1). The mean HBV DNA value of 4.0 log copies/mL corresponded to a maximal HBV DNA value of 5.7 by ROC analysis (AUC = 0.930, $P < 0.001$), and the mean ALT value of 30 IU/L corresponded to a maximal ALT value of 79 IU/L (AUC = 0.988, $P < 0.001$). These results suggested that patients having serum HBV DNA higher