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## Noninvasive assessment of liver fibrosis in patients with chronic hepatitis B

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### Abstract

Infection with hepatitis B virus is an important health problem worldwide: it affects more than 350 million people and is a leading cause of liver-related morbidity, accounting for 1 million deaths annually. Hepatic fibrosis is a consequence of the accumulation of extracellular matrix components in the liver. An accurate diagnosis of liver fibrosis is essential for the management of chronic liver disease. Liver biopsy has been considered the gold standard for diagnosing disease, grading necroinflammatory activity, and staging fibrosis. However, liver biopsy is unsuitable for repeated evaluations because it is invasive and can cause major complications, including death. Several noninvasive evaluations have been introduced for the assessment of liver fibrosis: serum biomarkers, combined indices or scores, and imaging techniques including transient elastography, acoustic radiation force impulse, real-time tissue elastography, and magnetic resonance elastography. Here, we review the recent progress of noninvasive assessment of liver fibrosis in patients with chronic hepatitis B. Most noninvasive evaluations for liver fibrosis have been validated first in patients with chronic hepatitis C, and later in those with chronic hepatitis B. The establishment of a noninvasive assessment of liver fibrosis is

urgently needed to aid in the management of this leading cause of chronic liver disease.

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**Key words:** Acoustic radiation force impulse; Biomarkers; Biopsy; Elastography; Fibrosis; Hepatitis B; Noninvasive evaluations

**Core tip:** The usefulness of noninvasive evaluations for predicting liver fibrosis remains to be fully evaluated in chronic hepatitis B. Few indices/scores based on combinations of serum biomarkers were originally proposed for use in patients with chronic hepatitis B. Transient elastography is less accurate for chronic hepatitis B than for chronic hepatitis C. Limited data are available regarding the usefulness of acoustic radiation force impulse, real-time tissue elastography, and magnetic resonance elastography in chronic hepatitis B. However, these methods are suitable for repeated evaluations and can be useful for assessing the clinical stage of disease, predicting complications of cirrhosis, and monitoring the response to treatment.

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### INTRODUCTION

Hepatic fibrosis, regardless of the underlying etiology, is a consequence of the accumulation of extracellular matrix components in the liver. This process is caused by persistent liver damage and consequent wound healing re-

action and can progress to cirrhosis, portal hypertension, and hepatocellular carcinoma (HCC), leading to increased morbidity and mortality<sup>[1,2]</sup>. An accurate diagnosis of liver fibrosis is thus essential for the management of chronic liver diseases.

Liver biopsy has been considered the “gold standard” for diagnosing chronic liver disease, grading necroinflammatory activity, and staging liver fibrosis<sup>[3,4]</sup>. However, sampling error can lead to underestimation of the degree of liver fibrosis, especially when the biopsy specimens are small or fragmented. In addition, interpretation of the results is subject to significant intraobserver and interobserver variability. Moreover, liver biopsy is unsuitable for repeated evaluations because it is invasive and can cause major complications, including death<sup>[5,6]</sup>. Therefore, several serum biomarkers, combined indices/scores, and imaging techniques for the noninvasive assessment of liver fibrosis have been introduced.

Infection with hepatitis B virus (HBV) is an important health problem worldwide: it affects more than 350 million people and is a leading cause of liver-related morbidity, accounting for 1 million deaths annually<sup>[7-9]</sup>. However, most noninvasive evaluations of liver fibrosis have been validated first in patients with chronic hepatitis C and only then in patients with chronic hepatitis B. Herein, we review the recent progress in the noninvasive assessment of liver fibrosis in patients with chronic hepatitis B.

## SERUM MARKERS

Several surrogate serum markers have been proposed as alternatives for the noninvasive assessment of liver fibrosis<sup>[10-12]</sup>. In general, markers of fibrosis can be divided into two groups: direct and indirect. Serum direct markers reflect extracellular matrix turnover. They include glycoproteins such as serum hyaluronate, laminin, and YKL-40; collagens such as procollagen III N-terminal propeptide and type IV collagen 7S; collagenases and collagenase inhibitors such as matrix metalloproteases and tissue inhibitory metalloprotease-1. Indirect markers reflect alterations in hepatic function rather than metabolism of the hepatic extracellular matrix. They include the platelet count, coagulation studies, and the levels of aspartate and alanine aminotransferases (AST and ALT). The advantages of biomarkers as measures of fibrosis include their high applicability and interlaboratory reproducibility and their wide availability for repeated assays. However, none is liver-specific: the results of all such tests can be influenced by comorbid conditions.

Several algorithms, indices, or scores derived from combinations of serum biomarkers have been proposed, namely, the Fibrotest<sup>[13]</sup>, Forns index<sup>[14]</sup>, AST-to-platelet ratio index (APRI)<sup>[15]</sup>, FIB-4<sup>[16]</sup>, Fibrometer<sup>[17]</sup>, FibroIndex<sup>[18]</sup>, and ELF<sup>[19]</sup>. Table 1 shows the diagnostic performance of several combined indices/scores in patients with chronic hepatitis B. Although most indices/scores were originally established using data from patients with chronic hepatitis C, their diagnostic accuracies have been

validated in patients with chronic hepatitis B<sup>[20-23]</sup>. Sebastiani *et al.*<sup>[20]</sup> reported that the area under the receiver operating characteristic (AUROC) curve values for identification of both significant fibrosis ( $\geq$  F2) and cirrhosis (F4) in patients with chronic hepatitis B were better for the Fibrotest than for the APRI and Forns index. Combined indices/scores including hepatic aminotransferase levels are unsuitable for monitoring liver fibrosis during treatment, as the serum levels of aminotransferases decrease rapidly after initiation of therapy. As the Fibrotest includes the serum haptoglobin, bilirubin, and  $\gamma$ -glutamyltranspeptidase levels, false-positive results may occur in patients with hemolysis or cholestasis and in those who have recently consumed alcohol<sup>[24]</sup>. Hui *et al.*<sup>[25]</sup> and Zeng *et al.*<sup>[26]</sup> have developed liver fibrosis scores specifically for patients with chronic hepatitis B, although their usefulness remains to be validated by other groups.

## TRANSIENT ELASTOGRAPHY

Transient elastography (FibroScan<sup>®</sup>, EchoSens, Paris, France), which has become a popular tool, is a rapid, objective, and promising technique for staging liver fibrosis by measuring the stiffness of the liver, expressed in units of kilopascals (kPa)<sup>[27,28]</sup>. This system is equipped with a probe including an ultrasonic transducer mounted on the axis of a vibrator. The vibration transmitted from the vibrator toward the tissue induces an elastic shear wave that propagates through the liver. These propagations are followed by pulse-echo ultrasound acquisition, and their velocity, which is directly related to tissue stiffness, is measured. In a morphometric analysis, Wong *et al.*<sup>[29]</sup> found that the liver stiffness as assessed by transient elastography correlates significantly with the proportion of the liver affected by fibrosis, particularly pericellular fibrosis rather than periportal or perivenular fibrosis.

Liver stiffness measurement has generally been considered reliable when it fulfills all of the following criteria:  $\geq$  10 valid measurements, a success rate of  $\geq$  60%, and an interquartile range/median ratio (IQR/M) of  $\leq$  0.30. However, in multivariate analyses of 1165 patients with various chronic liver diseases, Boursier *et al.*<sup>[30]</sup> recently reported that the reliability of measurement of liver stiffness depends on the IQR/M according to the median level of liver stiffness, thus defining three categories of reliability: “very reliable” (IQR/M  $\leq$  0.10), “reliable” (0.10 < IQR/M  $\leq$  0.30, or IQR/M > 0.30 with a median value of < 7.1 kPa), and “poorly reliable” (IQR/M > 0.30 with a median value of  $\geq$  7.1 kPa).

In a meta-analysis of 50 elastography studies performed mainly in patients with chronic hepatitis C, the mean AUROC curves for the diagnosis of significant fibrosis ( $\geq$  F2), severe fibrosis (F3/F4), and cirrhosis (F4) were 0.84, 0.89, and 0.94, respectively<sup>[31]</sup>. The diagnostic accuracy of transient elastography was generally high for cirrhosis but poorer for significant fibrosis<sup>[32]</sup>. The results of liver stiffness measurement can be affected by factors other than fibrosis, including necroinflammatory activ-

Table 1 Performance of serum fibrosis markers for identification of significant fibrosis ( $\geq$  F2) and cirrhosis (F4) in patients with hepatitis B

Ref.	Year	Patients (n)	Diagnosis for $\geq$ F2				Diagnosis for F4			
			Patients (%)	AUROC	Cutoff (kPa)	Se/Sp (%)	Patients (%)	AUROC	Cutoff (kPa)	Se/Sp (%)
Originally for patients with HCV										
Fibrotest (includes $\alpha$ 2-macroglobulin, haptoglobin, apolipoprotein A1, bilirubin, and GGT)										
Sebastiani <i>et al.</i> <sup>[20]</sup>	2007	110	68	0.85	F2	81/90	20	0.76	F4	56/96
<sup>1</sup> Bottero <i>et al.</i> <sup>[21]</sup>	2009	108	56	0.77	0.48	70/72	15	0.87	0.73	75/85
Forns index (includes age, platelet count, cholesterol, and GGT)										
Sebastiani <i>et al.</i> <sup>[20]</sup>	2007	110	68	0.63	4.20	58/78	20	-	-	-
Wong <i>et al.</i> <sup>[22]</sup>	2010	156 +	75	0.70	5.20	28/91	26	-	-	-
		82	59	0.72	8.40	43/93	20	-	-	-
<sup>1</sup> Bottero <i>et al.</i> <sup>[21]</sup>	2009	108	56	0.72	-	-	15	0.81	-	-
APRI (includes AST and platelet count)										
Sebastiani <i>et al.</i> <sup>[20]</sup>	2007	110	68	0.72	0.50	71/87	20	0.64	2.00	43/85
Kim <i>et al.</i> <sup>[23]</sup>	2010	668	79	0.70	-	-	34	0.73	-	-
<sup>1</sup> Bottero <i>et al.</i> <sup>[21]</sup>	2009	108	56	0.73	-	-	15	0.76	-	-
FIB-4 (includes age, AST, ALT, and platelet count)										
Kim <i>et al.</i> <sup>[23]</sup>	2010	668	79	0.86	1.00	91/73	34	0.93	1.60	88/84
<sup>1</sup> Bottero <i>et al.</i> <sup>[21]</sup>	2009	108	56	0.74	-	-	15	0.80	-	-
Fibrometer (includes age, platelet count, PT index, AST, $\alpha$ 2-macroglobulin, hyaluronate, and urea)										
<sup>1</sup> Bottero <i>et al.</i> <sup>[21]</sup>	2009	108	56	0.74	0.46	73/68	15	0.89	0.83	81/85
Originally for patients with HBV										
Hui score (includes body mass index, bilirubin, albumin, and platelet count)										
Hui <i>et al.</i> <sup>[25]</sup>	2005	235	25	0.79	0.15	88/50	-	-	-	-
Zeng score (includes age, $\alpha$ 2-macroglobulin, hyaluronate, and GGT)										
Zeng <i>et al.</i> <sup>[26]</sup>	2005	372	58	0.77	3.00	98/28	-	-	-	-

<sup>1</sup>Study subjects were coinfecting with human immunodeficiency virus. APRI: AST to platelet ratio index; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; AUROC: Area under the receiver operating characteristic curve; GGT:  $\gamma$ -Glutamyltranspeptidase; HBV: Hepatitis B virus; HCV: Hepatitis C virus; PT: Prothrombin.

ity<sup>[33-35]</sup>, obesity<sup>[36]</sup>, and extrahepatic cholestasis<sup>[37]</sup>.

The usefulness of transient elastography has been well validated in patients with chronic hepatitis C; however, limited data are available for its use in patients with other liver diseases<sup>[38]</sup>, including chronic hepatitis B. Table 2 shows the diagnostic performance of elastography for significant fibrosis ( $\geq$  F2) and cirrhosis (F4) in patients with chronic HBV infection<sup>[22,32,39-42]</sup>. In some studies<sup>[43,44]</sup>, measurement of liver stiffness has been less accurate in patients with chronic hepatitis B than in those with chronic hepatitis C. One possible explanation for the difference in diagnostic accuracy is that chronic hepatitis B is associated with acute exacerbations, in which severe/moderate necroinflammation can lead to overestimation of fibrosis, more frequently than is chronic hepatitis C<sup>[45]</sup>. On the other hand, liver stiffness measurement exhibits good diagnostic accuracy with a high negative predictive value<sup>[46]</sup>.

In previous studies that addressed both chronic hepatitis B and chronic hepatitis C<sup>[47,48]</sup>, the median liver stiffness at each stage of fibrosis was lower in patients with chronic hepatitis B than in those with chronic hepatitis C. The reported cutoff values for predicting cirrhosis in patients with chronic hepatitis B ranged from 9.4 to 12.9 kPa<sup>[32,39-42]</sup>; all of these values are lower than the optimal cutoff value of 13.0 kPa derived from a meta-analysis of 17 studies (mostly concerning chronic hepatitis C)<sup>[51]</sup>. The amount of fibrosis in the cirrhotic liver is generally lower

for chronic hepatitis B than for chronic hepatitis C because macronodular cirrhosis, characterized by large nodules delimited by thin septa, is more common in patients chronically infected with HBV.

Some studies have proposed the adoption of different cut-off values for each cause of liver disease<sup>[43]</sup>. To avoid overestimation of fibrosis, other studies have proposed basing the cut-off value on the ALT level<sup>[41]</sup>. Diagnostic algorithms using dual cut-offs, for positive and negative prediction of liver fibrosis, have also been proposed<sup>[49]</sup>.

## ACOUSTIC RADIATION FORCE IMPULSE

Acoustic radiation force impulse (ARFI; Siemens AG, Erlangen, Germany) imaging involves mechanical excitation of tissue using short-duration acoustic pulses to generate localized tissue displacement<sup>[50-52]</sup>. The harder the tissue, the faster the shear wave spreads. The displacement results in shear-wave propagation, which is tracked using correlation-based ultrasonic methods and recorded in m/s. This examination is performed during B-mode ultrasonography.

Sporea *et al.*<sup>[53]</sup> showed that the mean liver stiffness values obtained by ARFI were similar between patients with chronic hepatitis B and those with chronic hepatitis C at the same stage of fibrosis. Friedrich-Rust *et al.*<sup>[54]</sup> reported that the diagnostic accuracy of ARFI for the histological staging of liver fibrosis in patients with chronic hepatitis

**Table 2** Performance of transient elastography for identification of significant fibrosis ( $\geq$  F2) and cirrhosis (F4) in patients with hepatitis B

Ref.	Year	Patients (n)	Diagnosis for $\geq$ F2				Diagnosis for F4			
			Patients (%)	AUROC	Cutoff (kPa)	Se/Sp (%)	Patients (%)	AUROC	Cutoff (kPa)	Se/Sp (%)
Oliveri <i>et al</i> <sup>[39]</sup>	2008	188	26	0.97	7.5	94/88	20	0.97	11.8	86/96
Marcellin <i>et al</i> <sup>[40]</sup>	2009	173	50	0.81	7.2	70/83	8	0.93	11.0	93/87
Chan <i>et al</i> <sup>[41]</sup>	2009	161	-	-	-	-	25	0.93	12.0-13.4 <sup>2</sup>	98/75
Degos <i>et al</i> <sup>[32]</sup>	2010	284	42	0.78	5.2	89/38	10	0.85	12.9	52/93
Wong <i>et al</i> <sup>[22]</sup>	2010	156 + 82	68	0.80	9.0-12.0 <sup>2</sup>	54/99	23	-	-	-
<sup>1</sup> Mialhe <i>et al</i> <sup>[42]</sup>	2011	57	61	0.85	5.9	81/87	20	0.96	9.4	92/94

<sup>1</sup>Study subjects were coinfecting with human immunodeficiency virus; <sup>2</sup>Adapted to ALT values. AUROC: Area under the receiver operating characteristic curve; Se: Sensitivity; Sp: Specificity.

B was comparable to that of transient elastography.

## REAL-TIME TISSUE ELASTOGRAPHY

Real-time tissue elastography (Hitachi Medical Systems, Tokyo, Japan) is a new ultrasound-based diagnostic method for the evaluation of tissue elasticity and can be performed during routine B-mode screening of the liver<sup>[55-57]</sup>. This technology has already been proven diagnostically valuable for the detection of mass lesions in the breast, prostate, and pancreas. A computer-assisted apparatus is used to calculate the relative hardness of the tissue from the degree of tissue distortion and displays this information as a color image. Ultrasound elastography does not demonstrate physical elasticity directly but rather shows the relative degree of tissue strain under subtle compression.

A Chinese study of real-time tissue elastography in 71 patients with chronic hepatitis B found a strong negative correlation between the elastic strain ratio and the histological stage of fibrosis<sup>[58]</sup>. The AUROC curve for detection of significant fibrosis ( $\geq$  F2) was higher for real-time elastography than for blood parameters, such as the APRI and Forns index. Similar results were also reported in another Chinese study<sup>[59]</sup>.

## MAGNETIC RESONANCE ELASTOGRAPHY

Magnetic resonance (MR) elastography is a promising imaging technique that noninvasively measures the stiffness of the liver as well as that of other organs by analyzing the propagation of mechanical waves through tissue<sup>[60-62]</sup>. Its clear advantages include the potential to assess the entire liver parenchyma, the dispensability of an acoustic window, and operator independence. In addition, this method may be useful for quantifying hepatic fat content.

Venkatesh *et al*<sup>[63]</sup> examined 63 patients with chronic hepatitis B and reported that MR elastography was significantly more accurate for the detection of biopsy-confirmed significant fibrosis and cirrhosis than were serum fibrosis markers such as APRI. As there are only limited data on the accuracy of MR elastography in patients with chronic hepatitis B, further studies are required for validation.

## COMBINATIONS OF BIOMARKERS AND IMAGING METHODS

Combinations of serum markers and imaging studies can detect advanced fibrosis in patients with chronic hepatitis B with a high degree of accuracy. A Korean study developed a liver stiffness measurement-spleen diameter to platelet ratio index (LSPI) for the assessment of liver fibrosis: (liver stiffness measurement  $\times$  spleen diameter/platelet count)  $\times$  100<sup>[64]</sup>. Another Korean study established a model for predicting significant fibrosis, called the HALF index, consisting of liver stiffness values and the serum haptoglobin, apolipoprotein A1, and  $\alpha$ -2-macroglobulin levels<sup>[65]</sup>.

## CLINICAL APPLICATIONS

### Assessing the clinical stage of disease

The natural course of chronic HBV infection acquired perinatally or during infancy consists of four distinct phases: "immune tolerance," "immune reactivity," "inactive carrier state," and "reactivation"<sup>[7-9]</sup>. Assessment of the clinical stage of disease is usually based on the ALT activity, HBV DNA level, and titers of hepatitis e antigen (HBeAg) and anti-HBe antibodies; however, noninvasive evaluations could also be helpful to discriminate HBeAg-negative patients who have significant fibrosis despite normal ALT activity from inactive carriers of HBV<sup>[66-68]</sup>. Some patients may then require further assessment by liver biopsy for proper evaluation of indication of antiviral therapy.

### Predicting complications of cirrhosis

Noninvasive methods can be used to predict complications of cirrhosis. In a Korean prospective study of 1130 patients with chronic hepatitis B<sup>[69]</sup>, 57 patients developed HCC during the 24-51-mo follow-up period. Multivariate analysis showed that patients with higher liver stiffness measurements by transient elastography were at significantly greater risk of developing HCC, with the following hazard ratios: 3.07 for 8.1-13 kPa; 4.68 for 13.1-18 kPa; 5.55 for 18.1-23 kPa; and 6.60 for  $>$  23 kPa. Wong *et al*<sup>[70]</sup> proposed the LSM-HCC score, a liver stiffness-based HCC risk score based on transient elastography data

from 1555 consecutive patients with chronic HBV infection. This score was constructed from the liver stiffness measurement, age, serum albumin level, and HBV DNA level and ranges from 0 to 30. When a cutoff value of 11 was used, the score excluded future HCC with a high negative predictive value (99.4%-100%) after 5 years.

Liver stiffness values have also been shown to correlate with the presence and severity of esophageal varices. Using transient elastography data from 577 consecutive patients with B-viral cirrhosis, a Korean group developed a liver stiffness measurement-based model, the liver stiffness measurement-spleen diameter to platelet ratio score liver stiffness measurement (LSPS  $\times$  spleen diameter/platelet count), for assessment of the cumulative risk of future esophageal variceal bleeding<sup>[71]</sup>. Multivariate analysis found an LSPS of  $\geq 6.5$  ( $P = 0.003$ ), along with large variceal size and Child-Pugh classification B/C, to be a significant predictor of a first occurrence of esophageal variceal bleeding. A Chinese study found significant linear correlations between liver and spleen stiffness as measured by ARFI and the stage of fibrosis in 138 patients with hepatitis B-related cirrhosis<sup>[72]</sup>. As there was also a significant linear correlation between spleen stiffness and the varix grade, ARFI can be used as a noninvasive method for assessing the presence and severity of esophageal varices.

### Monitoring response to treatment

Several studies have reported significant decreases in liver stiffness and biomarker levels in patients with chronic hepatitis B who were treated with interferon- $\alpha$  or nucleos(t)ide analogues<sup>[73-81]</sup> as well as in patients with chronic hepatitis C who achieved sustained virologic response to interferon<sup>[82]</sup>. We studied the liver stiffness measurement by transient elastography in 20 patients with chronic hepatitis B<sup>[80]</sup>. The liver stiffness values in patients treated with entecavir decreased significantly from 11.2 kPa (range: 7.0-15.2 kPa) to 7.8 kPa (range: 5.1-11.9 kPa;  $P = 0.0090$ ) during 12 mo of treatment. Fung *et al.*<sup>[81]</sup> also repeated liver stiffness measurement during antiviral therapy in 58 chronic HBV infected patients with baseline ALT levels from  $\times 1$  to  $\times 10$ , the upper limit of the normal. The ALT level became normal after a median of 3 mo of antiviral therapy. The AUROC curve for diagnosis of significant fibrosis by liver stiffness was 0.68 in patients with an elevated ALT level at baseline versus 0.73 after ALT level normalization, suggesting that even mild-to-moderate elevation in the ALT level may increase liver stiffness. The decrease in liver stiffness during the first few months of nucleos(t)ide analogue treatment may be attributable to improvement in necroinflammation rather than regression of liver stiffness.

### CONCLUSION

The usefulness of noninvasive evaluations for predicting liver fibrosis has been less extensively studied and validated for chronic hepatitis B than for chronic hepatitis C.

Few algorithms or indices/scores based on combinations of serum biomarkers were originally proposed for use in patients with chronic hepatitis B. Transient elastography is less accurate in patients with chronic hepatitis B than in those with chronic hepatitis C. Limited data are available on the usefulness of ARFI, real-time tissue elastography, and magnetic resonance elastography in patients with chronic hepatitis B. In addition, these methods do not provide information on necroinflammatory activity, steatosis, iron deposition, or other findings that can be obtained by liver biopsy. However, they are suitable for repeated evaluations and can be useful for assessing the clinical stage of disease, predicting complications of cirrhosis, and monitoring the response to treatment.

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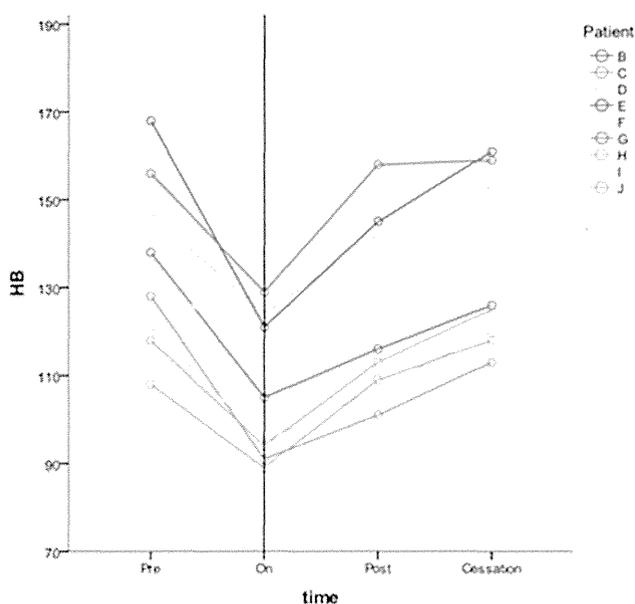


Fig. 1 Haemoglobin levels of renal transplant patients over time. Pre – Haemoglobin levels prior to initiation of dapsone; On – Haemoglobin levels 6–12 months after initiation of dapsone; Post – Haemoglobin levels 1–3 months after cessation of dapsone; Cessation – Haemoglobin levels 3–6 months after cessation of dapsone; HB – Haemoglobin g/dL.

related haemolysis and methaemoglobinaemia has been described in the paediatric population.<sup>4</sup> This is a likely risk in adult solid organ transplant recipients, with diminished renal function particularly in rapid hydroxylators who form toxic N-hydroxylated metabolites that are excreted in urine.<sup>5</sup>

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## SUCCESSFUL TREATMENT OF HEPATITIS B VIRUS-ASSOCIATED MEMBRANOUS NEPHROPATHY WITH ENTECAVIR AND IMMUNOSUPPRESSIVE AGENTS

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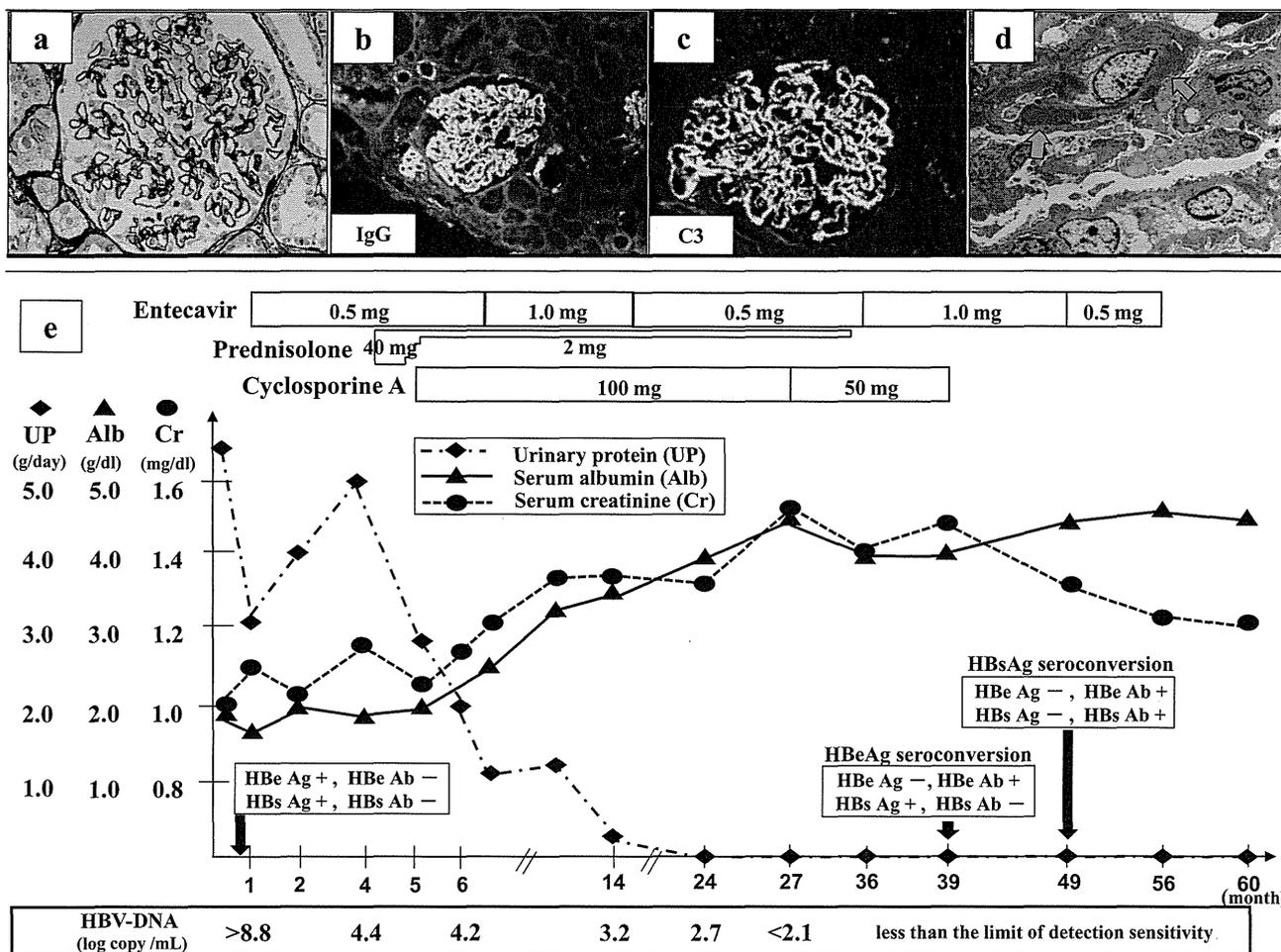
## CASE REPORT

A 57-year-old man with hepatitis B virus (HBV) infection was admitted due to oedema of the lower extremities. Laboratory data revealed nephrotic syndrome. While serum hepatitis B virus surface antigen (HBsAg) and hepatitis B virus envelope antigen (HBeAg) were positive, HBs antibody (HBsAb) and HBeAb were negative. Renal biopsy revealed membranous nephropathy (Fig. 1a–d). Clinically, we diagnosed this as HBV-associated membranous nephropathy (HBV-MN).

The clinical course is summarized in Figure 1e. Initially, entecavir was prescribed orally at 0.5 mg/day. However, the nephrotic syndrome worsened. At 4 months, oral prednisolone 40 mg/day was added. After the onset of severe steroid psychosis, the dose of prednisolone was gradually reduced. At 5 months, oral cyclosporine A 100 mg/day was added. At 24 months, the urinary protein excretion became negative. At 39 months, HBeAg seroconversion was achieved. We subsequently stopped the immunosuppressive therapy, and continued treatment with entecavir alone. HBV reactivation was not seen during the course. At 49 months, HBsAg seroconversion was achieved, and we stopped the entecavir. After cessation of the entecavir, the complete remission of nephritic syndrome was sustained.

## DISCUSSION

Entecavir monotherapy has recently been reported to be effective in the treatment of HBV-MN.<sup>1</sup> In this case, we initiated treatment with entecavir monotherapy. However, the nephrotic syndrome worsened. Thus, we added immunosuppressive agents to the entecavir treatment. HBV reactivation was not observed in this case. In Japan, the guideline for the prevention of immunosuppressive therapy or chemotherapy-induced reactivation of HBV infection<sup>2</sup> recommends the pre-emptive administration of entecavir. However, there has been one report of mild to moderate flare



**Fig. 1** (a) Light microscopic examination (PAM staining). Thickening of the glomerular basement membrane and spikes are shown in the glomeruli. (b,c) Immunofluorescence microscopic examination. Granular deposition of IgG and C3 are evident along the glomerular basement membrane. (d) Electron microscopic examination. Subepithelial electron-dense deposits are evident (arrows). (e) Clinical course. --♦--, Urinary protein (UP); --▲--, Serum albumin (Alb); --●--, Serum creatinine (Cr).

of hepatitis during chemotherapy, even with the pre-emptive use of entecavir.<sup>3</sup> Thus, it is necessary to administer immunosuppressive therapy carefully to HBV-MN patients in consultation with hepatologists.

This is the first report of the successful treatment of HBV-MN by combination therapy with entecavir and immunosuppressive agents. Pre-emptive use of entecavir may be useful to prevent HBV reactivation induced by immunosuppressive therapy in HBV-MN patients.

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# A Deep-Sequencing Method Detects Drug-Resistant Mutations in the Hepatitis B Virus in Indonesians

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## Key Words

Hepatitis B virus · Deep sequencing · Nucleos(t)ide analogue · Indonesia

## Abstract

**Objective:** The long-term administration of a nucleos(t)ide analogue (NA) for the treatment of chronic hepatitis B may encourage the emergence of viral mutations associated with drug resistance. Minor populations of viruses may exist before treatment, but are difficult to detect because of technological limitations. Identifying minor viral quasispecies should be useful in the clinical management of hepatitis B virus (HBV) infection. **Methods:** Six treatment-naïve Indonesian patients with chronic HBV infection participated in this study. The polymerase region of the HBV genome,

including regions with known drug-resistant mutations, was subjected to capillary sequencing and MiSeq sequencing (Illumina). Mutations were analyzed with Genomics Workbench software version 6.0.1 (CLC bio). **Results:** The mean mapping reads for the six samples was 745,654, and the mean number of amplified fragments ranged from 17,926 to 25,336 DNA reads. Several known drug-resistant mutations in the reverse transcriptase region were identified in all patients, although the frequencies were low (0.12–1.06%). The proportions of the total number of reads containing mutations I169L/M, S202R, M204I/L or N236S were >1.0%. **Conclusion:** Several known NA-resistant mutations were detected in treatment-naïve patients in Indonesia using deep sequencing. Careful management of such patients is essential to prevent drug-resistant mutations from spreading to other patients.

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## Introduction

Approximately 350 million people are infected with hepatitis B virus (HBV) worldwide, including asymptomatic carriers, making HBV infection a major global health problem. Chronic HBV infection often progresses to liver cirrhosis and hepatocellular carcinoma, and around 1 million deaths per year are thought to be related to HBV infection [1]. The prevalence of HBV infection varies considerably between countries. In Indonesia, it is reported that 4.0–20.3% of the population are infected with HBV, and Indonesia is considered to be a moderate-to-high endemic region [2].

The purpose of chronic hepatitis B (CHB) therapy is the sustained suppression of HBV replication and the remission of liver disease. The nucleos(t)ide analogues (NAs) used to treat CHB include lamivudine (LAM), telbivudine (L-deoxythymidine; LdT), entecavir (ETV), adefovir dipivoxil (ADV), tenofovir and emtricitabine. NAs mainly suppress viral proliferation by inhibiting HBV DNA polymerase activity. They are generally well tolerated and are easy to administer once daily. They also reduce liver damage, improve liver function and inhibit hepatocarcinogenesis in chronically HBV-infected patients with fewer adverse effects than conventional interferon therapy [3, 4]. However, the emergence of drug-resistant mutations during long-term treatment is a major limitation of these drugs. Drug-resistant mutations were found in approximately 70% of patients treated with LAM for up to 5 years and in 3% of patients treated with ADV for up to 3 years. Resistance to ETV was reported in 1.1% of patients treated for up to 4 years [5–7]. Numerous cases of severe reactivation, viral relapse and exacerbation of hepatitis after discontinuation of NA therapy have also been reported [8].

The HBV genome is a partially double-stranded circular DNA molecule of approximately 3,200 bp, consisting of four overlapping open reading frames that encode the surface protein, the core protein, a polymerase and region X, which encodes a multifunctional nonstructural protein. The polymerase region is an important target for NAs, and mutations in this region are sometimes associated with drug resistance. HBV replication is very fast, with up to  $10^{11}$  viral particles released per day. This high replication rate, coupled to the absence of a proofreading mechanism in the HBV polymerase, contributes to the appearance of mutations [9, 10]. Mutations in the reverse transcriptase (RT) region sometimes induce viral breakthrough. These mutations include drug-induced mutations after NA treatment, and natural mutations or viral quasispecies present prior to treatment [3, 5].

However, it is currently very difficult to detect viruses with drug-resistant mutations that are present in very low copy numbers because of technological limitations. Next-generation sequencing methods are used to sequence thousands to millions of sequences concurrently, allowing us to detect any preexisting mutations before antiviral therapy is commenced. Identifying minor viral quasispecies and low-frequency NA-resistant mutations in treatment-naïve patients should allow us to select the most appropriate NA regimen and to predict the future emergence of mutations, offering significant benefits in the management of patients with HBV infection. Although some studies have used next-generation sequencing methods to detect NA-resistant mutations, most of the patients had HBV genotype C [11, 12].

In this study, we sought to identify quasispecies in the RT region of the HBV genome and detect possible drug-resistant mutations in Indonesian treatment-naïve patients with CHB, using a next-generation sequencer. The results are expected to be useful for evaluating the risk of emerging drug-resistant mutations and selecting the most appropriate antiviral drug for patients with CHB.

## Materials and Methods

### Subjects

To detect preexisting mutations associated with NA resistance in Indonesian patients, we obtained serum samples at the inpatient and outpatient units of the Gastroenterology and Hepatology Department of Internal Medicine, Dr. Sardjito Hospital, Yogyakarta, Indonesia, from 6 treatment-naïve chronic HBV carriers. The patients were 22–77 years old (median 38 years). Three of the patients were asymptomatic carriers and the other 3 had advanced liver disease: hepatocellular carcinoma in 2 and liver cirrhosis in 1. Alanine aminotransferase and aspartate aminotransferase levels were measured immediately after blood collection. Informed consent was obtained from all of the patients. The study was approved by the Medical and Health Ethics Committees of Gadjah Mada University and Kobe University (Japan).

### DNA Extraction and Measurement of the HBV Viral Load

HBV DNA was extracted from 200 µl of sera using a Qiagen DNA Blood Mini Kit, according to the manufacturer's instructions (Qiagen, Tokyo, Japan). The HBV viral load was measured with real-time polymerase chain reaction (PCR) on an ABI Prism 7700 Genetic Analyzer (Applied Biosystems, Foster City, Calif., USA), using a previously described specific primer and probe set [13].

### Direct PCR Sequencing and Genotype Determination

The RT region was amplified and confirmed with a PCR assay using specific primers [14]. After the first round of PCR with the HB8F and HB6R primers, a nested PCR was performed with the HB2F and HB3R primers. The amplified products (694 bp) ob-

**Table 1.** Clinical characteristics of patients and results of next-generation sequencing

	Patient ID					
	B55	B77	B149	B13	B126	B143
Age, years	40	22	28	77	55	36
Sex	male	male	female	male	female	male
Diagnosis	AC	AC	AC	HCC	HCC	cirrhosis
Viral load, log copies/ml	7.7	7.7	2.7	<2.6	<2.6	3.9
AST, IU/l	74	152	31	74	31	115
$\gamma$ -GTP, IU/l	40	27	17	8	19	30
Genotype	B3	B3	B3	B3	B3	B3
Total reads	1,781,062	1,327,708	1,638,654	1,260,250	1,298,306	1,413,446
Mapping reads	914,396	679,886	837,533	648,284	667,297	726,531
Mean coverage per nucleotide	25,336	18,191	23,372	17,926	18,519	20,589

AC = Asymptomatic carrier; HCC = hepatocellular carcinoma; AST = aspartate aminotransferase;  $\gamma$ -GTP =  $\gamma$ -glutamyl transpeptidase.

tained with the nested PCR were directly sequenced using a Taq Dye Deoxy Terminator Cycle Sequencing Kit on an ABI Prism<sup>®</sup> 3100-Avant Genetic Analyzer (Applied Biosystems). The HBV genotypes were determined using a phylogenetic tree of the S region. Reference sequences were retrieved from the Japan/European Molecular Biology Laboratory, and GenBank DNA databases. Sequences were aligned using ClustalX software ([www.clustal.org](http://www.clustal.org)). Phylogenetic trees were constructed using the neighbor-joining method and bootstrap resampling was performed 1,000 times. The analyses were conducted with Molecular Evolutionary Genetics Analysis (MEGA) software [15].

#### PCR Products and Short-Read Sequencing

The concentration of each nested PCR product (694 bp) was measured using a Qubit dsDNA HS Assay Kit (Q32851; Invitrogen, Carlsbad, Calif., USA). A library of PCR products (<500 bp) of the viral genome (50 or 0.2 ng) was prepared using Nextera DNA Sample Prep Kit (Illumina, San Diego, Calif., USA) or Nextera XT DNA Sample Prep Kit (Illumina), according to the manufacturer's instructions. The PCR products were uniformly sheared into 500-bp fragments using these kits and the PCR product libraries were mixed with 1% 8 pM PhiX as controls, and then run on a MiSeq sequencer (Illumina) for paired-end 151-bp sequencing. The fluorescent images were analyzed using the MiSeq control software and reporter analysis (Illumina) to obtain FASTQ-formatted sequence data.

#### Sequence Read Mapping and Data Analysis

In accordance with the recommendation of Illumina, the sequences were used when the read quality estimated by the Q30 (quality score 30) was over 80% of the consensus sequence. After a quality check and data trimming, Genomics Workbench software version 6.0.1 (CLC bio, Aarhus, Denmark) was used to assemble the sequences. Sequence reads were mapped against the reference HBV genome (AB713528). Single-nucleotide polymorphisms were identified with probabilistic variant detection modules using the default parameters in the mapping algorithm.

The characteristics of the viral quasispecies were evaluated in terms of their genetic complexity based on the number of different sequences present in the population. To achieve this, we used the setting 'read conflicts' in Genomic Workbench. When mapping was completed, conflicts between the sequence reads were annotated on the consensus sequence. The definition of a conflict is a position at which at least one of the sequence reads encodes a different nucleotide.

#### Statistical Analysis

Continuous variables were compared with Student's t test or analysis of variance.  $p < 0.05$  was considered statistically significant. Statistical analyses were performed using SPSS software version 18.0 (SPSS Inc., Chicago, Ill., USA).

## Results

#### RT Genome Alignment

The characteristics of the subjects enrolled in this study are summarized in table 1. We sequenced and analyzed part (encoding amino acids 119–306) of the RT region. Figure 1 shows the deduced protein sequence alignment of the six samples obtained with the direct-sequencing method. The capillary-sequencing method detected no drug-resistant mutations. The phylogenetic tree revealed that all samples examined in this study belonged to genotype B3 (fig. 2).

#### NA-Resistant Mutations Detected with Next-Generation Sequencing

The number of mapping reads in the six samples ranged from 648,284 to 914,396, and the coverage



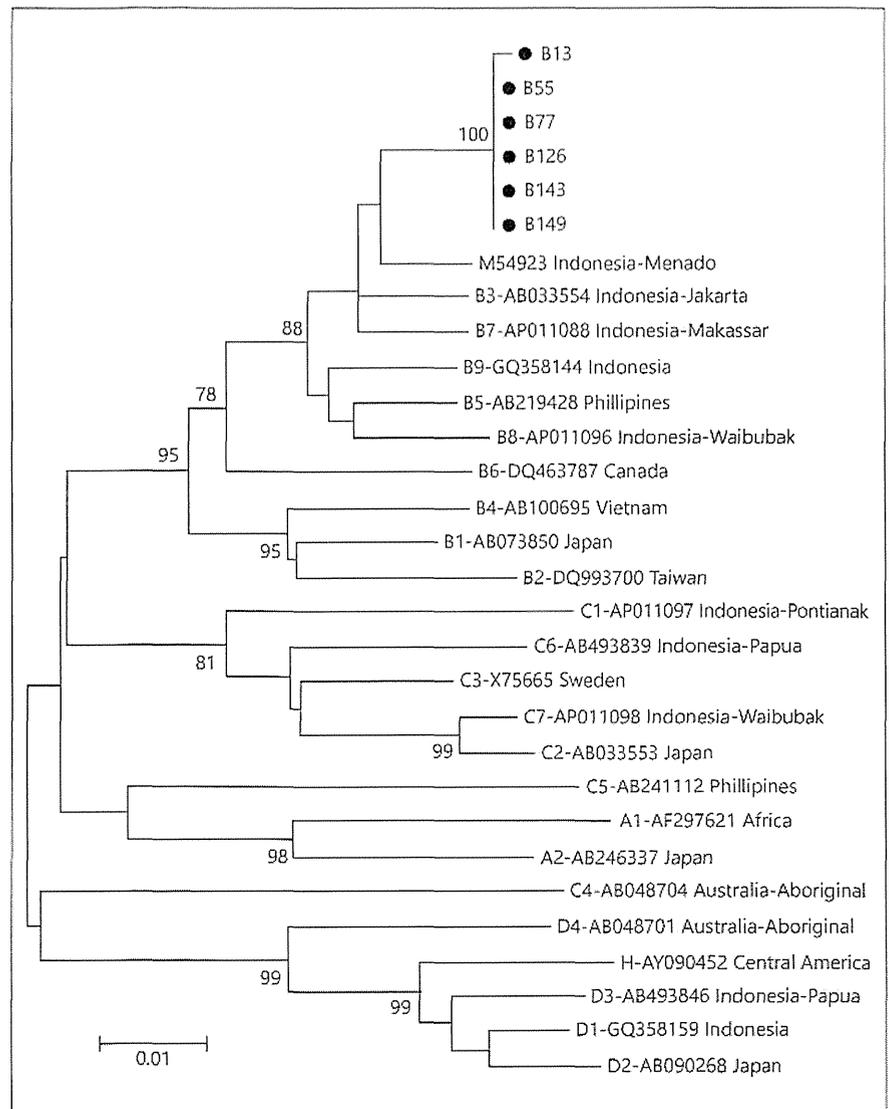
**Fig. 1.** Amino acid sequence alignment of the RT region of the HBV genome. The amino acid sequences were obtained by direct sequencing. The amino acid sequences obtained for the Indonesian treatment-naïve patients in this study (bold) were

compared with those of other Indonesian isolates (top row). The residues within squares show the nine known positions of the NA resistance (rt169, rt173, rt180, rt181, rt184, rt202, rt204, rt236 and rt250).

ranged from 17,926 to 25,366 (table 1). Preexisting NA-resistant mutations in nine known positions of the RT region (rt169, rt173, rt180, rt181, rt184, rt202, rt204, rt236 and rt250) were detected in all 6 patients, although the frequencies of these mutations were low (0.12–1.06%). The frequencies of the amino acid substitutions M204I (conferring resistance to LAM/ETV), A181T (to LAM/ADV), A181V (to ADV) and T184A (to ETV) ranged from 0.91 to 1.06%, and were greater than those of the other amino acid substitutions. The frequencies of M204V (conferring resistance to LAM/ETV), S202G (to ETV), T184S (to ETV), N236T (to ADV) and M250V (to ETV) ranged from 0.48 to 0.69%.

The frequencies of the LAM/ETV-resistant substitutions L180M and I169T ranged from 0.12 to 0.2% (table 2).

The next-generation sequencing method also revealed several other substitutions with frequencies  $\geq 1\%$ : I169M, I169L, V173A, S202R, M204L and N236S (table 3). These mutations have not previously been reported to be associated with NA resistance. Statistical analysis revealed that the prevalence of preexisting NA-resistant mutations was not significantly different between the chronic asymptomatic patients and the patients with advanced liver disease (table 4).



**Fig. 2.** Neighbor-joining phylogenetic tree for HBV strains in Indonesian treatment-naïve patients (indicated with solid circles). The phylogenetic analysis was carried out based on 694 bp of the partial S region. The bootstrap support for the consensus tree, inferred from 1,000 replicates, is indicated for each branch. The evolutionary distances were computed using the maximum composite likelihood method.

## Discussion

The emergence of drug resistance is a serious problem during treatment with NA. The inhibition of HBV replication with NA may lead to the emergence of several mutations that are translated into specific amino acid changes that confer antiviral resistance [16]. Drug-resistant viruses show increased levels of HBV DNA in the liver and blood, a phenomenon known as ‘virological breakthrough’ or ‘clinically induced hepatitis flare’ [17]. The presence of genomic quasispecies may facilitate the emergence of multidrug resistance. Several studies have demonstrated the higher relative sensitivity of ultra-deep

sequencing compared with conventional direct-population sequencing. Therefore, ultra-deep sequencing is useful for detecting low-frequency drug-resistant mutations that cannot be detected with standard methods [18–21]. Conventional sequencing based on the Sanger method can only detect mutations present in >20% of viral quasispecies. Although the line probe assay is another common and convenient method to detect mutations, it can only detect mutations present in >15% of viral quasispecies [20]. Cloning-based sequencing methods are also commonly used, but they usually detect less than 50% of the substitutions identified with ultra-deep sequencing [21]. The relationship between the viral dy-

**Table 2.** Known NA-resistant mutations in the RT region of the HBV genome detected with next-generation sequencing

Position/ substitution	NA resistance	Patient ID						Mean ± SD
		B13	B55	B77	B126	B143	B149	
rt169/I169T	ETV	0.19	0.19	0.2	0.18	0.19	0.19	0.19±0.01
rt173/V173L	LAM	0.31	0.28	0.29	0.24	0.26	0.27	0.28±0.24
rt180/L180M	LAM/ETV	0.12	0.12	0.12	0.11	0.13	0.12	0.12±0.01
rt181/ A181T	LAM/ADV/LdT	0.31	0.3	0.31	0.3	0.27	0.32	0.30±0.17
A181V	ADV/LdT	0.34	0.34	0.36	0.38	0.38	0.38	0.36±0.19
rt184/ T184A	ETV	0.96	0.98	0.99	0.92	0.91	0.94	0.95±0.32
T184S	ETV	0.5	0.49	0.51	0.50	0.48	0.58	0.51±0.01
rt202/S202G	ETV	0.51	0.53	0.54	0.54	0.52	0.53	0.53±0.11
rt204/ M204V	LAM/ETV	0.63	0.63	0.54	0.61	0.57	0.63	0.60±0.27
M204I	LAM/LdT	1.05	1.03	0.59	1.06	1.06	1.05	0.97±0.19
rt236/N236T	ADV	0.67	0.64	0.68	0.69	0.68	0.68	0.67±0.02
rt250/M250V	ETV	0.33	0.33	0.37	0.37	0.35	0.33	0.34±0.02

Values are percentages.

namics assessed by ultra-deep sequencing and the clinical course of the infection has been described in patients with chronic hepatitis C treated with NS3-4 protease inhibitors [22, 23]. In our study, the analysis focused on nine well-known amino acid positions in the HBV polymerase gene that are associated with NA-resistant mutations. Because no drug-resistant mutations were detected with capillary sequencing, it is reasonable to assume that there were no mutations before treatment commencement. However, with deep sequencing, we detected several major drug-resistant mutations, including rtI169T and rtM250V conferring resistance to ETV, rtV173L to LAM, rtL180M to LAM/ETV, rtA181T to LAM/ADV/LdT, rtA181V to ADV/LdT, rtT184A/S to ETV, rtS202G to ETV, rtM204V/I to LAM/ETV/LdT, and rtN236T to ADV. The frequencies of these mutations ranged from 0.1 to 1.06% in all six samples obtained from Indonesian treatment-naïve patients with CHB (table 2). One should be careful about what threshold to use to define a clinically meaningful population. In recent studies, minority variants were defined as differences greater than 0.5 and 1.0% of mutations detected by deep sequencing [24, 25]. In this study, the proportions of the total numbers of reads containing mutations I169L/M, S202R, M204I/L or N236S were >1.0%.

Interestingly, we found no significant differences in the mutation frequencies between the chronic asymptomatic patients and the patients with advanced liver disease ( $p >$

0.05; table 4). The mutation profiles revealed that these major NA-resistant mutations were present before treatment commenced and that the mutation profiles of all 6 patients were similar. In comparison with earlier studies using deep sequencing, the mutational profiles were somehow different. A study from Korea reported that minor populations present in >1% of viral quasiespecies were detected in I169L, L180M and S202G in treatment-naïve patients [11], and a study from the USA revealed other mutational profiles including V173L, T184S and S202G [26]. Mutational profile differences depend on the genotype differences. In general, it was thought that clinical outcomes and prognoses differed among HBV genotypes and sub-genotypes. HBV genotype B is associated with earlier HBeAg seroconversion and better response to interferon therapy than genotype C [27, 28]. Whereas HBV in our study was genotype B, other studies from Korea and the USA were genotypes C and G, respectively [11, 26].

It had been unclear whether genomic variations contributed to the emergence of preexisting NA-resistant mutations. A recent report using deep sequencing revealed that these preexisting mutations were related to therapeutic discontinuation [24]. The next-generation sequencer was first introduced in 2005 and represents a significant advance in DNA sequencing. This method reads several million to billion short DNA fragments per run [29] and more than 1 Gb of the genome can be read in one run [30, 31]. Although next-generation sequencing will inevitably

**Table 3.** Previously unreported mutations at nine positions associated with NA resistance detected with next-generation sequencing

Position/ substitution	Patient ID						Mean ± SD
	B13	B55	B77	B126	B143	B149	
rt169							
I169M	2.12	2.05	1.98	0.34	2.07	2.07	1.77±0.70
I169L	1.15	1.00	1.04	1.05	1.07	1.01	1.05±0.05
I169V	0.52	0.48	0.50	0.48	0.47	0.53	0.50±0.02
I169R	0.21	0.2	0.21	0.23	0.23	0.22	0.22±0.01
I169K	0.12	0.13	0.12	0.12	0.11	0.11	0.12±0.01
rt173							
V173A	0.95	0.98	0.98	0.91	0.96	0.93	0.95±0.02
V173E	0.38	0.35	0.38	0.39	0.42	0.38	0.38±0.02
V173G	0.35	0.35	0.36	0.33	0.35	0.36	0.35±0.01
V173M	0.25	0.26	0.26	0.24	0.24	0.25	0.25±0.01
rt180							
L180F	0.68	0.68	0.67	0.63	0.7	0.63	0.67±0.02
L180S	0.43	0.42	0.37	0.38	0.38	0.38	0.36±0.02
L180W	0.33	0.32	0.34	0.31	0.32	0.35	0.33±0.01
L180V	0.3	0.3	0.28	0.3	0.3	0.3	0.3±0.01
rt181							
A181S	0.32	0.32	0.33	0.31	0.36	0.32	0.33±0.02
A181D	0.18	0.19	0.18	0.21	0.18	0.18	0.19±0.01
A181P	0.16	0.18	0.16	0.17	0.16	0.17	0.17±0.01
A181G	0.14	0.16	0.12	0.16	0.11	0.14	0.14±0.02
rt184							
T184N	0.19	0.18	0.17	0.19	0.2	0.18	0.19±0.01
rt202							
S202R	1.63	1.72	1.75	1.8	1.77	1.72	1.73±0.06
S202I	0.23	0.23	0.25	0.23	0.26	0.23	0.24±0.01
S202N	0.18	0.15	0.18	0.18	0.13	0.18	0.16±0.02
S202T	0.11	0.08	0.1	0.11	0.09	0.11	0.10±0.01
rt204							
M204L	1.53	1.49	1.75	1.55	1.54	1.53	1.57±0.09
M204T	0.45	0.45	0.25	0.43	0.45	0.45	0.41±0.08
M204K	0.15	0.16	0.1	0.15	0.16	0.15	0.15±0.02
M204R	0.44	0.39	0.18	0.38	0.37	0.44	0.37±0.09
rt236							
N236S	1.04	0.95	0.95	0.95	0.97	0.98	0.97±0.03
N236D	0.45	0.45	0.44	0.45	0.42	0.43	0.44±0.01
N236I	0.05	0.43	0.45	0.49	0.42	0.49	0.39±0.17
N236H	0.33	0.36	0.35	0.34	0.34	0.35	0.35±0.01
rt250							
M250I	0.34	0.31	0.36	0.32	0.4	0.36	0.35±0.03
M250R	0.26	0.25	0.24	0.26	0.26	0.25	0.25±0.01
M250L	0.21	0.20	0.22	0.22	0.21	0.23	0.22±0.01

Values are presented as percentages.

make a significant contribution to human genomics, it is also effective in the detection of mutant populations occurring at low frequencies in specific viral genomes. In our study, the method sequenced more than 100,000 DNA fragments, allowing detailed analyses. Frequencies of

1–2% for the substitutions L180M or M204V/I in pretreatment sequences were reported for patients who subsequently developed resistance to LAM [11, 24]. Therefore, a frequency of approximately 1% for the preexisting mutations M204I (conferring resistance to LAM/LdT) and

**Table 4.** NA-resistant mutations in treatment-naïve patients with HBV according to clinical conditions

Position/ substitution	NA resistance	Asymptomatic CHB, %	Advanced liver disease, %	p value
rt169/I169T	ETV	0.193	0.187	0.23
rt173/V173L	LAM	0.280	0.275	0.67
rt180/L180M	LAM/ETV	0.120	0.123	0.19
rt181				
A181T	LAM/ADV/LdT	0.330	0.342	0.28
A181V	ADV/LdT	0.360	0.363	0.72
rt184				
T184A	ETV	0.970	0.930	0.14
T184S	ETV	0.523	0.493	0.49
rt202/S202G	ETV	0.533	0.523	0.35
rt204				
M204V	LAM/ETV	0.623	0.595	0.97
M204I	LAM/LdT	0.890	1.055	0.33
rt236/N236T	ADV	0.667	0.68	0.15
rt250/M250V	ETV	0.340	0.35	0.72

T184A (to ETV) might predict the emergence of mutations conferring resistance to NAs in these patients.

In conclusion, deep sequencing is an effective method to detect minor populations of antiviral drug-resistant mutations in treatment-naïve patients with CHB. Therefore, deep sequencing is a useful method for predicting the future emergence of antiviral drug-resistant HBV during long-term NA therapy. However, the threshold of a clinically meaningful population must be evaluated in future studies. This study shows that the profiles of NA-resistant mutations in treatment-naïve patients with HBV genotype B3 differ from those in similar studies of genotype C patients [11]. Nevertheless, our findings suggest that the genomic variations in HBV might play a crucial role in the subsequent evolution of NA resistance.

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## Disclosure Statement

The authors have no conflicts of interest to declare.

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