However, the chemical structure of entecavir is similar to lamivudine, which resulted in the cross-resistance between lamivudine and entecavir. Recent long-term follow up studies conducted in South Korea and Hong Kong revealed that entecavir reduced liver-related death and HCC[97,98]. Adefovir (Hepsera®) is effective against lamivudine-resistant mutants and add-on therapy of adefovir and lamivudine is common for suppression of lamivudine-resistant mutants. Tenofovir (Viread®) and telbivudine (Sebivo®) are also safe and effective drugs but their introduction to clinical use is still limited. Telbivudine has recently been approved and is being used as a first-line drug in Indonesia. Unlike IFN therapy, meta-analysis revealed no significant difference between genotypes and response to NA[88]. However, as entecavir and telbivudine were introduced recently in developing countries, further studies will be needed to assess their efficacy against the different HBV genotypes/subgenotypes prevailing in those countries.

CONCLUSION

HBV is widespread in Asian countries and contributes to the mortality from HCC. To reduce HBV infection and HCC mortality, appropriate national immunization programs are required in HBV-endemic countries, including Japan. Although HBV infection is predominant and a number of novel genotypes/subgenotypes have been discovered in Asian countries, studies have not been sufficient regarding disease prognosis and antiviral treatment. It is possible that certain genotypes or variants of HBV prevailing in these regions possess stronger pathogenicity and are associated with more severe outcomes of liver diseases. The studies on HBV genotypes related to their pathogenicity in chronic liver diseases, including liver cirrhosis and HCC, and their effects on treatment outcome are awaited with great interest, especially in Southeast Asia, which is the most endemic region of HBV in Asia with unique HBV genotypes/subgenotypes.

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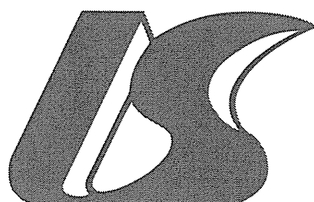

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Mutational diversity of NS5A and NS3 during triple therapy (telaprevir, pegylated-interferon-α 2b and ribavirin) for genotype 1b chronic hepatitis C: The Kobe Hepatitis Therapeutic Group

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Abstract. Telaprevir, a non-structural (NS)3/4A protease inhibitor, is a direct-acting antiviral drug that inhibits viral replication. Triple therapy with telaprevir, pegylated interferon, and ribavirin is a standard therapeutic regimen for patients with genotype 1b chronic hepatitis C virus (HCV) infection and a high viral load. Several factors, including mutations in the NS5A gene, are important predictors of the efficacy of interferon therapy. In this study, we examined the mutational diversity of NS5A and its impact on the efficacy of triple therapy. We enrolled patients with genotype 1b chronic HCV infection and a high viral load (31 males/17 females; mean age, 57.6 years), who were treated with triple therapy. This study was conducted at Kobe University Hospital and at three affiliated hospitals in Hyogo prefecture, Japan, between November 2011 and June 2013. A sustained viral response after 12 weeks (SVR12) was achieved in 37/48 patients (77%). Based on intent-to-treat analysis, SVR12 was significantly greater in patients with the major allele than in those with the minor allele for the IL28B single nucleotide polymorphism (SNP; 88 vs. 56%; $P<0.05$). The prevalence of the V2334I mutation in NS5A was significantly higher in patients who achieved SVR12, while that of G2356E was significantly higher in patients who did not achieve SVR12 ($P<0.05$). Mutations in the NS3 region that are thought to confer resistance to telaprevir were detected in 3/27 patients who achieved SVR12 (Val36, n=3) and in 5/10 patients who did not achieve SVR12 (Val36, n=4; Thr54, n=1). In conclusion, the IL28B SNP and mutations in the NS5A region were associated with the therapeutic response to triple therapy. Half of the patients who did not achieve SVR12 had mutations conferring resistance to telaprevir. However, pre-existing mutations in NS3 did not affect the efficacy of triple therapy.

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Key words: chronic hepatitis C, telaprevir, pegylated interferon/ribavirin therapy, NS5A

Introduction

Hepatitis C virus (HCV) is a major cause of chronic liver disease. An estimated 170 million individuals worldwide are infected with HCV, including 1.5 million individuals in Japan (1,2). The main goal of treatment for chronic HCV is to prevent the progression to cirrhosis and the development of hepatocellular carcinoma by eradicating the virus. Interferon (IFN)-based therapy was first used in 1992 (3), and the treatment regimens have improved since then. In Japan, a combination of pegylated interferon (PEG-IFN) and ribavirin (RBV) has been used since 2004 (4). However, almost half of the patients treated with this combination do not achieve a sustained viral response (SVR), despite long-term therapy (5).

Several factors have been reported to influence the efficacy of PEG-IFN/RBV therapy. Host factors associated with unfavorable outcomes, include advanced age, the female gender and advanced fibrosis. Additionally, the IL28B single nucleotide polymorphism (SNP) has been reported to be a strong genomic predictor of the efficacy of PEG-IFN/RBV therapy, such that the IL28B SNP is routinely examined before beginning any treatment regimen (6). As regards viral factors, a mutation at amino acid 70 of the core region of HCV (core 70) is an important predictor of the therapeutic efficacy (7). The

non-structural (NS)5A protein combines with the core protein in the formation of the viral particle. It has been reported that several regions in domain II of NS5A are associated with the therapeutic efficacy of IFN monotherapy and PEG-IFN/RBV combination therapy. For example, in 1996, it was reported that a large number of mutations in the IFN sensitivity-determining region (ISDR) (amino acids, 2209-2248) were associated with the SVR to IFN monotherapy in Japanese patients with genotype 1b HCV (8). In 2008, it was reported that mutations in the IFN-RBV resistance-determining region (IRRDR) (amino acids, 2334-2379) were also associated with the SVR to combined PEG-IFN/RBV therapy (9).

Telaprevir (TPV), an NS3/4A protease inhibitor, is the first commercially available direct-acting antiviral (DAA) that directly inhibits viral replication. Although TPV/PEG-IFN/RBV combination therapy has achieved a viral eradication rate of up to 70-80% (10), 20-30% of patients did not achieve SVR during treatment due to side-effects (e.g., skin rash and renal dysfunction), loss of compliance and the development of antiviral resistance.

Thus, taking the above data into consideration, we conducted a collaborative study in Kobe, Japan to identify which factors, including NS5A mutations, are associated with the SVR in patients with genotype 1b HCV and a high viral load who were treated with TPV/PEG-IFN/RBV.

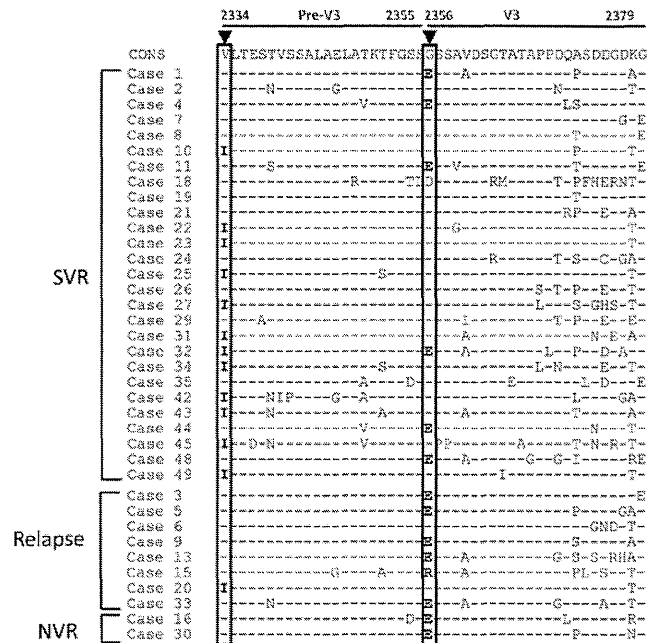

Figure 1. Amino acid alignment in the IFN-RBV resistance-determining region (IRRDR) region. V2334I and G2356E mutations were frequent in patients who achieved SVR12 and in those who did not achieve SVR12, respectively.

Materials and methods

Patients and sample collection. Serum samples were collected from patients with genotype 1b chronic HCV infection and a high viral load. Overall, 48 patients (31 males/17 females; mean ± standard deviation age, 57.7±8.3 years) were enrolled in this study. The patients were treated with TPV/PEG-IFN/RBV combination therapy for 12 weeks followed by PEG-IFN/RBV for 12 weeks. TPV (Mitsubishi Tanabe Pharma Corp., Osaka, Japan) was orally administered at doses of 750 or 500 mg 3 times daily (every 8 h). PEG-IFN (Pegintron®; Schering-Plough, Innishannon, County Cork, Ireland) was subcutaneously injected once weekly (1.5 μg/kg). RBV was orally administered at 400-800 mg daily.

The serum HCV-RNA status was assessed at 4 weeks, at the end of treatment, and at 6 months after the end of treatment. Serum samples were collected and stored at -80°C until virological examination. The rapid virological response (RVR) was defined as undetectable HCV-RNA at 4 weeks. SVR12 was defined as persistent undetectable serum HCV-RNA and normal serum alanine aminotransferase levels at 12 weeks after the end of treatment.

This study was conducted between November 2011 and June 2013 at Kobe University Hospital and at 3 affiliated hospitals in Hyogo prefecture. The study was approved by the Ethics Committee of Kobe University Hospital. Written informed consent was obtained from each patient prior to enrollment in the study.

NS3 and NS5A sequence analysis. HCV-RNA was extracted from 140 μl of serum using a commercial kit according to the manufacturer's instructions (QIAamp viral RNA kit; Qiagen, Tokyo, Japan). The NS3 and NS5A regions of the HCV genome were amplified and sequenced by nested RT-PCR using primer sets, as previously described (9,11). The amino acid sequences were deduced and aligned using Genetyx Win software version 7.0 (Genetyx Corp., Tokyo, Japan).

Statistical analysis. All statistical analyses were performed using SPSS software version 16 (SPSS Inc., Chicago, IL, USA). P-values of <0.05 were considered to indicate statistically significant differences.

Results

Baseline characteristics and on-treatment responses. Among the 48 patients, 22 were treatment-naïve and 26 were receiving IFN-based therapy. Of these latter 26 patients, 17 experienced relapse on prior therapy and 9 were non-responders. As regards the IL28B SNP (rs8099917), 32 patients had the major allele (TT) and 16 patients had the minor allele (TG/GG). Overall, 37/48 patients (77%) achieved SVR12 following triple therapy. The mean age of the patients who achieved SVR12 was less than that of those who did not achieve SVR12, although the difference was not significant. The SVR12 rate was significantly lower in non-responders to previous therapy (44%) than in relapsed patients (82%) and treatment-naïve patients (86%) (P<0.05). The SVR12 rate was significantly higher in patients with the IL28B major allele than in patients with the minor allele (88 vs. 56%; P<0.05).

Effect of mutations in the core protein or NS5A region on therapeutic outcomes. We then sought to identify factors associated with the SVR12 by intent-to-treat analysis. The frequency of mutations amino acid 70 in the core region of HCV and the number of mutations in the ISDR did not differ significantly between patients who achieved SVR12 and

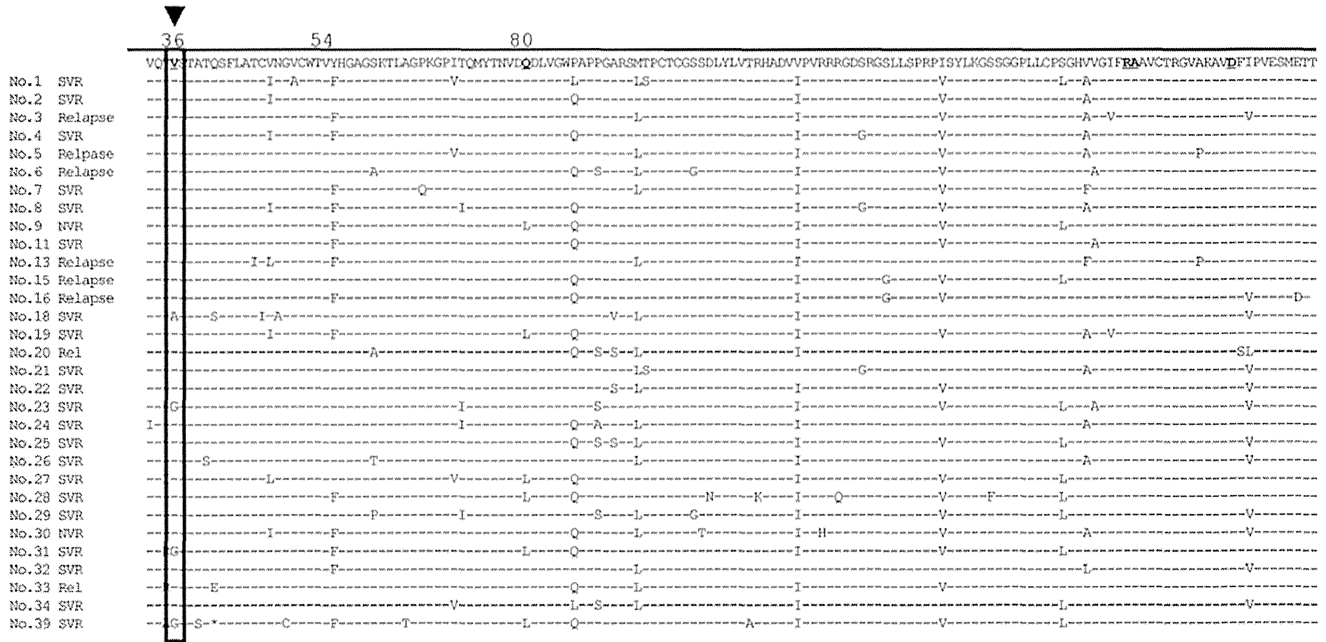

Figure 2. Amino acid alignment in the NS3 region before treatment. Three patients who achieved SVR12 had the Val36 mutation in the NS3 region.

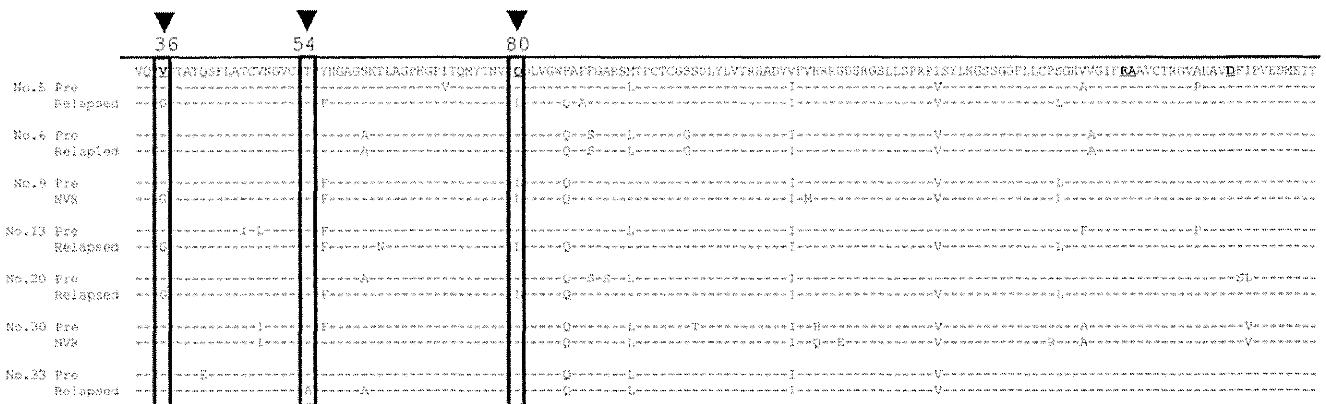

Figure 3. Amino acid alignment in the NS3 region of 7 patients is shown. Alignment before and after treatment was arranged in 2 lines. Four patients who did not achieve SVR12 (case nos. 5, 9, 13 and 20) had Val36 mutations and 1 patient (case 33) had a Thr54 mutation, which are thought to confer resistance to TVR.

those who did not achieve SVR12 (Table I). The amino acid alignment in IRRDR is shown in Fig. 1. The mean number of mutations in the IRRDR did not differ significantly between patients who achieved SVR12 and those who did not achieve SVR12 (5.1±2.9 vs. 4.4±2.2). However, the frequencies of 2 amino acid mutations differed significantly the 2 groups of patients. The V2334I mutation was significantly more frequent in patients who achieved SVR12 than in those who did not achieve SVR12 (44 vs. 10%; $P<0.05$), and the frequency of the G2356E mutation was lower in patients who achieved SVR12 than in those who did not achieve SVR12 (22 vs. 70%; $P<0.05$).

Mutations in the NS3 region before and after treatment. We then examined whether mutations in the NS3 region were associated with antiviral resistance, using sera obtained before and after therapy. Three patients who achieved SVR12 had the Val36 mutation in the NS3 region, which is thought to confer resistance to TVR (Fig. 2). In addition, 5/10 patients who did not achieve SVR12 had mutations conferring resistance to TVR, including 4 patients with Val36 and 1 with Thr54 (Fig. 3).

Discussion

In recent years, many DAAs have been developed and thus clinical trials are examining their efficacy for the treatment of chronic HCV infection (12,28). TPV was approved in Japan in November 2011, and the triple therapy (TVR/PEG-IFN/RBV) has become a standard regimen for patients with genotype 1b chronic HCV infection with a high viral load. In the US, TVR was approved by the Food and Drug Administration in May 2011 for use in combination with PEG-IFN/RBV for the treatment of genotype 1b chronic HCV infection (13). The SVR rate in patients with genotype 1 HCV treated with triple therapy was 61-69% in the PROVE1 and PROVE2 studies published in

Table I. Comparison between patients who achieved SVR and those who did not (no SVR).

Factor	SVR	No SVR	P-value
Number of patients	37 (77%)	11 (23%)	
Age	56.0±9.2	59.5±12.4	NS
Gender (M/F)	24/13	7/4	NS
Previous therapy			
Naïve	19 (86%)[a]	3 (14%)	
Relapse	14 (82%)[a]	3 (18%)	
NVR	4 (44%)	5 (56%)	
IL28B SNP			
TT	28 (88%)	4 (12%)	<0.05
TG/GG	9 (56%)	7 (44%)	
Core 70 wild	17/28	5/11	
ISDR ≥1	5/16 (31%)	4/9 (44%)	NS
IRRDR ≥4	19/27 (70%)	6/10 (60%)	NS
V2360A and/or K2378T	17/27 (63%)	5/10 (50%)	NS
V2334I	12/27 (44%)	1/10 (10%)	0.039
G2356E	6/27 (22%)	7/10 (70%)	0.016

[a]P<0.05, compared to NVR. SVR, sustained viral response; SNP, single nucleotide polymorphism; NS, not significant; NVR, no viral response; ISDR, IFN sensitivity-determining region; IRRDR, IFN-RBV resistance-determining region.

2009 (10,14). Since then, several clinical studies have confirmed the effectiveness of TVR-based therapy and it has now become a standard component of therapeutic regimens worldwide (15,16). In our study, the SVR12 rate was 77%, which is as high as that in earlier studies. However, 31% of patients classified as non-responders to prior therapy achieved SVR during treatment with a TVR-based regimen in the REALIZE trial published in 2012 (17). We obtained similar results in our study, as the SVR12 was significantly lower in non-responders (44%) than in relapsed patients (82%) and treatment-naïve patients (86%) (P<0.05).

The NS3 protein is approximately 67 kDa and has serine proteinase activity. The NS3 protein forms a complex with NS4 and serves to process NS4 and NS5 proteins. NS3/4A protease inhibitors exhibit strong antiviral effects as the NS3/4A protease activity is necessary for the lifecycle of HCV. Although TVR is very effective, it is frequently associated with side-effects, including skin rash and renal dysfunction, which lead to treatment discontinuation. Therefore, it is essential to increase compliance to improve the therapeutic efficacy of TPV. In this study, 7/48 patients discontinued treatment due to side-effects and 4 of these patients experienced disease relapse. Accordingly, it will be important to investigate how to maintain compliance, particulalry among older patients.

Although TVR-based therapy is highly effective, approximatley 30% of patients do not achieve SVR, despite triple therapy. In trials of boceprevir-based therapy, it was found that RVR and the IL28B SNP were predictive of SVR (18). Similarly, Chayama *et al* reported that RVR, the IL28B SNP, and the response to prior therapy were predictors of SVR during triple therapy (19). In terms of viral factors, it has been reported that substitutions of amino acid 70 in the core region of HCV genotype 1b were significant predictors of SVR (20). In our study, IL28B and the response to prior therapy were significant predictors of SVR.

Mutations in several amino acids in the NS5A protein have been reported and appear to play an important role in the response to IFN. HCV NS5A is a zinc-containing phosphoprotein that regulates HCV RNA replication and particle production. A previous study using bioinformatics-assisted modeling suggested that there were 3 principal domains (21), with domain I (amino acids, 1973-2185) located in the N-terminal region, and domain II (amino acids, 2222-2314) and domain III (amino acids, 2328-2419) in the C-terminal region. Another study revealed that domain III was important for HCV particle formation (22). We have also previously reported that IRRDR in domain III is strongly associated with SVR (23). Although the number of ISDRs and IRRDRs did not affect the therapeutic efficacy of triple therapy in this study, 2 novel mutations were potentially associated with SVR. V2334 is located in the putative nuclear localization signal sequence (PPRKKRTVV; amino acids, 2326-2334) within the C-terminal region of NS5A (24). This mutation may affect the sensitivity to antiviral therapy by changing the transition of HCV during intracellular localization. Another study suggested that a specific C-terminal region (amino acids, 2350-2419) is involved in basal phosphorylation (25). G2356 is located in this region and may affect cellular signaling mechanisms by altering NS5A phosphorylation.

TPV has a covalent linear structure and is a first-generation NS3/4A inhibitor. Several mutations, including V36A/M/L, T54A/S, R155K/M/S/T, A156S and A156T/Y, have been reported to confer resistance to TPV (26). In this study, we also examined mutations associated with resistance to antiviral drugs. Using direct sequencing analysis, we found that 3 patients had the Val36 mutation in NS3 before therapy, which may confer resistance to TVR. However, all of these patients achieved SVR12, which suggests that antiviral therapy should not be contraindicated in patients with mutations conferring low levels of resistance, such as Val36 and Thr54. Simeprevir, which should soon be available for the treatment of genotype 1 chronic HCV infection (27), is a non-covalent macrocyclic inhibitor and is classified as a second-generation inhibitor with a different resistant profile to first-generation inhibitors. While the Q80 and D168 mutations are specific to non-covalent peptidomimetic inhibitors, Arg155 and A156 confer cross-resistance to all proteinase inhibitors (28). Although the Val36 and Thr54 mutations were detected in relapsed patients and in non-responders of the present study, we found none of the cross-resistant mutations. These patients may benefit from second-generation DAAs, but it is important to determine the presence of mutations that may confer resistance to these drugs.

Although TVR-based IFN therapy is effective, this treatment regimen is often limited by the side-effects of IFN. It is necessary that future therapies should be associated with greater SVR, greater compliance, shorter treatment duration, less viral resistance and better safety profiles than existing

drugs. Previous studies demonstrated that dual oral therapy with daclatasvir, a NS5A inhibitor, and asnaprevir, an NS3 protease inhibitor, was well tolerated and the SVR was high (29,30). Based on these results, we predict that a combination of two or more DAAs could achieve complete viral clearance in all patients with chronic HCV infection.

In conclusion, the IL28B SNP is strongly associated with SVR in patients receiving TVR/PEG-IFN/RBV triple therapy. Mutations in V2334 and G2356 are potential viral factors associated with the therapeutic efficacy of this regimen. Mutations in NS3 were found in approximately half of patients who did not achieve SVR and may confer resistance to second-generation proteinase inhibitors.

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High rate of seronegative HCV infection in HIV-positive patients

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Abstract. Co-infection with human immunodeficiency virus (HIV) and hepatitis C virus (HCV) is a significant global health problem. The two viruses are transmitted with high efficacy via blood-to-blood contact, mainly intravenous drug use (IVDU), whereas HCV is less easily transmitted sexually. Antibody testing is the main screening method for HCV infection, although it may not be the optimal option for HIV infection. The aim of this study was to investigate HCV infection in HIV-positive patients, with and without a detectable anti-HCV antibody response. A total of 187 plasma samples were obtained from HIV-positive patients in Surabaya, Indonesia and examined for anti-HCV [HCV enzyme immunoassay (EIA) 3.0], HCV genotype/subtype [reverse transcription-polymerase chain reaction (RT-PCR) using primers targeting a part of NS5B/5'UTR followed by sequencing] and HCV viral load (quantitative RT-PCR). A total of 119 patients (63.6%) were found to be anti-HCV-positive and, among these, HCV RNA was detected in 73 (61.3%), with HCV-1a as the predominant subtype (31.5%). Of the 68 anti-HCV-negative samples, HCV RNA was detected in 26/68 (38.2%) mostly as the HCV-3a subtype (50%). High HCV viral loads were more common among the HCV-seropositive patients. The HCV-seropositive samples with detected HCV RNA were mostly obtained from HIV-positive patients with parenteral transmission (IVDU) (76.7%); however, the HCV-seronegative samples with detected HCV RNA were mostly from patients who had acquired HCV through heterosexual transmission (61.5%). In conclusion, HIV-positive patients were at high risk of becoming co-infected with HCV and several remained HCV-seronegative. Furthermore, there may exist differences in HCV seropositivity and subtypes between HIV-positive patients who acquired HCV sexually and those who acquired HCV parenterally.

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Introduction

The epidemic of human immunodeficiency virus (HIV) infection in Asia, including Indonesia, is rapidly expanding (1). The introduction of highly active antiretroviral therapy (HAART) has markedly reduced HIV-related morbidity and mortality. However, non-HIV-related conditions, particularly liver disease, currently constitute an increasingly high proportion of the causes of mortality among HIV-infected individuals (2). Hepatitis C virus (HCV) has emerged as an important cause of morbidity and mortality among HIV-positive individuals (3).

As the majority of individuals who acquire HCV are asymptomatic, it is difficult to determine some of the characteristics of acute infection (4). Early diagnosis is rare and the extent of this epidemic is unknown, since the majority of at-risk individuals are not tested for acute HCV infection (5). These and several other aspects of HCV infection may be further complicated by co-infection with HIV-1. In HIV-infected individuals, untreated acute HCV infection typically progresses to chronic HCV infection, a leading cause of non-AIDS-related morbidity and mortality among HIV-infected individuals in the HAART era (2).

HIV and HCV share common transmission pathways, which may explain the high rate of co-infection with the two viruses. Of the 33.4 million HIV-infected individuals worldwide in 2008, it is estimated that ≥5 million have concomitant HCV infection. Whereas the two viruses are transmitted with high efficacy via blood-to-blood contact [particularly in intra-

venous drug users (IVDUs)], HCV is less easily transmitted sexually and its risk remains controversial (6).

Antibody testing is the main screening method for HCV infection (7). However, serological screening in HIV-infected patients may not be the optimal screening method, possibly as a result of immunosuppression (8). Therefore, HCV RNA testing is recommended for the diagnosis of HCV infection (8,9).

The aim of this study was to investigate HCV infection in anti-HCV-positive and -negative HIV patients in Surabaya, Indonesia.

Materials and methods

Collection of field samples. Plasma samples were obtained from HIV-positive patients, who visited the Institute of Tropical Disease (ITD), Airlangga University, Surabaya, Indonesia, for an HIV viral load examination requested by a clinician. The majority of the patients (176/187, 94%) were on HAART with activity against AIDS (lamivudine+zidovudine+efavirenz or lamivudine+zidovudine+nevirapine) and exhibited no symptoms of acute hepatitis. The plasma samples were stored at -80˚C prior to examination. The study protocol was reviewed and approved by the Ethics Committees of Kobe University, Japan and Airlangga University, Indonesia and informed consent was obtained from all the patients. The HIV viral load data were retrieved from the patient database maintained at ITD, Airlangga University, Indonesia.

Anti-HCV tests. All the plasma samples were subjected to HCV enzyme immunoassay (EIA) 3.0 (Hepalisa Anti HCV; PT Indec Diagnostics, Jakarta, Indonesia) to detect anti-HCV, according to the manufacturer's instructions. The third-generation anti-HCV test which detects multiple antigenic determinants (core, NS3, NS4 and NS5) was used to increase sensitivity.

Viral RNA extraction, reverse transcription-polymerase chain reaction (RT-PCR) amplification and sequencing. HCV RNA was extracted from 140 μl plasma using a commercially available kit (QIAamp Viral RNA kit; Qiagen, Tokyo, Japan).

For the amplification of the NS5B region of the HCV genome, the extracted RNA was reverse-transcribed and amplified using SuperScript One-Step RT-PCR (Invitrogen, Tokyo, Japan) and a set of primers. The reaction was initially performed at 45˚C for 30 min for RT and at 94˚C for 2 min, followed by the first-round PCR over 40 cycles, with each cycle consisting of denaturation at 94˚C for 1 min, annealing at 45˚C for 1.5 min and extension at 72˚C for 2 min, using outer primers 166 (5'-TGGGGATCCCGTATGATACCCGCTGCTTTGA-3', nt 8230-8260, +) and 167R (5'-GGCGGAATTCCTGGTCATAGCCTCCGTGAA-3', nt 8601-8630, -). All the PCRs were performed using Platinum Taq DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA). The second-round PCRs were performed under the same conditions to amplify the HCV genome, using different sets of primers as follows: i) HC23 (5'-TTTGACTCAACCGTCACTGA-3', nt 8256-8275, +), HC24 (5'-CTCAGGCTCGCCGCATCCTC-3', nt 8577-8596, -) and HC26 (5'-CTCAGGTTCCGCTCGTCCTC-3', nt 8577-8596, -); ii) HC15 (5'-ACTGTCACTGAACAGGACAT-3', nt 8265-8284, +) and HC16 (5'-GCTCTATCCTCATCGACGCC-3', nt 8568-8587, -); iii) HC23 and HC28 (5'-CACGAGCATGGTGCAGTCCCGGAGC-3', nt 8507-8531, -); iv) HC23 and HC32 (5'-AGGTAGCACGTCAGCGTGTTTCC-3', nt 8454-8476, -); and v) HC23 and HC34 (5'-TAGCACGTCATGGTGTTTCCCAT-3', nt 8451-8473, -) (10). If the result of amplification of the NS5B region was negative, the extracted RNA was reverse transcribed and amplified using the same condition but using a different set of primers as follows: i) UTR1 (5'-CCGGGAGAGCCATAGTGGTC-3', +) and UTR2 (5'-AGTACCACAAGGCCTTTCGC-3', -) (first-round PCR); ii) UTR3 (5'-TGGTCTGCGGAACCGGTGAG-3', +) and UTR4 (5'-ACCCAACACTACTCGGCTAG-3', -) (second-round PCR) (11).

The PCR products were electrophoresed in a 2% agarose gel containing ethidium bromide and were visualized under UV illumination. Amplified cDNA fragments were sequenced by a direct sequencing method with the BigDye Terminator v1.1 Cycle Sequencing kit and an ABI Prism 310 sequencer (Applied Biosystems, Foster City, CA, USA).

Sequence analysis. Each obtained sequence was compared to those of the reported subtypes and on the basis of the percent homologies on the NS5B region, each isolate was assigned a subtype (10,12). When a subtype assignment was not possible due to the lack of NS5B amplification, the nucleotide sequences of the 5'UTR region were determined and compared with the consensus sequence motifs for each of the major genotypes previously reported (13).

Quantification of plasma HCV RNA titers. The quantification of plasma HCV RNA titers was performed with TaqMan® Gene Expression Master Mix (Applied Biosystems) using Applied Biosystems 7300 Real-Time PCR system.

Results

Samples. A total of 187 plasma samples were obtained from 187 HIV-positive patients (153 men and 34 women; mean age, 29.4 years; age range, 3 months-58 years). The risk factors for infection were IVDU (62.6%), heterosexual and homosexual intercourse (35.8 and 1.1%, respectively) and vertical transmission (0.5%) (Table I).

A total of 119 patients (63.6%) were anti-HCV-positive and the remaining 68 (36.4%) were anti-HCV-negative. The group of HCV-seropositive patients was most likely (73.1%) to have acquired HCV through parenteral transmission (IVDU), followed by heterosexual transmission (26.9%) (Table I).

HCV RNA detection. HCV RNA was detected in 73 of the 119 anti-HCV-positive samples (61.3%); among these, HCV-1a (31.5%) was the predominant subtype, followed by 3a (23.3%), 1 (16.4%), 1c (10.9%), 1b (9.6%), 3k (4.1%) and 2, 2a and 4a (1.4% each). Of the 68 anti-HCV-negative samples, HCV RNA was detected in 26 (38.2%), with HCV-3a being the most prevalent subtype (50%), followed by 3k (23.1%), 1c (15.4%) and 1b (11.5%) (Table II). An HCV viral load of >100,000 IU/ml was more commonly observed among the group of HCV-seropositive and HCV RNA-positive patients (33/73,

Table I. Modes of transmission according to the anti-HCV status.

	Modes of transmission (%)				
Anti-HCV	IVDU	Heterosexual	Homosexual	Vertical	Total (%)
Positive	87 (73.1)	32 (26.9)	-	-	119 (63.6)
Negative	30 (44.1)	35 (51.5)	2 (2.9)	1 (1.5)	68 (36.4)
Total	117 (62.6)	67 (35.8)	2 (1.1)	1 (0.5)	187 (100)

HCV, hepatitis C virus; IVDU, intravenous drug use.

Table II. Distribution of HCV genotypes/subtypes among samples with detected HCV RNA according to the anti-HCV status.

	HCV genotypes/subtypes (%)								
Anti-HCV	1	1a	1b	1c	2	2a	3a	3k	4a
Positive (n=73)	12 (16.4)	23 (31.5)	7 (9.6)	8 (10.9)	1 (1.4)	1 (1.4)	17 (23.3)	3 (4.1)	1 (1.4)
Negative (n=26)	-	-	3 (11.5)	4 (15.4)	-	-	13 (50)	6 (23.1)	-

HCV, hepatitis C virus.

Table III. Modes of transmission among samples with detected HCV RNA according to the anti-HCV status.

	Modes of HCV transmission (%)		
Anti-HCV	IVDU	Heterosexual	Homosexual
Positive (n=73)	56 (76.7)	17 (23.3)	-
Negative (n=26)	9 (34.6)	16 (61.5)	1 (3.9)

HCV, hepatitis C virus; IVDU, intravenous drug use.

Table IV. HIV viral load among anti-HCV-positive and HCV RNA-positive patients vs. anti-HCV-negative and HCV RNA-positive patients.

HIV viral load (copies/ml)	Anti-HCV (%)	
	Negative	Positive
≥100,000	14 (53.9)	41 (56.2)
<100,000	7 (26.9)	27 (37.0)
Undetected	5 (19.2)	5 (6.8)
Total	26	73

HIV, human immunodeficiency virus; HCV, hepatitis C virus.

45.2%) compared to the group of HCV-seronegative and HCV RNA-positive patients (1/26, 3.9%).

The majority of HCV-seropositive samples with detected HCV RNA were obtained from HIV-positive patients who had acquired HCV through parenteral transmission (IVDU) (56/73, 76.7%), followed by those with a history of heterosexual transmission (17/73, 23.3%). However, HCV-seronegative samples with detected HCV RNA were mostly obtained from HIV-positive patients with a history of heterosexual transmission (16/26, 61.5%), followed by those with parenteral (IVDU) (9/26, 34.6%) and homosexual transmission (1/26, 3.9%) (Table III).

HIV viral load. High HIV viral loads (≥100,000 copies/ml) were mostly detected among the group of HCV-seropositive and HCV RNA-positive patients (41/73, 56.2%) and the group of HCV-seronegative and HCV RNA-positive patients (14/26, 53.9%) (Table IV).

Discussion

HIV/HCV co-infection affects over one-third of HIV-infected individuals worldwide (14). Following the introduction of HAART, HCV infection has been considered as the principal cause of morbidity and mortality among HIV-infected individuals (15). Co-infected patients exhibit a higher mortality rate compared to singly HIV-infected and HCV infection is considered a predictor of mortality (16). Despite these emerging trends, screening for HCV in HIV-infected patients is not routinely performed, since HCV is perceived as a slowly progressive disease, which would be unlikely to affect the natural history of HIV and associated opportunistic infections (4). In Indonesia, the rapidly increasing number of new HIV infections makes the epidemic one of the fastest growing in Asia (1). However, the incidence rate of HCV infection among HIV-positive patients has not yet been determined in Indonesia. We examined repositoried blood specimens obtained from HIV-positive patients in Surabaya to identify and describe cases of newly acquired HCV infection.

Antibody testing is the main screening method for HCV infection in HIV-infected individuals (7). Tests available for the diagnosis of HCV infection in the acute phase vary in sensitivity and the third-generation antibody ELISA tests were developed to detect multiple antigenic determinants (core, NS3, NS4 and NS5) to increase the sensitivity. In this study, the result of anti-HCV tests using EIA 3.0 demonstrated that, of the 187 plasma samples obtained from HIV-positive patients, 119 (63.6%) were anti-HCV-positive and the remaining 68 (36.4%) were anti-HCV-negative (Table I). However, serological screening in HIV-infected patients may not be the optimal screening method, possibly as a result of immunosuppression (8).

In our study, the group of HCV-seropositive patients was more likely (73.1%) to have acquired HCV through parenteral transmission (IVDUs), followed by heterosexual transmission (26.9%) (Table I). Individuals infected with HIV are frequently co-infected with HCV due to the shared modes of transmission (9). The risk of HCV transmission is significantly higher for patients who acquire HIV infection parenterally compared to those who acquire it sexually. The role of homosexual and heterosexual transmission of HCV remains controversial; it is considered to occur, although with a low efficiency. The sexual route is a common mode of HIV transmission, although it is not as effective for HCV (17). A previous study reported that ≤90% of HIV-positive IVDUs tested positive for HCV antibodies (18), although only 4-8% of the individuals who acquire HIV through sexual contact have detectable HCV antibodies (19,20). In our study, the heterosexual transmission history in HCV-seropositive patients (26.9%) was significantly higher compared to previously reported findings, which may be due to certain mechanisms involved in HCV transmission between sexual partners, particularly those who engage in habits that are associated with a high risk of virus transmission. Transmission may also result from exposure to unreported parenteral risk factors or from sharing certain personal items, such as toothbrushes or razors, which may result in accidental exposure to the partner's blood (21).

In HIV/HCV co-infection, abnormal antibody and cellular immune responses to HCV have been described (22-24). Immunosuppression by HIV infection may impair antibody formation and false-negative HCV antibody tests have been reported in individuals co-infected with HIV (22,25). Loss of HCV antibodies is observed in rare cases with advanced immune deficiency in HIV/HCV co-infection and does not necessarily indicate viral clearance (23). Identifying HCV seroconversion in serial samples is suboptimal, since the antibody development may be delayed in HIV-infected individuals to >1 year after the initial infection (26), compared to HIV-negative patients, who generally produce antibodies to HCV within 6 weeks of infection (27). This delay in the formation of antibodies may result in a significant delay in the diagnosis of HCV in HIV-positive individuals and the patients may miss the opportunity to receive effective treatment for their infection (59 vs. 40% success rate) (28,29). The European AIDS Clinical Society co-infection guidelines endorse an approach that should be considered, with serological testing for HCV for all patients upon entry into HIV care and annually thereafter for HCV-uninfected individuals, with HCV RNA testing for all HCV antibody-negative patients exhibiting an unexplained increase in alanine transaminase levels and at high risk for HCV infection (IVDU, mucosal trauma during intercourse) (30). Matthews and Dore (31) suggested that consideration should be given to HCV RNA testing, despite a negative HCV antibody status, in cases of unexplained transaminase elevation in patients with CD4 cell counts <200/mm^3 when acute hepatitis C is suspected. Frequent serum HCV RNA testing is thus a possible screening strategy (32). Of note, the HCV antibody status may be negative, despite active HCV viremia, in 10-15% of immunosuppressed patients (31).

Our study demonstrated that HCV RNA was detected in 73 (61.3%) of the 119 anti-HCV-positive samples; of the 68 anti-HCV-negative samples, HCV RNA was detected in 26 (38.2%) (Table II). In the HCV-seropositive and HCV RNA-positive group of patients, a high HCV viral load (>100,000 IU/ml) was more frequently detected (33/73, 45.2%) compared to the HCV-seronegative and HCV RNA-positive group of patients (1/26, 3.9%). Higher HCV viral loads may also be responsible for the increased transmissibility, as has been noted in HIV-co-infected patients (17); in this study, they were mostly found among anti-HCV-positive patients. A previous study demonstrated that HCV RNA was detected more frequently among the 31 HCV-seropositive patients (84.4% of tests) compared to the 20 HCV viremic HCV-seronegative patients (51.5% of tests) (9). Another study reported that HCV RNA was detected in the blood of >80% of HIV-positive individuals who were positive for HCV antibodies (33). Those findings are consistent with those of our study, according to which HCV RNA was highly detected among HIV-positive and HCV-seropositive patients, but was also relatively highly detected among HIV-positive and HCV-seronegative patients. There was a predominantly high HIV viral load (≥100,000 copies/ml) among the HCV-seropositive and HCV RNA-positive patients (41/73, 56.2%) and also among HCV-seronegative and HCV RNA-positive patients (14/26, 53.9%) (Table IV), which may have contributed to these results. As previously reported, patients with higher HIV RNA titers tend to have lower CD4 cell counts. The impaired T-helper type 1 immune response in turn may alter the response of immune cells to HCV, permitting greater HCV replication (34,35).

IVDU continues to be the primary risk factor for acquisition of HCV/HIV co-infection, with HCV usually acquired within the first year of IVDU (36); however, patients with a sexual transmission history generally acquired HIV prior to HCV (3,37). It was previously suggested that the presence of HIV may increase the heterosexual transmission of HCV (20). HCV infection appears to occur more frequently among HIV-infected HCV-seronegative individuals than appreciated, particularly if HIV acquisition was through sexual as opposed to parenteral risk factors (9). Those findings confirmed the results of our study, demonstrating that the majority of the HCV-seronegative samples with detected HCV RNA were obtained from HIV-positive patients with heterosexual transmission history (61.5%), differed from HCV-seropositive samples with detected HCV RNA, which were mostly obtained from HIV-positive patients with parenteral transmission (IVDU) history (76.7%) (Table III). However, men engaging in sexual intercourse with other men did not appear to exhibit an overall increased risk for co-infection (38-40), although

epidemics of acute HCV were previously described among HIV-infected men engaging in sexual intercourse with other men exhibiting high-risk behaviors (41). The two HIV-infected homosexual males included in this study were anti-HCV-negative, although HCV RNA was detected in one of them (data not shown). HCV/HIV co-infection among homosexual men requires further investigation.

The distribution of HCV genotypes/subtypes in HIV-positive patients reflects the route of transmission. HCV RNA was detected in 73 (61.3%) of the 119 anti-HCV-positive samples and, among these, HCV-1a (31.5%) was the predominant subtype, followed by 3a (23.3%), 1 (16.4%), 1c (10.9%), 1b (9.6%), 3k (4.1%) and 2, 2a and 4a (1.4% each). Of the 68 anti-HCV-negative samples, HCV RNA was detected in 26 (38.2%), with HCV-3a being the most prevalent subtype (50%), followed by 3k (23.1%), 1c (15.4%) and 1b (11.6%) (Table II). HCV subtypes 1a and 3a were more commonly reported among IVDUs (42). The high incidence of HCV-3a in the group with a history of heterosexual transmission may be attributed to the sexual partners, who were IVDUs; however, this hypothesis requires further investigation. Considering the differences in the predominant transmission mode between HCV-seropositive and -seronegative individuals, it is likely that the mode of HCV transmission is associated with the HCV subtypes.

In conclusion, HIV-positive patients are at a high risk of becoming co-infected with HCV, several of whom may remain HCV-seronegative, partly due to their immunodeficiency status. These data may also suggest that there may exist differences in HCV seropositivity and subtypes between HIV-positive patients who acquired HCV sexually and those who acquired HCV parenterally. It seems prudent to consider HCV infection in HIV-positive patients who test negative for HCV antibodies, as delayed HCV seroconversion in HIV-positive patients may result in delayed diagnosis and treatment.

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Predominance of precore mutations and clinical significance of basal core promoter mutations in chronic hepatitis B virus infection in Indonesia

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Abstract. Chronic hepatitis B virus (HBV) infection is a major health problem worldwide, with a particularly high prevalence in the Asian-Pacific region. During chronic hepatitis B virus (HBV) infection, mutations commonly occur in the basal core promoter (BCP) and precore (PC) regions of HBV, affecting HBeAg expression, particularly following HBeAg seroconversion. Mutations in the B- and T-cell epitopes of the HBV core have also been observed during disease progression. The clinical significance of HBV genome variability has been demonstrated, however the results are a subject of controversy. Considering the characteristics of the virus associated with geographical location, the profiles of BCP, PC and core mutations and their clinical implications in patients with chronic HBV infection in Surabaya, Indonesia, were investigated. The BCP, PC and core mutations and HBV genotypes were detected by direct sequencing. The HBeAg/anti-HBe status and HBV DNA levels were also assessed. This study enrolled 10 patients with chronic HBV infection (UC) from Dr Soetomo General Hospital and Indonesian Red Cross, Surabaya, East Java, Indonesia, 10 patients with chronic hepatitis B and liver cirrhosis (LC) and 4 patients with chronic hepatitis B and hepatocellular carcinoma (HCC) from Dr Soetomo General Hospital. The PC mutation A1896 was predominant in all the groups (60-100%), together with the PC variant T1858, which was associated with HBV genotype B. The number of detected core mutations (Thr/Ser130) was higher in HCC patients (50%). However, the BCP mutations T1762/A1764 were predominant in LC patients (50-60%). The LC and HCC patients carried HBV isolates with additional mutations, at least at BCP or PC, mainly following HBeAg seroconversion. In the majority of anti-HBe-positive samples, the BCP T1762/A1764 mutations were associated with a high viral load, regardless of the PC 1896 status. In conclusion, the PC mutations were found to be predominant in all the groups. However, the BCP mutations were mainly detected in the LC group and may be considered as a critical indicator of a poor clinical outcome.

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Introduction

Chronic hepatitis B virus (HBV) infection is a major clinical problem worldwide. It is particularly important in the Asian-Pacific region where the prevalence of HBV infection is high (1), including Indonesia, which belongs to the moderate-to-high hepatitis B endemic regions (2,3). Chronically infected patients exhibit a wide spectrum of clinical presentations, ranging from an asymptomatic carrier state to chronic active hepatitis B with progression to liver failure, liver cirrhosis (LC) or hepatocellular carcinoma (HCC) (4,5). It has been hypothesized that the genetic variability of the virus, which affects its expression of viral antigens, may also affect the outcome of the infection (6). However, the results of a previous molecular study are a subject of controversy and these issues require further elucidation (7).

It has been suggested that HBeAg may serve as a decoy to buffer the anti-core protein immune response or to induce immune tolerance in perinatally infected individuals. However, the anti-HBe immune response results in an efficient reduction

of viral load and thereby provides a strong pressure toward viral variants with reduced or no HBeAg expression (8,9). Two major types of HBV variants that frequently occur and affect the expression of HBeAg are the precore (PC) and the basal core promoter (BCP) mutants (9). The most frequently detected PC mutation is a nucleotide (nt) transition at the codon 28 (A1896) which converts into a TAG stop codon and eliminates HBeAg expression (10,11). Previously reported evidence suggested that the pattern of PC mutation is restricted by the secondary structure requirements of the ε encapsidation signals which are essential for viral replication. In order to stabilize this ε structure, the nt at position 1,896 is paired with the nt at position 1,858, which is naturally specific for certain HBV genotypes (12,13). The most common BCP mutations, A1762T and G1764A, result in a decrease of HBeAg expression, mediated by reduced transcription of PC RNA, but enhance viral replication (8,14,15). Occurrence of these mutations and any additional BCP mutations may confer increased replication efficiency to the virus (9,16). These changes were previously considered to be associated with the HBeAg-negative phenotype. However, more recent studies demonstrated that they may also be found in certain HBeAg-positive patients, particularly those with chronic hepatitis. These BCP mutations may be detected with or without PC mutations (17-19). Core mutations, particularly amino acid (aa) at position 130 exposed on the surface of mature HBeAg, were observed in the course of disease progression and accumulate in the B- and T-cell epitopes (20,21). These findings emphasize the possibility that the mutations in the T- or B-cell epitopes exert a significant effect on T-cell function or subsequent cytokine release and on the association between the host immunological reaction and viral persistence.

A previous study suggested that HBeAg mutations are associated with chronic hepatitis, LC and HCC (13). However, conflicting evidence suggested that the HBeAg mutations are present in HBV carriers and in those individuals with mild forms of HBV infection or without liver disease (7). In addition, several core mutations have not been proven to result in loss of immune recognition (22).

The aim of this study was to analyze the profiles of BCP, PC and core mutations associated with the HBeAg/anti-HBe status and HBV DNA load in patients with chronic HBV infection at various phases in Surabaya, Indonesia. The characteristic of the HBV isolates associated with geographical location may play an important role in these results.

Materials and methods

Patients and controls. Three groups of patients were investigated: i) the UC group included patients who had been HBsAg-positive for >6 months, although without any clinical significance, from Dr Soetomo General Hospital, Surabaya, Indonesia and HBsAg-positive blood donors with asymptomatic liver disease from the Blood Transfusion Unit, Indonesian Red Cross, Surabaya; ii) the LC group included HBV-infected patients with LC; and iii) the HCC group included HBV-infected patients with HCC from Dr Soetomo General Hospital. Determination of the groups from Dr Soetomo General Hospital was performed by clinical and biochemical data; ultrasonographic data were added to confirm the LC and HCC groups. Blood donors were included in the UC group if the laboratory examinations verified immunoglobulin M (IgM) anti-hepatitis B core (HBc)-negativity and normal alanine transaminase levels. None of the patients had a history of antiviral drug use. Ethical clearance for the present study was obtained from the Ethics Committee of the Dr Soetomo General Hospital, Surabaya. All the participants signed a consent form.

Serological markers. HBsAg, HBeAg, anti-HBe antibody and other serological markers (anti-HCV and anti-HIV antibodies) were detected by a microparticle enzyme immunoassay (Abbott, Wiesbaden, Germany). Blood donors were screened for IgM anti-HBc to exclude acute HBV infection. The anti-HCV and anti-HIV antibodies were also tested to confirm the absence of exclusion criteria.

Viral DNA extraction, PCR amplification and sequencing. HBV DNA was extracted from each serum sample using the High Pure Viral Nucleic Acid kit (Roche Molecular Systems, Inc., Alameda, CA, USA) following the manufacturer's insructions. The extracted DNA was used as a template for the amplification of the respective gene regions. Polymerase chain reactions (PCRs) were performed with the High Fidelity PCR enzyme mix (Fermentas, Vilnius, Lithuania). The reactions included 2.5 μl High Fidelity PCR buffer (with $MgCl_2$), 2.5 μl dNTP with a concentration of 2 mM, 0.25 μl High Fidelity PCR enzyme mix, 10 μl DNA and 0.5 μl of each primer with a concentration of 100 pmol/μl, in a total reaction volume of 25 μl. The thermocycling conditions included a 5-min denaturation step at 94˚C, followed by 40 cycles of 1 min at 94˚C, 1 min at 55˚C and 2 min at 72˚C (23).

The BCP, PC and partial core genes were amplified in the first round using HBc1 (5'-TTA CAT AAG AGG ACT CTT GG-3', nt 1,650-1,669) and HB9R (5'-GGA TAG AAC TAG CAG GCA T-3', nt 2,654-2,635) (24). If the first-round PCR was negative, the second-round PCR was performed using primers HBc1 and HBc2 (5'-TAA AGC CCA GTA AAG TTT CC-3', nt 2,494-2,475) (25).

To elucidate the association between HBV genotype and PC mutations, part of the S gene was amplified in the first round using primers P7 (5'-GTG GTG GAC TTC TCT CAA TTT TC-3', nt 256-278) and P8 [5'-CGG TAW(A/T) AAA GGG ACT CAM(A/C) GAT-3', nt 796-776]. If the first-round PCR was negative, the second-round PCR was performed using primers HBs1 (5'-CAA GGT ATG TTG CCC GTT TG-3', nt 455-474) and HBs2 (5'-AAA GCC CTG CGA ACC ACT GA-3', nt 713-694) (23). The nt sequences of the amplified fragments were determined using the BigDye Terminator v1.1 Cycle Sequencing kit with an ABI Prism 310 (Applied Biosystems, Foster City, CA, USA).

Sequences analyses for the detection of BCP, PC and core mutations. All sequence data analyses were performed with Genetyx-Windows version 9 (Software Development Co., Ltd., Tokyo, Japan). After aligning the sequences obtained from this study and from International DNA databases (DDBJ/EMBL/GenBank), the variability of the BCP, PC and core was analyzed. The HBV genotypes were determined based on the homologous percentage of >96% in the S and/or core

Table I. Number of BCP, PC and core mutations in various clinical stages of chronic HBV infection.

		No. of HBV mutations/variants						
		BCP		PC			Core	
Clinical stage	No.	T1762 (%)	A1764 (%)	T1858 (%)	A1896 (%)	A1899 (%)	Leu97 (%)	Thr/Ser130 (%)
UC	10	2/10 (20)	2/10 (20)	10/10 (100)	6/10 (60)	3/10[b] (30)	3/10 (30)	1/10[d] (10)
LC	10	6/10 (60)	5/10 (50)	10/10 (100)	6/10 (60)	2/10[a] (20)	4/10 (40)	3/10[e] (30)
HCC	4	1/4 (25)	1/4 (25)	4/4 (100)	4/4 (100)	0/4 (0)	2/4 (50)	2/4[c] (50)
Total	24	9/24 (38)	8/24 (33)	24/24 (100)	16/24 (67)	5/24 (21)	9/24 (38)	6/24 (25)

Mutations of BCP and PC are presented at nucleotide positions, but core mutations are presented at amino acid (aa) positions. [a]One sample exhibited combined PC A1899 and PC A1896 mutations; [b]two samples exhibited combined PC A1899 and PC A1896 mutations; [c]all the core mutations at aa 130 were Thr130, however, 1 sample in the HCC group exhibited Ser130 mutation; [d]one sample exhibited combined Thr130 and Leu97 core mutations; [e]two samples exhibited combined Thr130 and Leu97 core mutations. BCP, basal core promoter; PC, precore; HBV, hepatitis B virus; UC, chronic HBV infection; LC, liver cirrhosis; HCC, hepatocellular carcinoma; Leu, leucine; Thr, threonine; Ser, serine.

Table II. Patterns of BCP, PC and core mutations in various clinical stages of chronic HBV infection.

		Patterns of BCP, PC and core mutations						
Clinical stage	No.	BCP+ PC- C-	BCP+ PC+ C-	BCP+ PC- C+	BCP+ PC+ C+	BCP- PC+ C-	BCP- PC+ C+	BCP- PC- C-
UC	10	-	2 (20%)	-	-	2 (20%)	3 (30%)	3 (30%)
LC	10	2 (20%)	1 (10%)	1 (10%)	2 (20%)	2 (20%)	2 (20%)	-
HCC	4	-	-	-	1 (25%)	1 (25%)	2 (50%)	-
Total	24	2 (8%)	3 (13%)	1 (4%)	3 (13%)	5 (21%)	7 (29%)	3 (13%)

BCP mutations, mutations of T1762/A1764 in the BCP region; PC mutations, mutations of A1896 and/or A1899 in the PC region; C mutations, mutations of Leu97 and/or Thr/Ser130 in the core region; +, mutant type; -, wild-type. BCP, basal core promoter; PC, precore; C, core; HBV, hepatitis B virus; UC, chronic HBV infection; LC, liver cirrhosis; HCC, hepatocellular carcinoma; Leu, leucine; Thr, threonine; Ser, serine.

gene sequences compared to HBV isolates from International DNA databases (DDBJ/EMBL/GenBank) (26,27).

Quantification of HBV DNA. The HBV DNA level was determined with the COBAS Amplicor HBV monitor (Roche Molecular Systems, Inc., Branchburg, NJ, USA).

INNO-LiPA PC assay. The INNO-LiPA PreCore kit (Innogenetics, Ghent, Belgium) was used to assess serum samples (with A1896 PC mutation but HBeAg-positive) which could not be assessed by direct sequencing. The kit is able to detect a wild-type/mutant mixed population of circulating virus.

Results

Profiles of BCP, PC and core mutations and HBV genotype. A total of 29 HBsAg-positive serum samples were obtained from patients and control subjects. In 24 of the 29 samples the sequences of BCP, PC and core regions were detected and confirmed. The samples were collected from 10 patients with UC, 10 with LC and 4 patients with HCC. All 24 subjects were aged 22-68 years (mean age, 43.8 years) and included 18 men and 6 women. Their descent was from Java (88%), Flores (4%), Sulawesi (4%) and Aceh-Batak (4%) and they resided in Surabaya.

In the 24 serum samples, the most frequently detected mutation in the PC region was A1896 (67%) and it was predominant in all the groups (60-100% of each group) (Table I). The PC A1896 mutation was encountered in all isolates identified as genotype B (data not shown). Based on part of the S and/or core genes, the 24 isolates were classified into HBV genotype B. The association between genotype B and the PC variant T1858 was 100%. The other PC mutation, A1899, was detected in 21% isolates (5/24) and in some of them it was observed in combination with A1896 (3/5, 60%). In the BCP region, T1762 and A1764 mutations were detected in 38% and 33% isolates, respectively, and these mutations were predominant in the LC group (50-60%). Mutations in the core region aa 130 were detected in six (25%) isolates (10-50% of each group), mostly in the HCC group. The obtained mutation types in core aa 130 included Thr130 (five isolates) and Ser130 (one isolate). The other core mutation, Leu97, was encountered in 38% isolates (30-50% of each group). The core mutations, Thr130 combined with Leu97, were observed in three isolates (3/5, 60%).

To simplify data analysis, this study focused on BCP T1762/A1764, PC A1896 and core Thr/Ser130 as the well-known hotspot mutations. The mutations of PC A1899 and core Leu97

Table III. Status of HBeAg/anti-HBe in various clinical stages of chronic HBV infection.

		Status of HBeAg/anti-HBe (%)		
Clinical stage	No.	HBeAg(+)/anti-HBe(-)	HBeAg(-)/anti-HBe(+)	HBeAg(-)/anti-HBe(-)
UC	10	3 (30)	6 (60)	1 (10)
LC	10	3 (30)	7 (70)	0
HCC	4	0	4 (100)	0
Total	24	6/24 (25)	17/24 (71)	1/24 (4)

HBV, hepatitis B virus; UC, chronic HBV infection; LC, liver cirrhosis; HCC, hepatocellular carcinoma; (+), positive; (-), negative.

Table IV. BCP, PC and core mutations of HBV prior to and following HBeAg seroconversion.

		BCP		PC				Core			
		nt 1762/1764		nt 1896		nt 1899		aa 97		aa 130	
Time point	No.	WT (%)	MT (%)	WT (%)	MT (%)	WT (%)	MT (%)	WT (%)	MT (%)	WT (%)	MT (%)
Prior to SC	6	4 (66.7)	2 (33.3)	4 (66.7)	2 (33.3)	6 (100)	0	4 (66.7)	2 (33.3)	4 (66.7)	2 (33.3)
Following SC	17	10 (58.9)	7 (41.2)	3 (23.5)	14 (76.5)	12 (70.6)	5 (29.4)	10 (58.9)	7 (41.2)	13 (76.5)	4 (23.5)

BCP, basal core promoter; PC, precore; HBV, hepatitis B virus; nt, nucleotide; aa, amino acid; WT, wild-type; MT, mutant type; SC, seroconversion.

were also included, since they could be found in combination and share certain effects with the mutations mentioned above (PC A1896 with A1899 and core Leu97 with Thr/Ser130) (Table II). There were several basic pattern possibilities, i.e., no mutations (BCP- PC- C-), BCP mutation only (BCP+ PC- C-), BCP and PC mutations (BCP+ PC+ C-), BCP and C mutations (BCP+ PC- C+), PC mutation only (BCP- PC+ C-), PC and core mutations (BCP- PC+ C+), core mutation only (BCP- PC- C+) and triple mutations (BCP+ PC+ C+). The pattern of core mutation only (BCP- PC- C+) was not detected in this study. The LC and HCC groups carried HBV isolates with additional mutations, at least BCP or PC mutations. The LC group mainly carried HBV with BCP mutations (60%), whereas the HCC group commonly exhibited PC mutations (100%). The triple mutation pattern (BCP+ PC+ C+) was only detected in the LC and HCC groups; however, the BCP- PC- C- pattern was absent in the UC group.

Status of HBeAg/anti-HBe and HBV DNA level associated with BCP, PC and core mutations. Of the 24 serum samples, HBeAg-negative/anti-HBe-positive was the predominant type (71%), followed by HBeAg-positive/anti-HBe-negative (25%) and HBeAg-negative/anti-HBe-negative (4%). Anti-HBe-positive was predominant in the HCC group (100%), followed by the LC (70%) and UC (60%) groups (Table III).

In the six HBeAg-positive serum samples (prior to seroconversion), the number of wild-types of BCP (A1762/G1764), PC (G1896 and G1899) and core (Ile97 and Pro130) was higher (range, 66.7-100%) compared with the number of mutant types of BCP (T1762/A1764), PC (A1896 and A1899) and core (Thr/Ser130) (Table IV). As determined by the INNO-LiPA assay, the two samples from patient 7RS (UC group) and patient 17RS (LC group) that were HBeAg-positive in the presence of the PC A1896 mutation, had mixed strains of mutant and wild-types of the PC region.

Following HBeAg seroconversion, the number of the mutant types of BCP nt 1,762/1,764 was lower compared to the wild-types, although the number was increased compared to prior to seroconversion. However, the number of core mutations Thr/Ser130 was lower following compared to prior to seroconversion. Of note, the number of the mutant type of PC A1896 mutations was significantly higher (82.4%) in anti-HBe-positive serum samples compared to that of wild-type mutations (17.7%) (Table IV).

Following HBeAg seroconversion, the mutant types of BCP (T1762/A1764) were frequently associated with a high level of HBV DNA (57.1%). However, the number of serum samples with the mutant type of PC A1896 exhibiting low HBV DNA levels and that of samples with high HBV DNA levels were identical. Of the three samples with the mutant type of combined PC A1899 and PC A1896, two were associated with a low level of HBV DNA (Table V).

Discussion

HBV mutations in the PC, BCP and core regions have been reported to exert various effects on the clinical course of patients with HBV-related liver diseases (28). In this study, these mutations were assessed in various phases of chronic hepatitis B infection in Surabaya, Indonesia.