recently reported that HCV promotes hepatic gluconeogenesis via an NS5A-mediated, forkhead box O1 (FoxO1)-dependent pathway, resulting in increased cellular glucose production in hepatocytes (Deng et al., 2011). This paper discusses our current model for HCV-induced glucose metabolic disorders.

## HCV REPLICATION DOWN-REGULATES CELL SURFACE EXPRESSION OF GLUT2

The uptake of glucose into cells is conducted by the facilitative glucose carrier, glucose transporters (GLUTs). GLUTs are integral membrane proteins that contain 12 membrane-spanning helices. To date, a total of 14 isoforms have been identified in the GLUT family (Wu and Freeze, 2002; Macheda et al., 2005; Godoy et al., 2006). Glucose is transported into hepatocytes by GLUT2. We previously reported that HCV J6/JFH1 infection suppresses hepatocytic glucose uptake through down-regulation of surface expression of GLUT2 in human hepatoma cell line, Huh-7.5 cells (Kasai et al., 2009). We also demonstrated that GLUT2 expression in hepatocytes of the liver tissues from HCV-infected patients was significantly lower than in those from patients without HCV infection. Our data suggest that HCV infection down-regulates GLUT2 expression at transcriptional level. We are currently analyzing transcriptional control of human GLUT2 promoter in HCV replicon cells as well as in HCV J6/JFH1-infected cells.

## HCV INFECTION PROMOTES HEPATIC GLUCONEOGENESIS

Then we analyzed hepatic glucose production and expression of transcription factors using HCV replicon cells and HCVcc system in order to clarify a role of HCV infection in glucose metabolic changes. Hepatic glucose production is usually regulated by gluconeogenesis and glycolysis. Therefore, we examined whether HCV infection induces gluconeogenesis or glycolysis. We found that the PEPCK and G6Pase genes were transcriptionally up-regulated in J6/JFH1-infected cells (**Figure 1**). On the other hand, the GK gene was transcriptionally down-regulated in HCV-infected cells. We obtained similar data in HCV replicon cells (both in subgenomic replicon cells and full-genomic replicon cells). When HCV replication was suppressed by IFN treatment, the up-regulation of PEPCK and G6Pase gene expression as well as the down-regulation of GK gene expression were canceled. From these results, HCV infection selectively up-regulates PEPCK and G6Pase genes, whereas HCV infection down-regulates GK gene (Deng et al., 2011).

Both HCV replicon cells and HCV-infected cells produced greater amounts of glucose than the control cells. IFN treatment canceled the enhanced glucose production in HCV replicon cells as well as in HCV-infected cells. G6P is an important precursor molecule that is converted to glucose in the gluconeogenesis pathway (**Figure 1**). Our metabolite analysis showed that a significantly higher level of G6P was accumulated in HCV-infected cells than in the control cells, suggesting that HCV indeed promotes hepatic gluconeogenesis to cause hyperglycemia. There is a trend toward an increase in gluconeogenesis in HCV-infected cells (**Figure 1**).

## HCV SUPPRESSES FoxO1 PHOSPHORYLATION AT Ser319, LEADING TO THE NUCLEAR ACCUMULATION OF FoxO1

It has been reported that G6Pase, PEPCK, and GK are regulated by certain transcription factors, including FoxO1 (Hirota et al., 2008), hepatic nuclear factor 4α (HNF-4α; Hirota et al.,

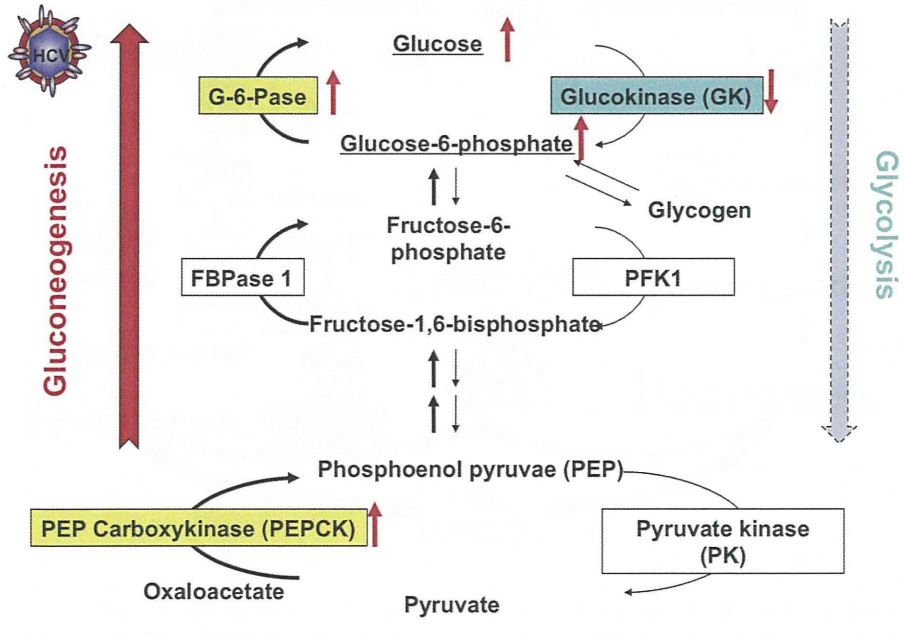


**FIGURE 1 | Regulation of Gluconeogenesis and Glycolysis in the HCV-infected cells.** HCV infection promotes gluconeogenesis via transcriptional up-regulation of the genes for PEPCK and G6Pase, the rate-limiting enzymes for hepatic gluconeogenesis, and transcriptional down-regulation of the gene for GK, the rate-limiting enzyme for hepatic glycolysis.

2008), Krüppel-like factor 15 (KLF15; Takashima et al., 2010), and cyclic AMP (cAMP) response element binding protein (CREB; Rozance et al., 2008). While we were analyzing these factors in both HCV replicon cells and HCV J6/JFH1-infected cells, we found the involvement of the FoxO1 in the transcriptional activation of G6Pase and PEPCK (Deng et al., 2011). It is known that the FoxO1 enhances gluconeogenesis through the transcriptional activation of various genes, including G6Pase and PEPCK (Gross et al., 2008). The function of FoxO1 is regulated by post-translational modifications, including phosphorylation, ubiquitylation, and acetylation (Tzivion et al., 2011). The phosphorylated form of FoxO1 is exported from the nucleus to the cytosol, resulting in loss of its transcriptional activity (**Figure 2**). Phosphorylation status of FoxO1 at Ser319 is critical for FoxO1 nuclear exclusion (Zhao et al., 2004). Although the total amounts of FoxO1 protein were unchanged, FoxO1 phosphorylation at Ser319 was markedly suppressed in HCV-infected cells compared to that in the mock-infected cells. It is known that the FoxO1 is phosphorylated by the protein kinase Akt and is exported from the nucleus to the cytosol, resulting in loss of its transcriptional activity (Tzivion et al., 2011). The majority of FoxO1 was accumulated in the nuclear fraction in HCV-infected cells, whereas in control cells FoxO1 was distributed in both the nuclear and cytoplasmic fractions. Akt phosphorylation was enhanced in HCV-infected cells, although the protein levels of total Akt protein were comparable, which is consistent with the report by Burdette et al. (2010). Our findings suggest an interesting scenario in which the HCV-mediated suppression in FoxO1 phosphorylation is caused by an unknown mechanism independent of Akt activity.

## HCV-INDUCED JNK ACTIVATION IS INVOLVED IN THE SUPPRESSION OF FoxO1 PHOSPHORYLATION

It is known that the stress-sensitive serine/threonine kinase JNK regulates FoxO at multiple levels (van der Horst and Burgering, 2007; Karpac and Jasper, 2009). We demonstrated that HCV infection induces phosphorylation and activation of JNK in a time-dependent manner, which is similar to that observed for the suppression of FoxO1 phosphorylation. As a result, c-Jun, a key substrate for JNK, got phosphorylated and activated in HCV-infected cells. The JNK inhibitor SP600125 clearly prevented the phosphorylation of c-Jun, and concomitantly recovered the suppression of FoxO1 phosphorylation in HCV-infected cells, suggesting that HCV activates the JNK/c-Jun signaling pathway, resulting in the nuclear accumulation of FoxO1 by reducing its phosphorylation status. The detailed mechanisms of HCV-induced suppression of FoxO1 phosphorylation via the JNK/c-Jun signaling pathway remain to be explored. There are at least two possibilities. The JNK/c-Jun signaling pathway (1) suppresses a protein kinase, or (2) activates a protein phosphatase to reduce phosphorylation of FoxO1.

## HCV-INDUCED MITOCHONDRIAL REACTIVE OXYGEN SPECIES PRODUCTION IS INVOLVED IN INCREASED GLUCOSE PRODUCTION THROUGH JNK ACTIVATION

Hepatitis C virus infection increases mitochondrial reactive oxygen species (ROS) production (Deng et al., 2008). *N*-acetyl cysteine (NAC; a general antioxidant) clearly prevented the phosphorylation of JNK, and concomitantly canceled the suppression of FoxO1 phosphorylation in HCV-infected cells, suggesting that

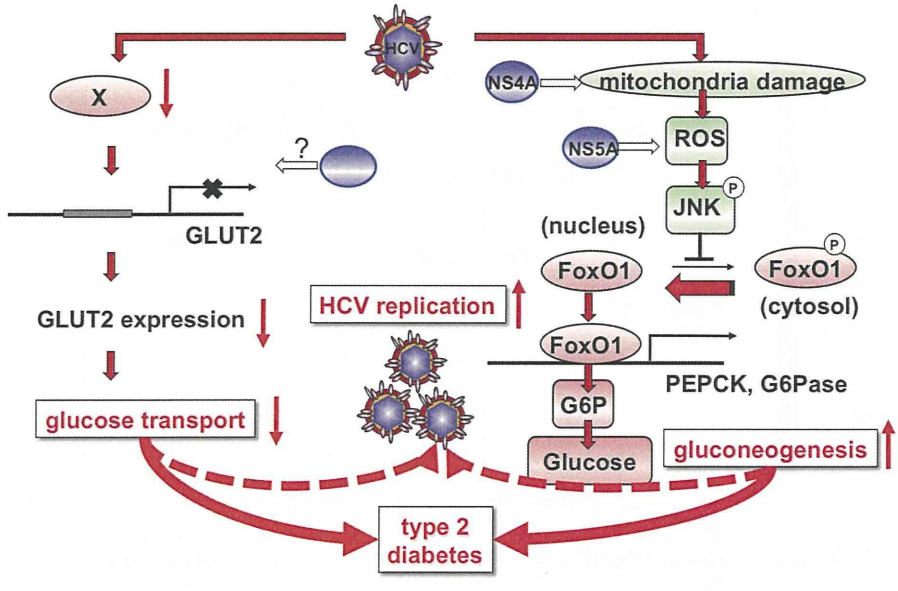


**FIGURE 2 | A proposed mechanism of HCV-induced glucose metabolic disorders.** HCV infection down-regulates cell surface expression of GLUT2 in hepatocytes at the transcriptional level. HCV down-regulates a transcription factor involved in GLUT2 gene expression through an unknown mechanism. HCV infection induces mitochondria damage and ROS production, leading to JNK activation. HCV NS4A protein is involved in mitochondrial damage. HCV NS5A protein is involved in ROS production. HCV-induced ROS production causes JNK activation, resulting in the decreased phosphorylation and nuclear accumulation of FoxO1. Nuclear accumulation of FoxO1 up-regulates gene expression of PEPCK and G6Pase, leading eventually to increased glucose production by gluconeogenesis. High glucose levels in the hepatocytes may confer an advantage in efficient replication of HCV.

HCV-induced ROS production is involved in the JNK activation. There was no significant difference in HCV RNA replication or infectious virus release between SP600125- or NAC-treated HCV-infected cells and non-treated HCV-infected cells. These results suggest that ROS-mediated JNK activation plays a key role in the suppression of FoxO1 phosphorylation, nuclear accumulation of FoxO1, and enhancement of glucose production in HCV-infected cells (Deng et al., 2011).

## HCV NS5A IS INVOLVED IN THE ENHANCEMENT OF GLUCOSE PRODUCTION

Then we sought to determine which HCV protein(s) is involved in the enhancement of glucose production. Transient expression of NS5A protein in Huh-7.5 cells significantly promoted the gene expression levels of G6Pase and PEPCK determined by real time quantitative RT-PCR. Promoter assay revealed that the level of PEPCK promoter activity was significantly higher in NS5A-expressing cells than in the control cells. Our results suggest that NS5A activate both the PEPCK promoter and the G6Pase promoter, leading to an increase in glucose production (Deng et al., 2011). The study by Banerjee et al. (2010) suggests that the HCV core protein modulates FoxO1 and FoxA2 activation and affects insulin-induced metabolic gene regulation in human hepatocytes. Our results, however, suggest that the HCV core protein is not significantly involved in the increased gluconeogenesis (Deng et al., 2011). The difference between these two studies needs to be explored.

There were previous reports suggesting that ROS production is induced in NS5A-expressing cells (Dionisio et al., 2009) or in hepatocytes of NS5A transgenic mice (Wang et al., 2009). We therefore sought to determine whether NS5A contributes to increased hepatic gluconeogenesis through the induction of ROS production. NS5A-expressing cells displayed a much stronger signal of ROS than in control cells. NS5A-expressing cells promoted phosphorylation level at Ser63 of c-Jun and suppressed FoxO1 phosphorylation at Ser319, suggesting that NS5A mediates JNK/c-Jun activation and FoxO1 phosphorylation suppression. These results suggest that NS5A play a role in the HCV-induced enhancement of hepatic gluconeogenesis through JNK/c-Jun activation and FoxO1 phosphorylation suppression.

## CONCLUSION AND FUTURE PERSPECTIVES

Taken together, we propose a model of HCV-induced glucose metabolic disorders as shown in **Figure 2**. HCV infection down-regulates cell surface expression of GLUT2 in hepatocytes at the transcriptional level. HCV down-regulates a transcription factor involved in GLUT2 gene expression through an unknown mechanism. As GLUT2 is a facilitative GLUT, it ensures large bidirectional fluxes of glucose in and out the cell due to its low affinity and high capacity (Leturque et al., 2009). Down-regulated cell surface expression of GLUT2 results in disruption of bidirectional transport of glucose in hepatocytes. Even in the fasting state, down-regulation of GLUT2 may result in low glucose uptake of hepatocytes, causing hyperglycemia. In the fed state, glucose secretion from hepatocytes may be suppressed due to low level cell surface expression of GLUT2, as GLUT2 is a bidirectional transporter.

Hepatitis C virus infection induces mitochondria damage and ROS production, leading to JNK activation. HCV NS4A protein is involved in mitochondrial damage (Nomura-Takigawa et al., 2006). HCV NS5A protein is involved in ROS production (Dionisio et al., 2009; Wang et al., 2009; Deng et al., 2011). HCV-induced ROS production causes JNK activation, which results in the decreased phosphorylation and nuclear accumulation of FoxO1 by an unidentified mechanism. Nuclear accumulation of FoxO1 up-regulates gene expression of PEPCK and G6Pase, leading eventually to increased glucose production by gluconeogenesis (Deng et al., 2011).

These two pathways, HCV-induced down-regulation of GLUT2 expression and up-regulation of gluconeogenesis, may contribute to development of type 2 diabetes in HCV-infected patients at least to some extent. HCV-induced down-regulation of GLUT2 expression and up-regulation of gluconeogenesis may result in high concentration of glucose in HCV-infected hepatocytes. As suggested in a recent study, low glucose concentration in the hepatocytes inhibits HCV replication (Nakashima et al., 2011). Therefore, high glucose levels in the hepatocytes may confer an advantage in efficient replication of HCV.

Our understanding of HCV-induced glucose metabolic disorders will require much more work to fully unfold this pathway. Further investigation including the mechanism of HCV-induced GLUT2 downregualtion, JNK-mediated decreased phosphorylation of FoxO1, and the possible effect(s) of the dysregulation of hepatic gluconeogenesis on the HCV life cycle and host cells are currently under way.

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# Polymorphisms of the Core, NS3, and NS5a Proteins of Hepatitis C Virus Genotype 1b Associate with Development of Hepatocellular Carcinoma

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**Hepatocellular carcinoma (HCC) is one of the common sequels of hepatitis C virus (HCV) infection. It remains controversial, however, whether HCV itself plays a direct role in the development of HCC. Although HCV core, NS3, and NS5A proteins were reported to display tumorigenic activities in cell culture and experimental animal systems, their clinical impact on HCC development in humans is still unclear. In this study we investigated sequence polymorphisms in the core protein, NS3, and NS5A of HCV genotype 1b (HCV-1b) in 49 patients who later developed HCC during a follow-up of an average of 6.5 years and in 100 patients who did not develop HCC after a 15-year follow-up. Sequence analysis revealed that Gln at position 70 of the core protein (core-Gln$^{70}$), Tyr at position 1082 plus Gln at 1112 of NS3 (NS3-Tyr$^{1082}$/Gln$^{1112}$), and six or more mutations in the interferon/ribavirin resistance-determining region of NS5A (NS5A-IRRDR≥6) were significantly associated with development of HCC. Multivariate analysis identified core-Gln$^{70}$, NS3-Tyr$^{1082}$/Gln$^{1112}$, and α-fetoprotein (AFP) levels (>20 ng/L) as independent factors associated with HCC. Kaplan-Meier analysis revealed a higher cumulative incidence of HCC for patients infected with HCV isolates with core-Gln$^{70}$, NS3-Tyr$^{1082}$/Gln$^{1112}$ or both than for those with non-(Gln$^{70}$ plus NS3-Tyr$^{1082}$/Gln$^{1112}$). In most cases, neither the residues at position 70 of the core protein nor positions 1082 and 1112 of the NS3 protein changed during the observation period. *Conclusion*: The present results suggest that HCV isolates with core-Gln$^{70}$ and/or NS3-Tyr$^{1082}$/Gln$^{1112}$ are more closely associated with HCC development compared to those with non-(Gln$^{70}$ plus NS3-Tyr$^{1082}$/Gln$^{1112}$). (HEPATOLOGY 2012;00:000–000)**

Hepatitis C virus (HCV) is a major etiologic agent of chronic hepatitis worldwide, with the estimated number of infected individuals being more than 180 million. Approximately 15% to 20% of chronically infected individuals undergo liver cirrhosis in a decade or so after infection, with hepatocellular carcinoma (HCC) arising from cirrhosis at an estimated rate of 1% to 4% per year.[1-3] Several host factors such as male gender, older age, elevated α-fetoprotein (AFP) level, advanced liver fibrosis as well as nonresponsiveness to interferon (IFN) therapy have been reported as important predictors of HCC development.[4,5] Recently, a host genetic factor, i.e., the *DEPDC5* locus polymorphism, was reported to be associated with progression to HCC in HCV-infected individuals.[6] On the other hand, it remains controversial as to whether HCV itself plays a direct role in the development of HCC. Experimental data suggest that HCV contributes to HCC by modulating pathways that promote malignant transformation of hepatocytes. HCV core, NS3, and NS5A proteins were shown to be involved in a number of potentially oncogenic pathways in cell culture and experimental animal systems.[7] HCV core protein rendered cultured cells more resistant to apoptosis[8,9] and promoted *ras* oncogene-mediated transformation.[10,11]

*Abbreviations: HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFN, interferon; IRRDR, interferon/ribavirin resistance-determining region; ISDR, interferon sensitivity-determining region.*

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Moreover, transgenic mice expressing the HCV core protein in the liver developed HCC.[12] However, the clinical impact of HCV proteins on HCC development in humans and whether all HCV isolates are equally associated with HCC is yet to be determined. In a clinical setting, HCV core protein mutations at positions 70 ($Gln^{70}$) and/or 91 ($Met^{91}$) were closely associated with HCC development.[13-16] $Gln^{70}$ and/or $Met^{91}$ were also linked to resistance to PEG-IFN/ribavirin (RBV) treatment.[17-20] In addition, we and other investigators reported that an N-terminal part of the NS3 protein has the capacity to transform NIH3T3 and rat fibroblast cells[21,22] and to render NIH3T3 cells more resistant to DNA damage-induced apoptosis, which is thought to be a prerequisite for malignant transformation of the cell.[23] Also, the NS5A protein is a pleiotropic protein with key roles in both viral RNA replication and modulation of the host cell functions.[24] In particular, the links between NS5A and the IFN responses have been widely discussed. It was proposed initially that sequence variations within a region in NS5A spanning from amino acids (aa) 2209 to 2248, called the IFN sensitivity-determining region (ISDR), were correlated with IFN responsiveness.[25] Subsequently, in the era of PEG-IFN/RBV combination therapy, we identified a new region near the C-terminus of NS5A spanning from aa 2334 to 2379, which we referred to as the IFN/RBV resistance-determining region (IRRDR).[26,27] The degree of sequence variations within the IRRDR was significantly associated with the clinical outcome of PEG-IFN/RBV therapy. In the context of HCC, several retrospective studies suggested that IFN-based therapy might reduce the risk of HCC development.[4,28-30]

In an attempt to clarify whether viral factors, in particular those within the core, NS3, and NS5A proteins, are involved in HCC development, we carried out a comparative analysis of the aa sequences obtained from HCV patients who developed HCC and those who did not. In addition, we studied the sequence evolution of these genes in the interval between chronic hepatitis C and HCC development over a period of 15 years.

## Patients and Methods

***Ethics Statement.*** The study protocol, which conforms to the provisions of the 1975 Declaration of Helsinki, was approved beforehand by the Ethic Committees in Akashi City Hospital and Kobe University Graduate School of Medicine, and written informed consent was obtained from each patient enrolled in this study.

***Patients.*** A total of 49 HCV-infected patients who developed HCC (HCC group) were retrospectively examined. They were followed up (from 1988 to 2003) with an average period until HCC development being 6.5 ± 2.9 years. Paired serum samples at the time of chronic hepatitis C (pre-HCC sample) and HCC development (post-HCC sample) were collected. As a control group, 100 HCV-infected patients who were followed up over a period of 15 years (from 1988 to 2003) without HCC development were retrospectively examined. Serum samples of the control group were available at the time of first visit to the clinic. All patients enrolled in this study were chronically infected with HCV genotype 1b (HCV-1b). HCV subtype was determined as reported previously.[31] Serum HCV RNA titers were quantitated by reverse-transcription polymerase chain reaction (RT-PCR0 with an internal RNA standard derived from the 5′ noncoding region of HCV (Amplicor HCV Monitor test, v. 2.0, Roche Diagnostics, Tokyo, Japan). All patients underwent liver biopsy and were diagnosed as chronic hepatitis. All HCC and 68% (68/100) of non-HCC patients received IFN-monotherapy, either natural IFN alpha (Sumiferon, Dainipponsumitomo Pharmaceutical, Osaka, Japan) at a dose of 6 million units (MU) or recombinant IFN alpha 2b (Intron A; Schering-Plough, Osaka, Japan) at a dose of 10 MU, 3 times a week for 6 months. All HCC patients were nonresponders (NR), who had detectable viremia during the entire course of IFN treatment. On the other hand, 18 (26%) of the 68 non-HCC patients treated with IFN achieved HCV RNA negativity at the end of treatment followed by rebound viremia within 6 months after the treatment and, therefore, they were referred to as relapsers. The other 50 IFN-treated, non-HCC patients were NR. The remaining 32 non-HCC patients did not receive IFN. All patients were seen every 2 months and tested for liver function markers during the follow-up period.

***Sequence Analysis of HCV Core, NS3, and NS5A Proteins.*** HCV RNA was extracted from 140 μL of serum using a commercially available kit (QIAmp viral

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RNA kit; Qiagen, Tokyo, Japan). The core, NS3, and NS5A regions of the HCV genome were amplified as described elsewhere.[26,32-34] The sequences of the amplified fragments were determined by direct sequencing. The aa sequences were deduced and aligned using GENETYX Win software version 7.0 (GENETYX, Tokyo, Japan). The numbering of aa was according to the polyprotein of the prototype of HCV-1b; HCV-J.[35]

***Statistical Analysis.*** Statistical differences in the baseline parameters of HCC and control groups were determined by Student's *t* test for numerical variables and Fisher's exact probability or chi-square tests for categorical variables. Likewise, statistical differences in viral mutations between HCC and control groups were determined by Fisher's exact probability test. Kaplan-Meier analysis was performed to estimate the cumulative incidence of HCC. The data obtained were evaluated by the log-rank test. Univariate and multivariate logistic analyses were performed to identify variables that independently associated with HCC development. Variables with $P < 0.1$ in univariate analysis were included in a backward stepwise multivariate logistic regression analysis. The odds ratios and 95% confidence intervals (95% CI) were calculated. All statistical analyses were performed using SPSS v. 16 software (Chicago, IL). Unless otherwise stated, $P < 0.05$ was considered statistically significant.

***Nucleotide Sequence Accession Numbers.*** The sequence data reported in this article have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB719460 through AB719842.

## Results

***Demographic Characteristics of HCC and Control Groups.*** The clinical characteristics of HCC and control groups are shown in Table 1. The HCC group had significantly higher titers of ALT, AST, and AFP, and higher fibrosis staging score than that of the control group. There was no significant difference in viremia titers between the two groups.

***Correlation Between Core Protein Sequence Polymorphism and HCC Development.*** HCV core protein sequences were obtained from all (49/49) and 94% (94/100) of pre-HCC and control patients' sera, respectively. Comparative sequence analysis revealed that 22 (45%) of 49 HCV isolates in the pre-HCC sera (pre-HCC isolates) and 59 (63%) of 94 HCV isolates from the control group (control isolates) had wild-core ($Arg^{70}/Leu^{91}$) (Table 2). The difference between HCC and control groups was hovering at a statistically significant level ($P = 0.05$). When the sequence pattern at position 70 alone was examined, a stronger association with HCC was observed. We found that 21 (43%) of 49 pre-HCC isolates had $Gln^{70}$ while only 13 (14%) of 94 control isolates did ($P = 0.0002$). On the other hand, there was no significant correlation between sequence pattern at position 91 and HCC. Thus, a single mutation at position 70 ($Gln^{70}$) was the only polymorphic factor within core protein that was significantly associated with HCC development. It should be noted that there was no significant correlation between $Gln^{70}$ and the degree of fibrosis progression (data not shown).

**Table 1. Demographic Characteristics of HCC and Control Groups**

| Factor | HCC | Control | *P*-value |
|---|---|---|---|
| Age | 57.3 ± 7.0* | 56.4 ± 8.3 | 0.54 |
| Sex (male/female) | 31/18 | 54/46 | 0.29 |
| ALT (IU/L) | 159.4 ± 79.8 | 129.7 ± 51.5 | 0.007 |
| AST (IU/L) | 113.0 ± 62.2 | 91.6 ± 44.1 | 0.017 |
| AFP (ng/L) | 29.1 ± 33.7 | 18.4 ± 4.4 | 0.002 |
| Platelets (x $10^4/mm^3$) | 16.2 ± 2.8 | 16.2 ± 2.4 | 0.88 |
| Inflammation grading score | 8.7 ± 0.9 | 8.4 ± 1.2 | 0.05 |
| Fibrosis staging score | 2.4 ± 0.5 | 2.2 ± 0.5 | 0.02 |
| HCV-RNA (KIU/mL) | 593.4 ± 112.3 | 618.1 ± 95.9 | 0.17 |

*Mean ± SD. HCC, hepatocellular carcinoma; ALT, alanine aminotransferase; AST, aspartate transaminase; AFP; α-fetoprotein.

***Correlation Between NS3 Protein Sequence Polymorphism and HCC Development.*** Sequences of NS3 serine protease domain (aa 1027 to 1146) were obtained from 94% (46/49) and 93% (93/100) of pre-HCC and control isolates, respectively. We found that 29 (63%) of 46 pre-HCC isolates had Tyr and Gln at positions 1082 and 1112, respectively ($Tyr^{1082}/Gln^{1112}$), while 39 (42%) of 93 control isolates did (Table 2). The difference in the proportion between pre-HCC and control isolates was statistically significant ($P = 0.029$). On the other hand, there was no significant correlation between $Tyr^{1082}/Gln^{1112}$ and the degree of fibrosis progression (data not shown).

***Correlation Between NS5A Protein Sequence Polymorphism and HCC Development.*** NS5A protein sequences were obtained from 92% (45/49) and 74% (74/100) of pre-HCC and control isolates, respectively. Twenty-four (53%) of 45 pre-HCC isolates had IRRDR of 6 or more mutations (IRRDR≥6) while only 15 (20%) of 74 control isolates did (Table 2; $P = 0.0003$). We also found that pre-HCC isolates tended to have a higher degree of sequence heterogeneity in ISDR than control isolates, although not statistically significant due probably to the small number of

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**Table 2. Correlation Between HCC and Sequence Polymorphic Factors of Core, NS3 and NS5A**

| HCV Protein | Factor | No. of Subjects / No. of Total* HCC | Control | P-value |
|---|---|---|---|---|
| Core | Wild-core ($Arg^{70}$/$Leu^{91}$) | 22/49 (45%) | 59/94 (63%) | 0.05 |
| | Non-wild-core | 27/49 (55%) | 35/94 (37%) | |
| | $Gln^{70}$ | 21/49 (43%) | 13/94 (14%) | 0.0002 |
| | Non-$Gln^{70}$ | 28/49 (57%) | 81/94 (86%) | |
| | $Leu^{91}$ | 37/49 (76%) | 70/94 (74%) | 1.0 |
| | Non- $Leu^{91}$ | 12/49 (24%) | 24/94 (26%) | |
| NS3 | $Tyr^{1082}$ / $Gln^{1112}$ | 29/46 (63%) | 39/93 (42%) | 0.029 |
| | Non-($Tyr^{1082}$ / $Gln^{1212}$) | 17/46 (37%) | 54/93 (58%) | |
| NS5A | IRRDR≥6 | 24/45 (53%) | 15/74 (20%) | 0.0003 |
| | IRRDR≤5 | 21/45 (47%) | 59/74 (80%) | |
| | ISDR≥3 | 11/45 (24%) | 8/74 (11%) | 0.07 |
| | ISDR≤2 | 34/45 (76%) | 66/74 (89%) | |
| | $Asn^{2218}$ | 11/45 (24%) | 3/74 (4%) | 0.002 |
| | Non-$Asn^{2218}$ | 34/45 (76%) | 71/74 (96%) | |

*Number of subjects with a given factor / total number of HCC or control. HCC, hepatocellular carcinoma; $Arg^{70}$, arginine at position 70 of the core protein; $Leu^{91}$, leucine at position 91 of the core protein; $Gln^{70}$, glutamine at position 70 of the core protein; $Tyr^{1082}$, tyrosine at position 1082 of NS3; $Gln^{1212}$, glutamine at position 1212 of NS3; IRRDR, interferon/ribavirin resistance-determining region; ISDR, interferon sensitivity-determining region; $Asn^{2218}$, asparagine at position 2218 of NS5A-ISDR.

cases examined; 11 (24%) of 45 pre-HCC isolates and 8 (11%) of 74 of control isolates had ISDR with three or more mutations ($P = 0.07$). Moreover, Asn at position 2218 ($Asn^{2218}$) within the ISDR was found in 24% (11/45) of pre-HCC isolates and only in 4% (3/74) of the control isolates ($P = 0.002$), suggesting that $Asn^{2218}$ is significantly associated with development of HCC.

***Cumulative HCC Incidence on the Basis of Core-$Gln^{70}$, NS3-$Tyr^{1082}$/$Gln^{1112}$, NS5A-IRRDR≥6, and NS5A-$Asn^{2218}$.*** Follow-up study revealed that the cumulative HCC incidence in patients infected with HCV-1b isolates with core protein of $Gln^{70}$ and those of non-$Gln^{70}$, respectively, was 29% and 5% at the end of 5 years, 56% and 23% at the end of 10 years, and 63% and 26% at the end of 15 years (Fig. 1A), with the differences between the two groups being statistically significant ($P < 0.0001$; Log-rank test). Likewise, the cumulative HCC incidence in patients infected with HCV-1b isolates with NS3 of $Tyr^{1082}$/$Gln^{1112}$ and those of non-($Tyr^{1082}$/$Gln^{1112}$), respectively, was 15% and 7% at the end of 5 years, 37% and 24% at the end of 10 years, and 45% and 24% at the end of 15 years ($P = 0.02$) (Fig. 1B). Also, the cumulative HCC incidence in patients infected with HCV-1b isolates of IRRDR≥6 and those of IRRDR≤5, respectively, was 18% and 10% at the end of 5 years, 59% and 22% at the end of 10 years, and 63% and 27% at the end of 15 years ($P = 0.0002$) (Fig. 1C). Similarly, the cumulative HCC incidence in patients infected with HCV-1b isolates of $Asn^{2218}$ and those of non-$Asn^{2218}$, respectively, was 31% and 9% at the end of 5 years, 77% and 28% at the end of 10 years, and 77% and 33% at the end of 15 years ($P = 0.0003$) (Fig. 1D).

***Identification of Independent Factors Correlated with HCC Development by Univariate and Multivariate Logistic Regression Analyses.*** In order to identify significant independent factors associated with HCC development, all available data of baseline patients' parameters and core, NS3, and NS5A polymorphic factors were first analyzed by univariate logistic analysis. This analysis yielded eight factors that were significantly associated with HCC development: core-$Gln^{70}$, NS3-($Tyr^{1082}$/$Gln^{1112}$), NS5A-IRRDR≥6, NS5A-$Asn^{2218}$, increased levels of ALT (>165 IU/L), AST (>65 IU/L), and AFP (>20 ng/L), and fibrosis staging score (≥3). Subsequently, those eight factors were entered in multivariate logistic regression analysis. This analysis identified two viral factors, core-$Gln^{70}$ and NS3-($Tyr^{1082}$/$Gln^{1112}$), and a host factor, AFP levels (>20 ng/L), as independent factors associated with HCC development (Table 3).

The vast majority of pre-HCC isolates (85%; 39/46) had core-$Gln^{70}$ and/or NS3-$Tyr^{1082}$/$Gln^{1112}$ and only 15% (7/46) had non-($Gln^{70}$ plus NS3-$Tyr^{1082}$/$Gln^{1112}$). By contrast, about a half of control isolates (52%; 46/89) had non-($Gln^{70}$ plus NS3-$Tyr^{1082}$/$Gln^{1112}$) (Fig. 2A). The difference in the proportion between HCC and control groups was statistically significant ($P < 0.0001$). Furthermore, the cumulative HCC incidence after 15-year follow-up was highest (63%) among patients with core-$Gln^{70}$ plus NS3-($Tyr^{1082}$/$Gln^{1112}$), whereas it was lowest (11%) among patients with non-($Gln^{70}$ plus NS3-$Tyr^{1082}$/$Gln^{1112}$) (Fig. 2B), with the difference being statistically significant ($P < 0.0001$; Log-rank test).

***Evolution of the Sequences of the Core, NS3, and NS5A Proteins During the Follow-up Period from Chronic Hepatitis to HCC Development.*** Finally, we investigated sequence evolution of the core protein, NS3 and NS5A (IRRDR and ISDR) during the follow-up period from chronic hepatitis to HCC development by comparing the sequences between pre- and post-HCC isolates. The residue at position 70 of the core protein was conserved in 91% (41/45) of sequence pairs analyzed. The substitutions observed at this position were from $Arg^{70}$ and $His^{70}$ each to $Gln^{70}$ in two cases and from $Gln^{70}$ to $Arg^{70}$ in the other two cases. The residues at positions 1082 and 1112 of

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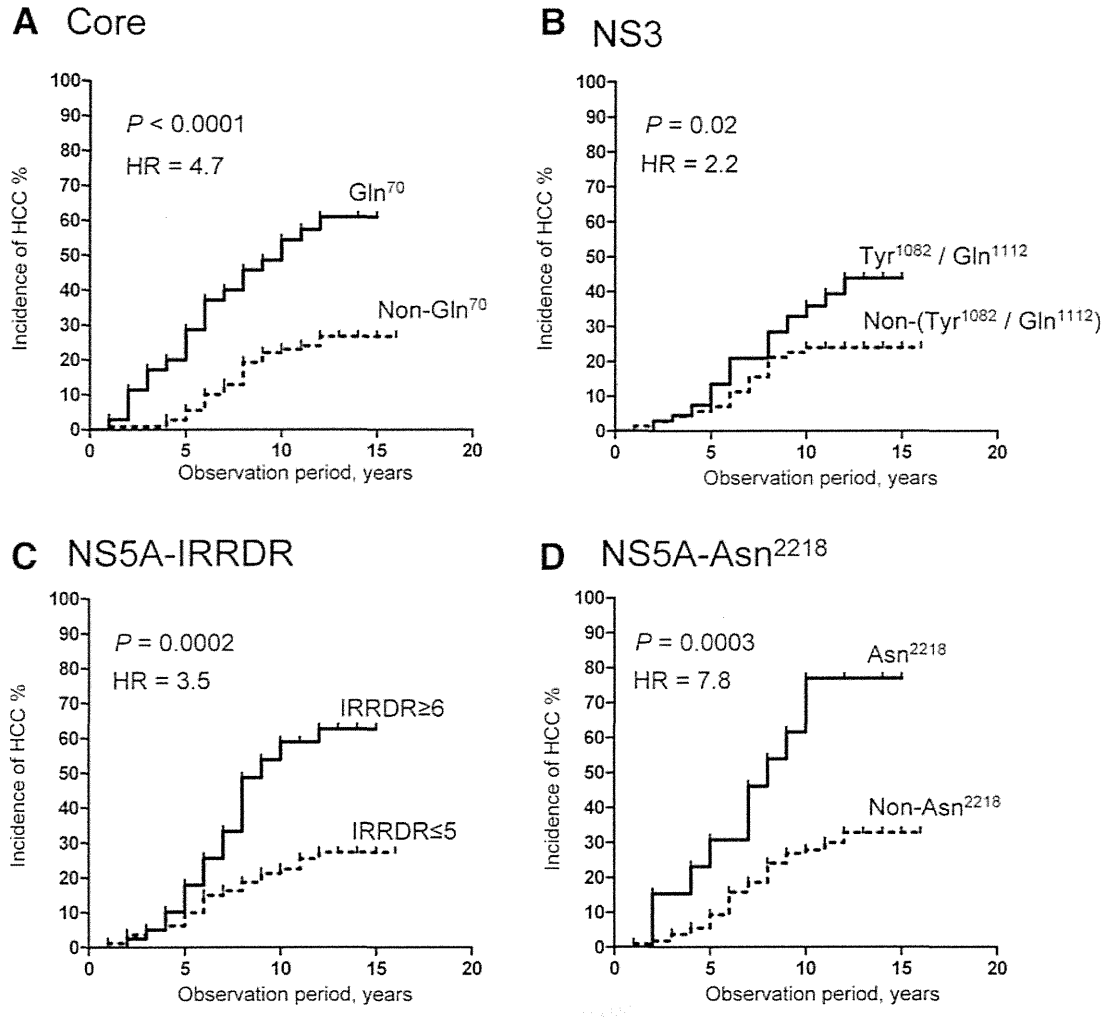


Fig. 1. Cumulative HCC incidence on the basis of HCV-1b sequence patterns. (A) Position 70 of the core protein. The numbers of core-$Gln^{70}$ and non-$Gln^{70}$ analyzed were 34 and 109, respectively. (B) Positions 1082 and 1112 of NS3. The numbers of NS3-($Tyr^{1082}$/$Gln^{1112}$) and non-($Tyr^{1082}$/$Gln^{1112}$) analyzed were 68 and 71, respectively. (C) NS5A-IRRDR. The numbers of NS5A-IRRDR≥6 and IRRDR≤5 analyzed were 39 and 80, respectively. (D) NS5A-$Asn^{2218}$. The numbers of NS5A-$Asn^{2218}$ and non-$Asn^{2218}$ analyzed were 14 and 105, respectively.

NS3 were conserved in 95% (41/43) and 100% (43/43), respectively, of the sequence pairs analyzed.

IRRDR and ISDR showed a high degree of sequence evolution. IRRDR sequences were different between pre- and post-HCC isolates in 66% (25/38) of cases analyzed (Fig. 3). IRRDR sequences tended to be more polymorphic at the time of HCC occurrence. Frequency of HCV isolates with IRRDR≥6 was F3

**Table 3. Univariate and Multivariate Regression Analyses to Identify Independent Factors Associated with HCC**

| | Univariate | | Multivariate | |
|---|---|---|---|---|
| Variable | Odds Ratio (95% CI) | *P*-value | Odds Ratio (95% CI) | *P*-value |
| Core-$Gln^{70}$ | 0.23 (0.10 - 0.52) | 0.0004 | 6.8 (2.1 - 23.0) | 0.001 |
| NS3-$Tyr^{1082}$ / $Gln^{1212}$ | 2.4 (1.1 - 4.9) | 0.029 | 3.4 (1.1 - 10.0) | 0.03 |
| NS5A-IRRDR≥6 | 4.5 (2.0 - 10.0) | 0.0003 | | |
| NS5A-$Asn^{2218}$ | 7.7 (2.0 - 29.0) | 0.002 | | |
| AFP (>20 ng/L) | 12 (5.1 - 30.0) | 0.0001 | 19.5 (4.7 - 80.0) | 0.0001 |
| ALT (>165 IU/L) | 4.0 (1.8 - 8.6) | 0.0006 | | |
| AST (>65 IU/L) | 3.9 (1.5 - 10.0) | 0.003 | | |
| Fibrosis staging score (≥3) | 2.4 (1.1 - 4.9) | 0.02 | | |

$Gln^{70}$, glutamine at position 70 of the core protein; $Tyr^{1082}$, tyrosine at position 1082 of NS3; $Gln^{1212}$, glutamine at position 1212 of NS3; IRRDR, interferon/ribavirin resistance-determining region; $Asn^{2218}$, asparagine at position 2218 of NS5A-ISDR, ALT, alanine aminotransferase; AST, aspartate transaminase; AFP; α-fetoprotein; IFN; interferon.

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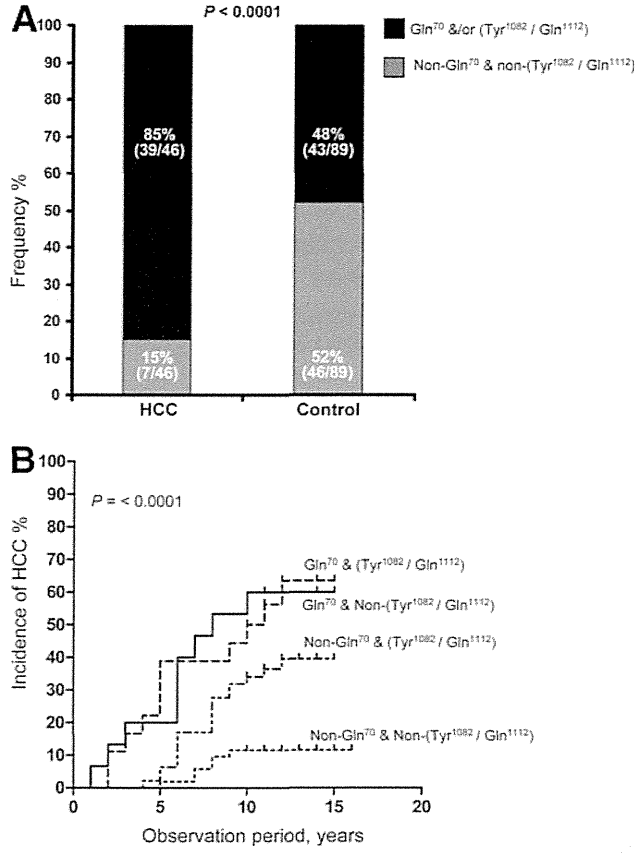


Fig. 2. (A) Proportions of HCV-1b isolates of the HCC high-risk group (core-$Gln^{70}$ and/or NS3-[$Tyr^{1082}$/$Gln^{1112}$]) and the low-risk group (non-$Gln^{70}$ and non-[$Tyr^{1082}$/$Gln^{1112}$]) among HCC and control groups. (B) Cumulative HCC incidence on the basis of different combined sequence patterns of position 70 of the core protein and positions 1082 and 1112 of NS3. Core-$Gln^{70}$ and NS3-($Tyr^{1082}$/$Gln^{1112}$), n = 18; core-$Gln^{70}$ and non-($Tyr^{1082}$/$Gln^{1112}$), n = 16; non-$Gln^{70}$ and NS3-($Tyr^{1082}$/$Gln^{1112}$), n = 48; non-$Gln^{70}$/non-($Tyr^{1082}$/$Gln^{1112}$), n = 53.

significantly higher in post-HCC isolates than in pre-HCC isolates; IRRDR≥6 was found in 47% (18/38) of post-HCC isolates compared to 24% (9/38) of pre-HCC isolates ($P = 0.03$). On the other hand, ISDR≥3 was found in 21% (8/38) of post-HCC isolates compared to 11% (4/38) of pre-HCC isolates, with the difference between the two groups being not statistically significant ($P = 0.3$).

## Discussion

HCC is one of the common long-term complications of HCV infection. However, whether HCV itself plays a direct role in the development of HCC and whether all HCV isolates are equally associated with HCC development remain to be determined. HCV core, NS3, and NS5A proteins have been reported to affect a wide variety of potentially oncogenic pathways in cell culture and experimental animal systems.[7] In the present study, we demonstrated that HCV isolates with core-$Gln^{70}$, NS3-$Tyr^{1082}$/$Gln^{1112}$ or NS5A-IRRDR≥6 were closely associated with HCC development. In addition, a follow-up study revealed that sequence patterns at position 70 of the core protein and positions 1082 and 1112 of NS3 did not significantly alter during the progression from chronic hepatitis to HCC while NS5A-IRRDR showed a significantly higher degree of sequence heterogeneity in post-HCC than in pre-HCC isolates.

Correlation between polymorphisms at positions 70 and 91 of HCV-1b core protein and IFN-based treatment outcome was extensively studied, especially in a Japanese population.[17-20] Interestingly, the same mutations were also associated with progression to HCC in the Japanese population with HCV-1b infection.[13] Results obtained in the present study confirmed and emphasized the significant association between the mutation at position 70 (core-$Gln^{70}$), but not at position 91, and HCC development (Tables 2, 3; Fig. 1A). Despite the clinical evidence that strongly supports the correlation between core-$Gln^{70}$ and HCC development, the molecular mechanism underlying this correlation is still obscure. Delhem et al.[36] found that tumor-derived HCV core proteins, but not nontumor-derived ones, interact with and activate double-stranded RNA-dependent protein kinase (protein kinase R or PKR), which might modulate viral persistence and carcinogenesis. $Gln^{70}$ was found in two of the three tumor-derived sequences, whereas $Arg^{70}$ was found in two of the three nontumor-derived ones.

As for the NS3 protein of HCV, the possible link between an N-terminal portion of NS3 encoding viral serine protease (aa 1027 to 1146) and hepatocarcinogenesis was reported.[21,22] However, information about the relationship between NS3 sequence diversity and HCC development is still limited. We previously reported a significant correlation between predicted secondary structure of an N-terminal portion of NS3 and HCC development.[34] In the present study, we demonstrated that HCV patients infected with HCV isolates with NS3-($Tyr^{1082}$/$Gln^{1112}$) were at a higher risk to develop HCC than those infected with HCV isolates with non-$Tyr^{1082}$/$Gln^{1112}$ (Tables 2, 3; Fig. 2B). Computer-assisted secondary structure analysis of NS3 revealed that $Tyr^{1082}$ was associated with the presence of a turn structure at around position 1083 while $Phe^{1082}$ was associated with the absence of the turn structure.[34] Notably, the catalytic triad of NS3 serine protease consists of $His^{1083}$, $Asp^{1107}$, and $Ser^{1165}$.[37] Since positions 1082 and 1112 are in close vicinity of the catalytic triad, sequences diversity at these positions

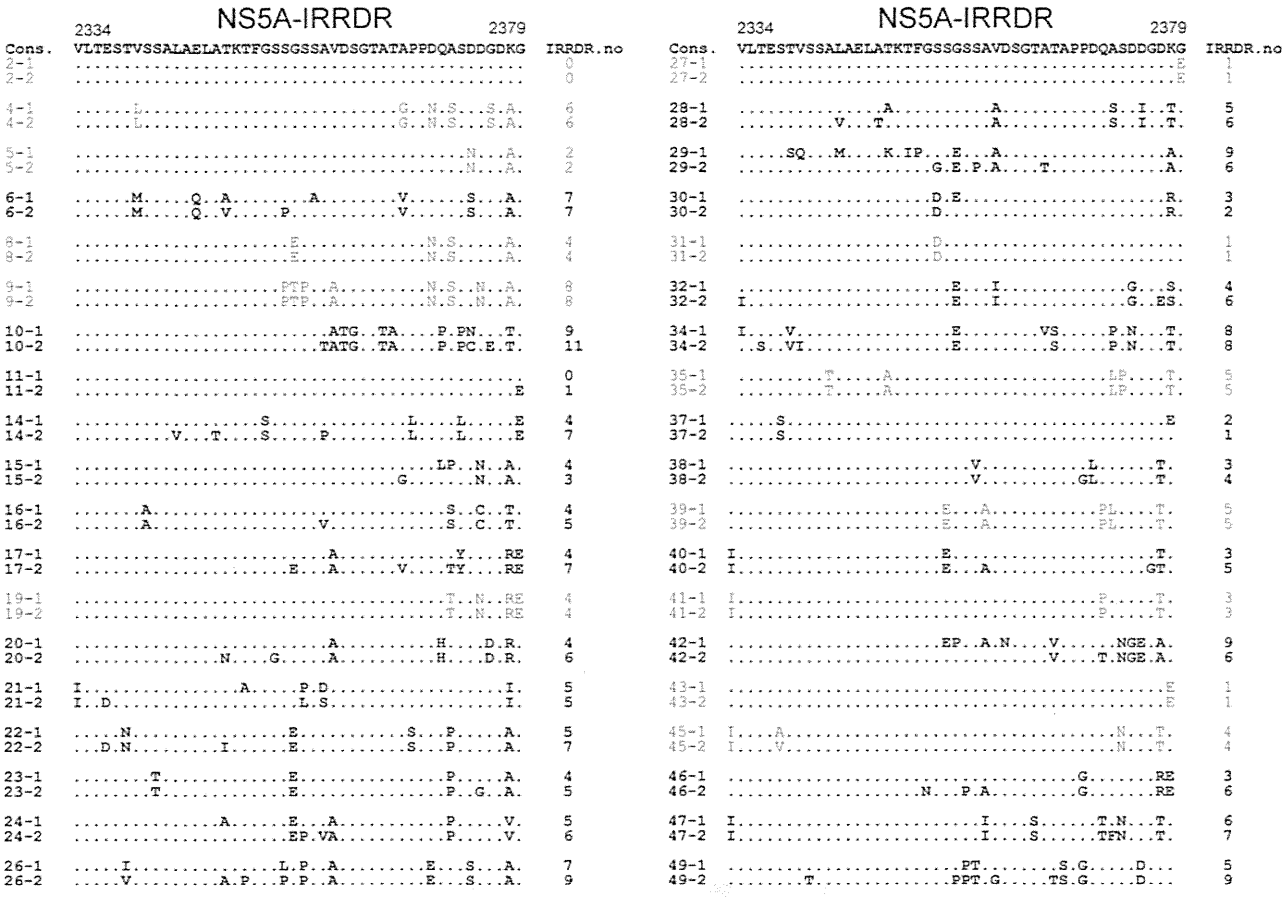


Fig. 3. Pairwise comparison of IRRDR sequences of HCV-1b during the follow-up period between chronic hepatitis and HCC development. Sequence pairs that differ between pre- (numbered with -1) and post-HCC isolates (numbered with -2) are shown. The consensus sequence (Cons.) is shown at the top. The numbers along the sequence indicate the aa positions. Dots indicate residues identical to those of the Cons. sequence. The numbers of IRRDR mutations are shown on the right.

might influence the serine protease activity and also pathogenicity of HCV. Large-scale, multicenter clinical studies as well as more detailed experimental studies at the molecular and cellular levels are needed to clarify the importance of sequence diversity at positions 1082 and 1112 of NS3 in HCV-mediated hepatocarcinogenesis.

HCV heterogeneity in NS5A-ISDR and NS5A-IRRDR are correlated with IFN-responsiveness.[17,18,25,26] As IFN-based therapy reduces the risk of HCC development,[4,28-30] we were interested to investigate whether there is a correlation between sequence heterogeneity in NS5A and development of HCC. Our present results revealed that a high degree of sequence heterogeneity in IRRDR (IRRDR≥6) was closely associated with HCC development (Table 2). We previously reported that IRRDR≥6 was significantly associated with good responses to PEG-IFN/RBV combination therapy.[26,27] These results collectively suggest that oncogenic properties and PEG-IFN/RBV responsiveness are independent viral characteristics and that PEG-IFN/RBV therapy helps eliminate oncogenic HCV isolates, thus reducing the risk of HCC development.

Position 2218 of NS5A, located within ISDR, appears to tolerate a wide range of aa substitutions as observed in different HCV-1b isolates.[25,38,39] Interestingly, Asn at position 2218 ($Asn^{2218}$) was detected significantly more frequently in pre-HCC isolates than in the control isolates. Further studies are needed to determine the possible importance of this residue in hepatocarcinogenesis.

Another focus of attention is how the sequences of the core protein, NS3, and NS5A-IRRDR evolve during the interval between chronic hepatitis and HCC development. One of the significant advantages of the present study was that we could conduct a longitudinal investigation by analyzing the target sequences of pre- and post-HCC isolates. We found that core-$Gln^{70}$ and NS3-($Tyr^{1082}$/$Gln^{1112}$) were well conserved in each paired sample. This indicates that core-$Gln^{70}$ and NS3-($Tyr^{1082}$/$Gln^{1112}$) were already present before the

development of HCC. Non-$Gln^{70}$ of the core protein and non-$Tyr^{1082}$ and non-$Gln^{1112}$ of NS3 were also well conserved in each paired sample. These results imply the possibility that these sequence patterns were not a result of HCC but, rather, they were a possible causative factor for the development of HCC. We hypothesize, therefore, that HCV isolates with core-$Gln^{70}$ and/or NS3-($Tyr^{1082}$/$Gln^{1112}$) are highly oncogenic, whereas those with non-($Gln^{70}$ plus NS3-$Tyr^{1082}$/$Gln^{1112}$) are less oncogenic. It is not clear yet as to whether these oncogenic mutations were present from the very beginning of HCV infection or if they emerged at a certain timepoint (before the initiation of follow-up) during the long-term persistence through an adaptive viral evolution in the host. More comprehensive follow-up study is needed to address this issue. In any case, the core-$Gln^{70}$ and NS3-($Tyr^{1082}$/$Gln^{1112}$) would be considered an index for prediction of HCC development. On the other hand, IRRDR in NS5A is more tolerant for sequence evolution. IRRDR in post-HCC isolates showed a significantly higher degree of sequence heterogeneity compared with that in pre-HCC isolates. This observation suggests that IRRDR is under strong selective pressure during the course of HCV infection and that the high degree of IRRDR heterogeneity (IRRDR$\geq$6) in HCV isolates from patients with HCC may not be a causative factor for development of HCC.

In conclusion, the present results suggest the possibility that patients infected with HCV isolates with core-$Gln^{70}$ and/or NS3-($Tyr^{1082}$/$Gln^{1112}$) are at a higher risk to develop HCC compared to those with non-($Gln^{70}$ plus NS3-$Tyr^{1082}$/$Gln^{1112}$).

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***Original Article***

# Detection of highly prevalent hepatitis B virus co-infection with HIV in Indonesia

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*Aim:* The prevalence of hepatitis B virus (HBV) co-infection with HIV is increasing worldwide because of shared transmission routes. This study aimed to assess the prevalence of HBV and HIV co-infection in Indonesia, and its molecular and clinical characteristics.

*Methods:* A total of 118 serum samples from HIV-infected patients (age 33.3 ± 8.9 years, 99 male, 19 female) collected in 2009 were serologically examined. HBV DNA was assessed by polymerase chain reaction (PCR) analysis targeting the S region.

*Results:* Overall, 15.3% (18/118) of the patients were hepatitis B surface antigen (HBsAg) positive, whereas 27.1% (32/118) were HBsAg negative but HBV DNA positive, and were considered to have occult HBV infection. HBsAg antibodies and/or HBV core antibodies were detected in 45.6% (31/68) of HBV DNA negative patients.

*Conclusion:* HBV co-infection, including occult HBV infection, was common in Indonesian HIV patients. Hepatic damage by the interaction of host immunity and HBV is still a remaining issue in these immunosuppressive patients, and further study will be needed.

**Key words:** co-infection, hepatitis B virus, HIV, Indonesia

## INTRODUCTION

THERE ARE AN estimated 400 million people worldwide with chronic hepatitis B virus (HBV) infection and an estimated 40 million people are infected with HIV. Co-infection with HBV and HIV is common because of shared blood-borne transmission routes, particularly injection drug use (IDU), with estimates for the prevalence of co-infection ranging 4–23%.[1–3] In areas of highly endemic HBV infection, co-infection with HBV was reported in 50% of HIV patients.[4]

In this setting, the mortality rate was higher in patients with HBV and HIV co-infection than for HIV or HBV infection alone.[5] Since the introduction of highly active antiretroviral therapy (HAART) in many countries, including Indonesia, the mortality rate attributed to AIDS has declined. Liver disease, however, has emerged as a significant cause of morbidity and mortality among HIV-infected individuals[6–9] because HIV promotes HBV replication and the progression of hepatic damage.[10] These changes cause prolonged elevations in alanine aminotransferase (ALT) levels,[11] shorten the time to the onset of cirrhosis[12] and increase the risk of developing hepatocellular carcinoma. The risk of death attributable to liver diseases in patients with HBV and HIV co-infection is 14-times higher than that of patients infected with either virus alone.[12,13] The prevalence of HBV-related liver diseases is expected to increase in HIV-infected patients because HAART has now been introduced in HBV endemic areas and the incidence of AIDS-related death is decreasing.

Occult HBV infection is defined as the presence of HBV DNA in serum and/or liver tissue of individuals with HBV core antibodies (anti-HBc) without hepatitis B surface antigen (HBsAg).[14] The prevalence of occult HBV infection varies greatly in HIV-infected patients and its clinical significance is unclear.[15] Occult HBV infection is possibly caused by changes in antigenicity,

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but it may also occur following suppression of viral replication, gene expression and virus secretion.[16]

Indonesia is experiencing one of the fastest increases in the incidence of HIV in Asia,[17] with a concentrated HIV epidemic in populations characterized by high-risk behaviors. In HBV endemic countries, such as Indonesia (5–20%), transmission mostly occurs perinatally or in early childhood. Therefore, HBV infection usually precedes HIV infection by several decades.[2] The main route of HIV transmission is IDU, followed by sexual contact.[17–19] The aims in this study were to assess the prevalence of HBV, including occult HBV infection, in HIV-infected patients in Surabaya, Indonesia, and determine its molecular and clinical characteristics, to understand the interaction between these two chronic viral infections.

## METHODS

### Study population

SERUM SAMPLES WERE collected from 118 HIV-infected individuals (mean age, 33.3 ± 8.9 years; 99 male, 19 female) with unknown HBsAg status who routinely visited a private clinic in Surabaya, the second biggest city in Indonesia, in 2009. Most of the patients were male (83.9%). The main routes of HIV transmission were IDU (63.8%; 74/118), sexual contact (35.6%; 42/118) and unknown exposure (1.7%; 2/118). Ninety-four percent of patients (109/118) were on HAART with activity against AIDS (lamivudine, zidovudine, and nevirapine or efavirenz). The study protocol was reviewed and approved by the Ethics Committees of Kobe University in Japan and Airlangga University in Indonesia. Informed consent was obtained from all patients. Demographic, clinical and laboratory data were also retrieved from the patient database maintained at the clinic.

### Serological assays

Serological tests for HBV markers were performed on all 118 serum samples from HIV-infected patients. Commercially available kits were used to assess HBsAg by reverse passive hemagglutination (Mycell II HBsAg; Institute of Immunology, Tokyo, Japan), HBsAg antibodies (anti-HBs) and anti-HBc by passive hemagglutination (Mycell II anti-HBs and Mycell anti-rHBc; Institute of Immunology). An enzyme immunoassay (EIA) (Espline HBsAg; Fuji Rebio, Tokyo, Japan) and an enzyme-linked immunosorbent assay (ELISA) (Surase B-96; General Biologicals, Hsin Chu, Taiwan) were used to confirm HBsAg positivity in samples positive for HBV DNA on polymerase chain reaction (PCR).

### HBV amplification and sequencing

After assaying HBV serological status, DNA was extracted from 200 μL of serum that had been stored at −80°C using a DNA extractor kit (QIAamp DNA Blood Mini Kit; QIAGEN, Tokyo, Japan) from all 118 HIV-infected individuals. The presence of HBV DNA was assayed by PCR with primer pairs for the precore/core (nt. 1611–2072) and S (nt. 18–557 and/or 414–989) regions. PCR amplification was performed as previously described.[20] The amplified fragments were directly sequenced using a Big Dye Deoxy Terminator cycle sequencing kit with an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

### Phylogenetic analysis

The HBV genotypes/subgenotypes were determined using the phylogenetic tree of 13 strains in the S (nt. 84–465) region. If the genotypes/subgenotypes could not be determined using these methods, the amplified fragments (nt. 406–646) of the small S region detected by real-time PCR were genotyped. Reference sequences were retrieved from the DNA Data Bank of Japan/European Molecular Biology Laboratory/GenBank database. Alignments were performed using CLUSTAL X software, and phylogenetic trees were constructed by the neighbor-joining method. To confirm the reliability of phylogenetic tree analysis, bootstrap resampling and reconstruction were carried out 1000 times. These analyses were carried out using Molecular Evolutionary Genetics Analysis (MEGA) software (available at www.megasoftware.net).

### Detection of HBV mutations and measurement of the HBV viral load

Hepatitis B virus nucleotide sequences encoding the *a* determinant region of HBsAg were translated to amino acid sequences (124–147) and aligned with reference sequences. Lamivudine-resistance mutations, within the amino acid sequence 125–213 of HBV polymerase, were assessed as previously described.[21] Viral load was assessed by real-time PCR using an ABI 7500 real-time PCR system (Applied Biosystems). HBV DNA was amplified with a primer and probe set, as described previously.[22]

### Statistical analysis

Statistical analysis was performed using the $\chi^2$-test or Fisher's exact test for categorical variables. The indepen-

dent Student's *t*-test was used for continuous variables. Values of $P < 0.05$ were considered to be significant.

## RESULTS

### HBsAg seroprevalence in HIV-infected individuals

AMONG 118 HIV infected patients, 15 samples were HBsAg positive by all three assays. Three HBV DNA positive samples were HBsAg positive on the EIA and/or ELISA alone. Overall, 18 patients (15.3%) were considered to be positive for HBsAg.

### Prevalence of HBV DNA

Hepatitis B virus DNA was detected in 50 out of 118 HIV-infected patients (42.4%). Of these, 32 patients (64% [27.1% of all patients]) were HBsAg negative, indicating occult HBV infection. Meanwhile, seven patients with occult HBV infection were serologically positive for the anti-HBc antibody,[23] 12 patients were negative for all serological markers, and three patients were positive for anti-HBc and anti-HBs (data not shown). HBV DNA was detected in all HBsAg positive subjects, and the mean HBV viral load was 6.1 ± 1.6 log copies/mL.

### Characteristics of patients with HBV and HIV co-infection according to HBsAg status

The virological, serological and clinical characteristics of patients positive for HBsAg and patients with occult HBV infection and HIV co-infection are shown in Table 1. High HBV viral loads (≥5 log copies/mL, $P = 0.0002$) and high aspartate aminotransferase (AST) levels (≥40 IU/L, $P = 0.047$) were significantly more frequent in HBsAg positive carriers than those in occult HBV infected patients.

### Serological status and clinical characteristics of HBV DNA negative patients

The serological status and clinical characteristics of HBV DNA negative patients are summarized in Table 2. AST levels were 40 IU/L or more in 15 out of 31 patients (48.4%) positive for anti-HBs and/or anti-HBc but negative for HBV DNA. The prevalence of AST of 40 IU/L or

**Table 1** Characteristics of patients with HBV and HIV co-infection according to HBsAg status

| Characteristic | HBsAg positive patients ($n = 18$) | Occult HBV-infected patients ($n = 32$) | All patients ($n = 50$) |
|---|---|---|---|
| Males, *n* (%) | 17 (94.4) | 28 (87.5) | 45 (90.0) |
| Age, years (mean ± SD) | 30.9 ± 7.7 | 33.2 ± 8.3 | 33.3 ± 8.9 |
| CD4 count <200 cells/mm$^3$, *n* (%) | 11 (61.1) | 25 (78.1) | 36 (72.0) |
| CD4 cell count, cells/mm$^3$ (mean ± SD) | 160.9 ± 116.9 | 141.1 ± 151.0 | 148.3 ± 138.7 |
| HBV viral load ≥5 log copies/mL, *n* (%) | 12 (66.7)* | 4 (12.5)* | 16 (32.0) |
| AST ≥40 IU/L, *n* (%) | 12 (66.7)** | 12 (37.5)** | 24 (48.0) |
| AST level (mean ± SD) | 74.3 ± 102.4 | 38.44 ± 24.9 | 51.4 ± 65.8 |
| ALT ≥40 IU/L, *n* (%) | 12 (66.7) | 14 (43.8) | 26 (52.0) |
| ALT, IU/L (mean ± SD) | 70.4 ± 71.9 | 41.7 ± 32.8 | 52.0 ± 51.7 |
| HBV subgenotypes, *n* | 13 | 29 | 42 |
| HBV/B3, *n* (%) | 11 (84.6) | 28 (96.6) | 39 (92.9) |
| HBV/C1, *n* (%) | 2 (15.4) | 1 (3.4) | 3 (7.1) |
| Transmission route, *n* (%) | | | |
| IDU | 13 (72.2) | 22 (66.8) | 35 (70.0) |
| Sexual contact | 5 (27.8) | 10 (31.3) | 15 (30.0) |
| Anti-HBs antibody, *n* (%) | 1 (5.6) | 12 (37.5) | 13 (26.0) |
| Mutation in the *a* determinant region, *n* | 17 | 31 | 48 |
| T123A, *n* (%) | 0 | 2 (6.5) | 2 (4.2) |
| Q129H, *n* (%) | 2 (11.8) | 0 | 2 (4.2) |
| M133L, *n* (%) | 6 (35.3) | 19 (61.3) | 25 (52.1) |

*$P = 0.0002$, **$P = 0.047$. ALT, alanine aminotransferase; anti-HBc, HBV core antibodies; anti-HBs, hepatitis B surface antigen antibodies; AST, aspartate aminotransferase; HBV, hepatitis B virus; IDU, injection drug use; SD, standard deviation.

**Table 2** Serological and clinical characteristics of HBV DNA negative patients ($n = 68$)

| | Anti-HBs positive patients | Anti-HBc positive patients | Anti-HBs and/or anti-HBc positive patients |
|---|---|---|---|
| Number (%) | 18 (26.5) | 17 (25.0) | 31 (45.6) |
| Males, *n* (%) | 14 (77.8) | 15 (88.2) | 26 (83.9) |
| Age, years (mean ± SD) | 33.2 ± 6.8 | 36.3 ± 10.3 | 35.1 ± 8.7 |
| CD4 cell count <200 cells/mm$^3$, *n* (%) | 13 (72.2) | 13 (76.5) | 24 (77.4) |
| CD4 cell count, cells/mm$^3$ (mean ± SD) | 129.1 ± 151.6 | 131.1 ± 154.8 | 115.3 ± 137.7 |
| AST ≥40 IU/L, *n* (%) | 5 (7.4) | 8 (11.9) | 15 (48.4) |
| AST, IU/L (mean ± SD) | 42.7 ± 35.4 | 49.4 ± 27.3 | 47.4 ± 33.1 |
| ALT ≥40 IU/L, *n* (%) | 7 (10.3) | 10 (14.7) | 18 (26.5) |
| ALT, IU/L (mean ± SD) | 48.9 ± 31.0 | 58.7 ± 38.3 | 53.6 ± 35.9 |

ALT, alanine aminotransferase; anti-HBc, HBV core antibodies; anti-HBs, hepatitis B surface antigen antibodies; AST, aspartate aminotransferase; HBV, hepatitis B virus; SD, standard deviation.

more was not significantly different between HBV DNA negative and HBV DNA positive patients. Previous HBV infection was recorded in 45.6% (31/68) of HBV DNA negative patients.

## CD4 cell count

The mean CD4 cell count was 148.7 ± 149.9 cells/mm$^3$ in all 118 HIV-infected patients, 161.0 ± 116.9 in HBsAg positive patients and 141.1 ± 151.0 in patients with occult HBV infection and HIV co-infection. The CD4 cell count was less than 200 cells/mm$^3$ in 83 out of 118 HIV-infected patients (70.3%). Among HBV DNA positive patients, 36 out of 50 (72.0%) had a CD4 cell count of less than 200 cells/mm$^3$. The prevalence of ALT of 40 IU/L or more was much higher in patients with a CD4 cell count of less than 200 cells/mm$^3$. Among HBsAg positive patients, the mean AST and ALT levels tended to be higher in those with a CD4 cell count of less than 200 cells/mm$^3$ compared with those with a cell count of 200 cells/mm$^3$ or more, although this was not statistically significant (see Table S1 in the supplemental material).

## Identification of HBV genotypes

Consensus nucleotide sequences were generated and aligned with reference sequences. Based on the pre-S2-S region (nt. 84–465), HBV subgenotypes B3 (HBV/B3) ($n = 7$) and HBV/C1 ($n = 2$) were identified in HBsAg positive patients, while HBV/B3 ($n = 3$) and HBV/C1 ($n = 1$) were identified in patients with occult HBV infection (Fig. 1). In the S region (nt. 406–646), HBV/B3 ($n = 4$) was identified in HBsAg positive patients and HBV/B3 ($n = 25$) was identified in patients with occult HBV infection. Similar to Indonesian patients with HBV infection alone, HBV/B3 was the most prevalent subgenotype in patients with HIV co-infection regardless of HBsAg positivity.

## Mutational analysis in relation to HBsAg status and drug resistance

The amino acid substitutions in the *a* determinant region (amino acids 124–147) are shown in Figure 2. A single amino acid substitution (T123A) was found in two patients with occult HBV infection. G145R and K12R, common escape mutations, were not detected in this study. One hundred and nine subjects (92.4%) were on HAART (lamivudine, zidovudine, and nevirapine or efavirenz) to control HIV. Amino acid mutations in the polymerase region were detected in two HBsAg positive patients; one was rtM204I and the other was rtL180M plus rtM204I. The patient with rtM204I had high ALT levels (80 IU/L), while HBV viral loads were relatively high in both of these patients with mutations in the polymerase region.

# DISCUSSION

INDONESIA IS A country with a moderate to high prevalence of HBV. It was reported that co-infection with HBV and HIV was prevalent in countries with highly endemic HBV infection because of shared routes of transmission.[24] In this study, 15% of the HIV-infected patients were HBsAg positive, which is extremely high compared with the prevalence of HBsAg carriers in the general population in Indonesia. It is also noteworthy that 42.2% of the HIV-infected patients were considered to have HBV and HIV co-infection, if we included

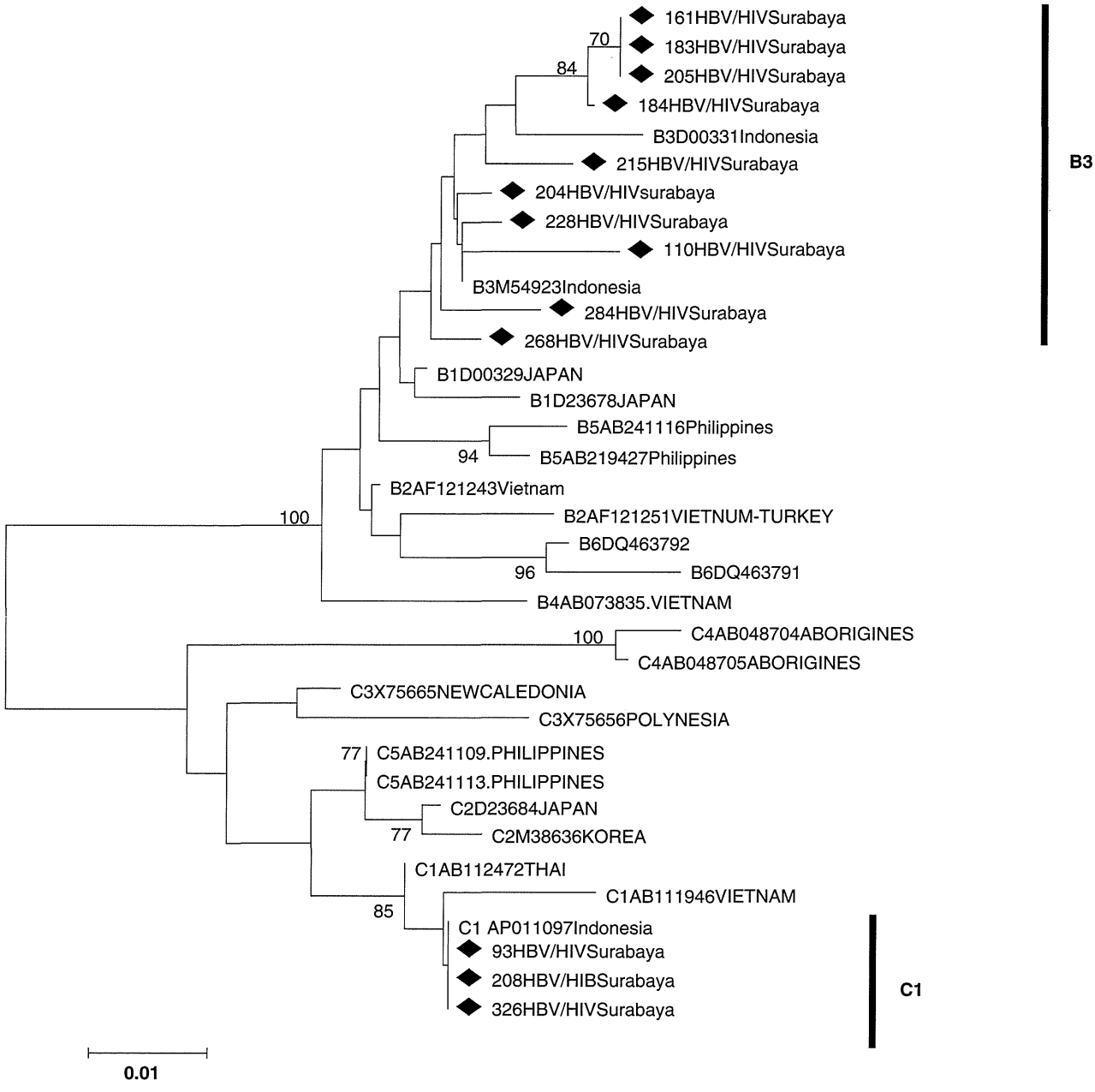


**Figure 1** Phylogenetic tree constructed with the neighbor-joining method based on the partial nucleotide sequence of the S region of 22 hepatitis B virus (HBV) reference strains. Reference isolates are indicated with their accession numbers and the country of origin is reported for each HBV/B and HBV/C strain. The number inside the tree indicates the bootstrap reliability.

occult HBV infection. Moreover, 68.6% of the HIV-infected patients in this study had current or previous HBV infection.

The clinical significance of occult HBV infection in HIV-infected patients is still controversial.[25,26] Therefore, we evaluated whether occult HBV infection contributes to liver damage in HIV-infected patients. It was previously reported that mutations in a determinant region, such as G145R, may induce HBsAg immune escape.[27] In this study, only two patients had an amino acid substitution (T123A) in this region, which suggests that the escape mutants including G145R and K122R are rarely

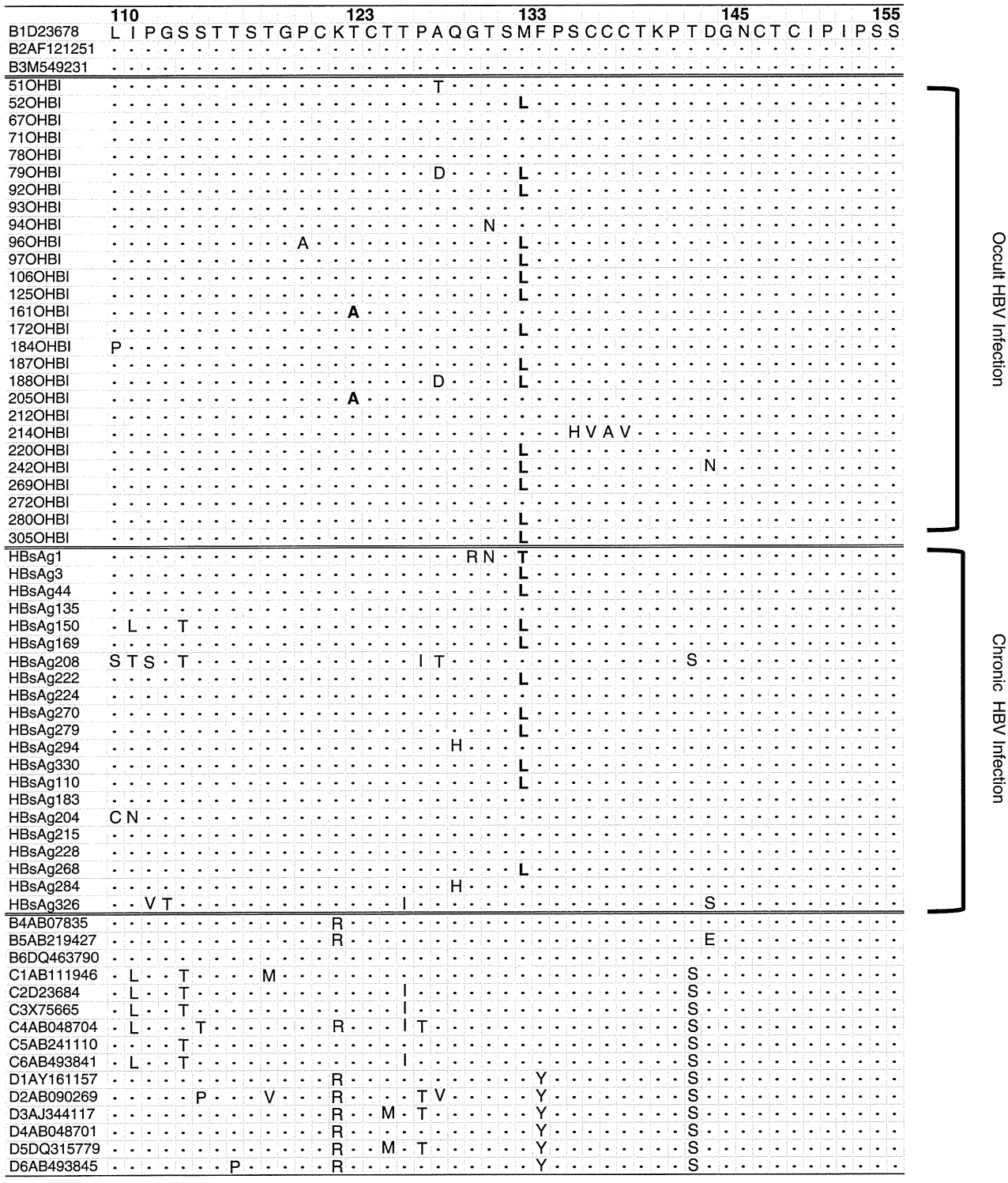


**Figure 2** Surface antigen *a* determinant amino acid sequence alignment for hepatitis B virus (HBV). The first-line sequence is the consensus sequence corresponding to the HBV subgenotype (B1; accession no. D23678) reference strain retrieved from the Japan/GenBank database. Dots indicate positions with amino acids identical to the HBV/B1 consensus sequence.

detected in Indonesian HBV patients and it is probably because of the presence of different HBV subgenotypes in Indonesian patients compared with those in other countries. We also found that the AST level and HBV viral load were significantly higher in HBsAg positive patients compared with occult HBV-infected patients, suggesting that hepatic damage is partly dependent on HBV viral factors.

In general, the hepatic damage associated with HBV infection is dependent on viral factors and the host's immune response to HBV-infected hepatocytes. In this study, the AST and the ALT level in HBsAg positive patients tended to be higher in those with a CD4 cell count of less than 200 cells/mm$^3$ than in patients with a CD4 cell count of 200 cells/mm$^3$ or more. This also suggests that hepatic damage is partly derived from host immunity in the severe immunosuppressive state.[28] In addition, the prevalence of occult HBV infection detected in this study was significantly higher than that previously found in Indonesian blood donors (8.1%).[29] This observation may be due to an advanced stage of HIV disease (70.3% with a CD4 cell count <200 cells/mm$^3$ in this study).[30]

In this study, the mode of transmission was mainly IDU, unlike in the USA and Europe where HIV is mainly transmitted by sexual contact followed by IDU.[31] HBV genotyping revealed that several strains were of subgenotype C1, which originated in southeastern Asian countries, including the Philippines, Vietnam and Thailand. These results suggest that HIV also spread through the same mode of transmission. Indonesia is facing one of the most rapidly growing HIV epidemics in Asia, except for Papua, and this increase is mainly driven by IDU.[17] The prevalence of HIV in the general population is still quite low (0.2%), but its prevalence among IDU exceeds 50%,[32] increasing the risk of HIV and HBV co-infection. A significant increase in IDU among young adults was recently reported in Indonesia, and IDU was identified as the main cause of the rapid spread of HIV/AIDS in southeastern Asia.[33]

Highly active antiretroviral therapy is used to treat patients worldwide, and HIV is now well controlled in many countries. Consequently, viral hepatitis caused by HBV and liver toxicity caused by HAART are issues for patients with HBV and HIV co-infection undergoing HAART therapy.[34] Lamivudine frequently induces multidrug resistance in the RT domain of the polymerase region. M204I and L180M are common amino acid mutations that cause multidrug resistance. We found two HBsAg positive patients with high HBV viral loads and lamivudine resistance, even though lamivudine was used for the treatment of HIV. The combination of tenofovir plus emtricitabine or lamivudine should therefore be preferred in this setting.[35] Clearly, studies are needed to examine HBV DNA status and to establish individualized treatment plans for patients with multidrug resistance.

In conclusion, HBV co-infection, including occult HBV infection, was common in Indonesian HIV positive patients. Additional studies are needed to understand how prolonged HIV infection accelerates the course of chronic HBV. Moreover, clinicians and health workers should consider the national guidelines for prophylaxis, screening and treatment.

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