Original Paper

Intervirology

Intervirology 2008;51:410–416
DOI: 10.1159/000205515

Received: April 29, 2008
Accepted after revision: January 19, 2009
Published online: March 4, 2009

Hepatitis C Virus Genotype in Blood Donors and Associated Liver Disease in Indonesia

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Key Words
Hepatitis C virus · Genotype · Chronic hepatitis C · Liver cirrhosis · Hepatocellular carcinoma · Indonesia

Abstract
Objective: The aim of this study was to investigate the distribution of hepatitis C virus (HCV) genotype and the possible association between genotype and HCV-associated liver disease in Indonesia. ***Methods:*** 32 anti-HCV-positive asymptomatic carriers (AC), 55 chronic hepatitis (CH), 41 liver cirrhosis (LC), and 35 hepatocellular carcinoma (HCC) patients were included in this study. HCV genotyping was performed by phylogenetic analysis of the NS5B and 5′-UTR regions. ***Results:*** The HCV subtype 1b (36.5%), based on NS5B region, was the most prevalent, followed by subtypes 3k (15.4%), 2a (14.4%), 1a (12.5%) and 1c (12.5%), and 2e (4.8%). Subtypes 2f, 3a, 3b, and 4a were also found in some of the samples. HCV subtypes 3k (40.0%) and 1a (35.0%) were the two major subtypes in AC. HCV subtype 1b was not found in AC, but it was common in CH (31.3%), LC (50.0%), and HCC (57.1%). ***Conclusion:*** HCV subtype 1b was prevalent in samples of HCV-associated liver disease patients, including CH, LC and HCC. The percentage of subtype 1b was increased with the disease severity (AC < CH < LC < HCC).

Introduction

Hepatitis C virus (HCV) infection is known to be a major contribution to chronic liver disease such as chronic hepatitis (CH) and liver cirrhosis (LC), often leading to hepatocellular carcinoma (HCC). Worldwide, more than 170 million people are infected with HCV [1]. HCV is an enveloped virus with a single-stranded, positive-sense, nonsegmented RNA genome of approximately 9,500 nucleotides, encoding a polyprotein precursor consisting of about 3,000 amino acids [2]. The polyprotein is cleaved by the host signal peptidase and two intrinsic viral encoded proteases to generate at least 10 viral proteins, including the core protein (C), the envelope 1 glycoprotein (E1) and two types of envelope 2 glycoprotein (E2), and 6 nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) [3, 4].

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Analysis of the HCV genome has revealed high heterogeneity of the virus. Relatively well-conserved regions of the genome (5′-UTR, C, E1, and NS5B) have been extensively studied and used as the basis for the classification of at least six different genotypes in human [5]. The varied genotypes differ in distribution both by geographical region and by mode of transmission. Genotypes 1–3 occur globally, while HCV genotype 4 is common in the Middle East and Africa [6–8], genotypes 5 and 6 are found in South Africa [9] and Southeast Asia [10], respectively. Subtypes 1a and 3a are associated with intravenous drug use, whereas subtype 1b is common in blood donors in Europe [11–13]. In Indonesia, the genotypes 1–3 are found in both blood donors and patients diagnosed with CH, LC, and HCC [14–16].

HCV infection becomes chronic in about 85% of individuals as adjudged by the persistence of HCV RNA in serum [17]. CH may progress to worsening stages of fibrosis and cirrhosis which can ultimately lead to the development of HCC [18]. Several case-control and cohort studies of HCC and LC patients in Europe and Asia found a weak, albeit consistently, increased relative risk of HCV subtype 1b with the development of severe and advanced liver disease including cirrhosis and HCC [19–23]. However, some studies did not confirm these findings [24, 25]. Thus, the association of HCV genotype and pathogenesis of liver disease including HCC remains controversial and there is still limited information about the association of HCV genotype and HCC in Indonesian patients. In this study, we investigated the distribution of HCV genotype in blood donor carriers and patients of CH, LC, and HCC, analyzing the possible association between HCV genotype and HCC development.

Materials and Methods

Samples

Serum samples were obtained from 163 blood donors and HCV-associated liver disease patients. There were 32 asymptomatic carriers (AC), 55 patients with CH, 41 patients having LC, and 35 HCC patients. AC sera were collected from blood donors in the Blood Transfusion Unit, Red Cross Makassar, South Sulawesi, between January 2007 and January 2008. Sera of CH, LC, and HCC patients were collected from Cipto Mangunkusumo Hospital and Klinik Hati, Jakarta, from the period of May 2005 until January 2008. All sera were positive for anti-HCV antibody as determined by using a third-generation HCV enzyme immunoassay (AXSYM, Abbott Laboratories, Chicago, Ill., USA). Blood samples were collected from each patient at the time of their clinical evaluation, then separated into sera and stored at –70° until use for viral RNA extraction. The study was approved by the Institutional Ethic Committee and informed consent was obtained from each patient.

Viral RNA Extraction

Serum samples that had been stored at –70° were retrieved for analysis. HCV RNA was extracted from 200 μl serum using High Pure Viral RNA kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. The RNA was finally eluted in 50 μl of ribonuclease-free water and stored at –70° until further analysis.

Reverse Transcriptase-Nested PCR

NS5B Region

The viral RNA was used for genome amplification of partial NS5B region by a nested reverse transcriptase-PCR (RT-PCR). First-round RT-PCR was performed using the Access RT-PCR System (Promega, Madison, Wisc., USA) in 25-μl aliquots containing 5 μl RNA, 0.2 mM of each dNTP, 1× AMV buffer, 2 mM $MgSO_4$, 2.5 units of AMV reverse transcriptase, 2.5 units of Tfl polymerase, and 1 μM of NS5B-1 and NS5B-2 primers (table 1). The following cycling parameters were used for the first-round RT-PCR: cDNA synthesis at 45° (45 min), enzyme inactivation at 95° (5 min), DNA amplification: denaturation at 95° (30 s), annealing at 80–55° (30 s) and elongation at 72° (1 min). For the first 6 cycles, the annealing temperature was reduced by 5° per cycle (touchdown PCR), and 55° for the rest of 30 cycles. Two microliters of PCR product from first-round PCR was used as a template for second-round PCR. The second-round PCR was performed using the Go Taq PCR Core System (Promega) in 25-μl aliquots containing 2 mM $MgCl_2$, 1× Green Go Taq Flexi Buffer, 0.625 units of Go Taq DNA polymerase, and 0.5 μM of NS5B-3 and NS5B-4 primers (table 1). The following cycling parameters were used for 30 cycles of second-round PCR: denaturation at 94° (1 min), annealing at 50° (2 min) and elongation at 72° (2 min).

5′-UTR Region

A nested RT-PCR was devised to amplify the 5′-UTR region. First-round RT-PCR was performed using Access RT-PCR System (Promega) in 25-μl aliquots containing 5 μl RNA, 0.2 mM of each dNTP, 1× AMV buffer, 2 mM $MgSO_4$, 2.5 units of AMV reverse transcriptase, 2.5 units of Tfl polymerase, and 1 μM of 5′-UTR-1 and 5′-UTR-2 primers (table 1). The following cycling parameters were used for the first-round RT-PCR: cDNA synthesis at 45° (60 min), enzyme inactivation at 95° (5 min), 35 cycles of DNA amplification: denaturation at 95° (1 min), annealing at 57.7° (30 s) and elongation at 72° (1 min). Two microliters of PCR product from the first-round PCR was taken as a template for the second-round PCR with Go Taq PCR Core System (Promega) using 0.5 μM of 5′-UTR-3 and 5′-UTR-4 primers (table 1). The following cycling parameters were used for 30 cycles in the second-round PCR: denaturation at 95° (1 min), annealing at 53.7° (30 s) and elongation at 72° (1 min).

Sequencing

PCR product of NS5B and 5′-UTR regions were purified from agarose gel using Wizard SV Gel and PCR Clean Up system kit (Promega), according to the manufacturer's protocol. Purified DNA fragments were directly sequenced employing an ABI 3130 xl Genetic Analyzer (Applied Biosystems, Inc., Foster City, Calif., USA) with the Big Dye Terminator V3.1 Cycle Sequencing kit (Applied Biosystems, Inc.).

Table 1. Primers used in this study

Primer	Nucleotide sequence (5′→3′)	Polarity	Reference
NS5B			
NS5B-1	TATGAYACCCGYTGCTTTGAC	forward	Cantaloube et al., 2005
NS5B-2	GAGGAGCAAGATGTTATCAGCTC	reverse	
NS5B-3	GATACCCGCTGCTTTGACTC	forward	
NS5B-4	GAATACCTGGTCATAGCCTCCG	reverse	
5′-UTR			
5′-UTR1	CCCTGTGAGGAACTWCTGTCTTCACGC	forward	Stuyver et al., 1996
5′-UTR2	TCTAGCCATGGCGTTAGTAYGAGTGT	reverse	
5′-UTR3	CACTCGCAAGCACCCTATCAGGCAGT	forward	
5′-UTR4	GCTCATGRTGCACGGTCTACGAGACCT	reverse	

Table 2. Positivity of RT-PCR and sequencing of the NS5B region of samples from different clinical diagnoses

Clinical diagnosis	n	PCR-positive n (%)	Sequenced n (%)
AC	32	20 (62.5)	20 (100)
CH	55	32 (58.2)	32 (100)
LC	41	25 (61.0)	24 (96.0)
HCC	35	28 (80.0)	28 (100)
Total	163	105 (64.4)	104 (99.0)

Phylogenetic Analysis

The nucleotide sequences of the HCV and a panel of sequences retrieved from the GenBank were aligned using BioEdit Sequence Aligment Editor. Phylogenetic analysis of NS5B (367 bp) and 5′-UTR (236 bp) regions was performed with ClustalW version 1.83.

Statistical Analysis

Statistical analyses were performed using SPSS 15.0 for Windows (SPSS, Inc., Chicago, Ill., USA). χ^2 test, unpaired t test, and ANOVA were used to assess the statistical significance of the difference between groups. p values <0.05 were considered statistically significant.

Results

HCV Genotype Distribution

The NS5B region was used for HCV genotyping and 105 out of the 163 samples (64.4%) were positive by RT-PCR (table 2). The number of samples that could be amplified was higher in HCC (80.0%) compared with other groups; 62.5% in AC, 58.2% in CH, and 61.0% in LC patients. Of the 105 RT-PCR positive samples, 104 samples (99.0%) could be sequenced and classified not only into genotype, but also subtype. By phylogenetic analysis of partial NS5B region (367 bp), the HCV strains from 104 samples were classified into the genotype 1 (with subtypes 1a, 1b, and 1c), 2 (with subtypes 2a, 2e, and 2f), 3 (with subtypes 3a, 3b, and 3k), and 4 (subtype 4a) (table 3). The subtype 1b was the most prevalent, accounting for 36.5% of the total samples, followed by subtypes 3k (15.4%), 2a (14.4%), 1a (12.5%) and 1c (12.5%), and 2e (4.8%) (table 3). One of each subtype 2f, 3a, 3b, and 4a was also found in the samples.

HCV Genotype and Clinical Diagnosis

When the genotype distribution was compared with clinical diagnosis, it was found that subtype 3k (40.0%) and 1a (35.0%) were dominantly detected in the AC group (table 3). The HCV subtype 1b was prevalent in CH (31.3%), LC (50.0%), and HCC (57.1%) groups. In the CH group, HCV genotype 1 was common (62.5%), which composed of subtypes 1a (9.4%), 1b (31.3%), and 1c (21.9%), in addition the genotypes 2 (subtypes 2a and 2e) and 3 (subtypes 3a and 3k) were also detected. Similarly, genotype 1 was also the major genotype in LC (70.8%), which composed of subtypes 1a (8.3%), 1b (50.0%), and 1c (12.5%). A high percentage of genotype 1 (57.1%) was found in HCC. Particular to subtype 1b, the prevalence of this subtype was increased from AC to HCC (0.0% in AC, 31.3% in CH, 50.0% in LC, and 57.1% in HCC) (table 3). Statistical analysis revealed a significant difference of HCV subtype 1b among all clinical stages ($p < 0.001$); however, it was not significant across all clinical stages. For instance, there was no significant difference between

Table 3. HCV genotype prevalence in different clinical diagnosis based on NS5B region

Genotype	AC (n = 20)	CH (n = 32)	LC (n = 24)	HCC (n = 28)	Total
Genotype 1	8 (40.0)	20 (62.5)	17 (70.8)	19 (67.9)	64 (61.5)
1a	7 (35.0)	3 (9.4)	2 (8.3)	1 (3.6)	13 (12.5)
1b	0 (0.0)	10 (31.3)	12 (50.0)	16 (57.1)	38 (36.5)
1c	1 (5.0)	7 (21.9)	3 (12.5)	2 (7.1)	13 (12.5)
Genotype 2	2 (10.0)	6 (18.8)	5 (20.8)	8 (28.6)	21 (20.2)
2a	2 (10.0)	5 (15.6)	3 (12.5)	5 (17.9)	15 (14.4)
2e	0 (0.0)	1 (3.1)	1 (4.2)	3 (10.7)	5 (4.8)
2f	0 (0.0)	0 (0.0)	1 (4.2)	0 (0.0)	1 (1.0)
Genotype 3	9 (45.0)	6 (18.8)	2 (8.3)	1 (3.6)	18 (17.3)
3a	0 (0.0)	1 (3.1)	0 (0.0)	0 (0.0)	1 (1.0)
3b	1 (5.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.0)
3k	8 (40.0)	5 (15.6)	2 (8.3)	1 (3.6)	16 (15.4)
Genotype 4	1 (5.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.0)
4a	1 (5.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.0)
Grand total	20 (100.0)	32 (100.0)	24 (100.0)	28 (100.0)	104 (100.0)

Table 4. HCV genotype prevalence in different clinical diagnoses based on the 5′-UTR region

Genotype	AC (n = 20)	CH (n = 31)	LC (n = 24)	HCC (n = 28)	Total
Genotype 1	9 (45.0)	18 (58.0)	17 (70.8)	20 (71.4)	64 (62.1)
Genotype 2	1 (5.0)	6 (19.4)	5 (20.8)	6 (21.4)	18 (17.5)
Genotype 3	9 (45.0)	7 (22.6)	2 (8.3)	2 (7.1)	20 (19.4)
3a	0 (0.0)	1 (3.2)	0 (0.0)	0 (0.0)	1 (1.0)
3b	1 (5.0)	1 (3.2)	0 (0.0)	0 (0.0)	2 (2.0)
3k	8 (40.0)	5 (16.1)	2 (8.3)	2 (7.1)	17 (16.5)
Genotype 4	1 (5.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.0)
Grand total	20 (100.0)	31 (100.0)	24 (100.0)	28 (100.0)	103 (100.0)

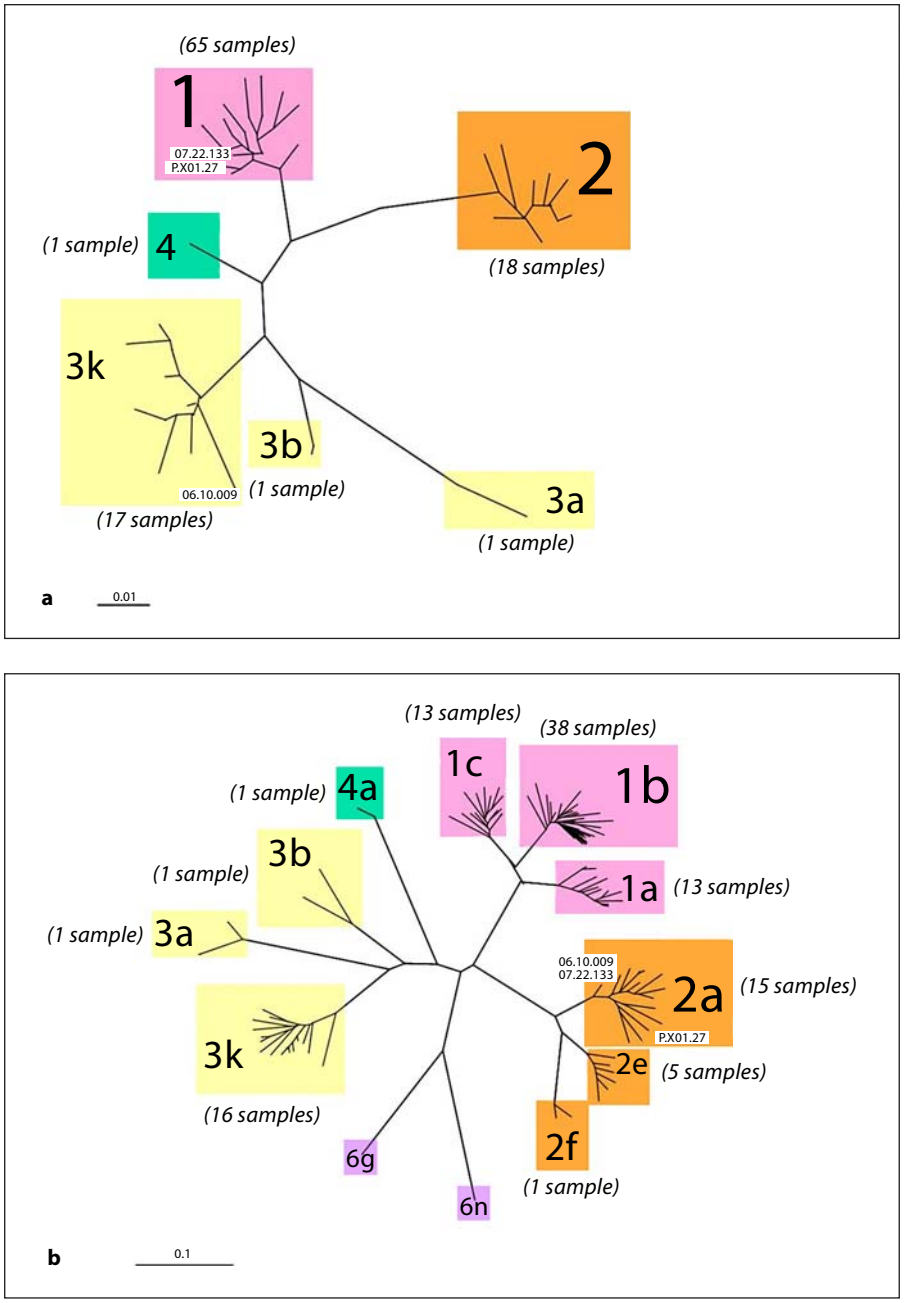
Table 5. Samples which showed different genotypes from an analysis based on 5′-UTR and NS5B regions

No.	Report No.	Clinical diagnosis	HCV genotype based on	
			5′-UTR	NS5B
1	07.22.133	AC	1	2a
2	P.X01.27	LC	1	2a
3	06.10.009	HCC	3k	2a

CH and LC ($p = 0.121$), and between LC and HCC ($p = 0.565$), but there was significant difference between AC and CH ($p = 0.015$). Moreover, the prevalence of subtype 1b was significantly higher in HCC compared to both CH ($p < 0.001$) and AC ($p < 0.001$).

Comparison of NS5B and 5′-UTR Regions

In samples where the NS5B region could be amplified, the 5′-UTR region was explored to confirm the genotype. Phylogenetic analysis of the 5′-UTR region showed that it could identify the genotype, but not the subtype of HCV, particularly for genotypes 1 and 2 as compared to



Color version available online

Fig. 1. Unrooted neighbor-joining phylogenetic tree from (**a**) partial 5′-UTR and (**b**) NS5B. Samples which are different genotypes in 5′-UTR and NS5B regions are highlighted.

analysis based on NS5B region (fig. 1); however, it could distinguish the subtype of genotype 3 (fig. 1a). By phylogenetic analysis of the 5′-UTR region (236 bp), the HCV strains from 103 samples were classified into genotypes 1 (62.1%), 2 (17.5%), 3 (19.4%) and 4 (1.0%) (table 4), and were identical to genotyping based on NS5B region (table 3). However, the genotyping results based on 5′-UTR and NS5B regions were different in three samples (07.22.133, P.X01.27, and 06.10.009 from the AC, LC, and HCC groups, respectively) (table 5). These three samples were identified as subtype 2a based on NS5B region, but 07.22.133 and P.X01.27 were classified as HCV genotype 1 and 06.10.009 was identified as subtype 3k based on the 5′-UTR region (table 5, fig. 1). These findings indicate that the HCV isolates in those samples might be recombinant viruses resulted from recombination of HCV with different genotypes.

Discussion

Phylogenetic analysis of partial NS5B region demonstrated that subtype 1b was the most prevalent in our samples, although other subtypes (3k, 2a, 2e, 1a, and 1c) were also detected. Generally, our findings are similar with previous data of HCV genotype in Indonesia. Hotta et al. [14] identified HCV subtypes 1b, 1d, and 2a from CH and LC patients and subtype 1b was found in 45.0 and 34.0% samples of CH and LC, respectively. In a study of blood donors, hemodialysis and HCC patients, subtypes 1a, 1b, 1d, 2a, and genotype 4 were found [15]. The subtype 2a was dominant in blood donors (51.9%), whilst subtype 1b was more common in hemodialysis and HCC patients (31.3 and 57.1%, respectively). HCV genotyping of 64 blood donor samples found that the predominant genotype is 1b (57.8%); this followed by 2a (17.2%), and 3b (10.9%) [16]. In contrast with this finding, our results did not identify genotype 1b from the blood donor (AC) group. In addition, we also found one subtype 4a, the genotype that is common in the Middle East and Africa, in AC samples.

When genotype distribution was analyzed based on clinical diagnosis, it was determined that subtype 1b was not found in AC, but was prevalent in CH and increased in LC and HCC. Statistical analysis did not show a significant difference between CH and LC, and between LC and HCC. It is suggested that there is an association between HCV subtype 1b and CH development, but no association with development of HCC from LC or CH. A weak but consistently increased relative risk of HCC in patients with subtype 1b has been found in several case-control and cohort studies of HCC and LC patients in Europe and Asia [19–23], which is different to our findings. However, further studies have not confirmed these findings [24, 25]. Therefore, the association of HCV genotype and pathogenesis of liver disease, particularly HCC, is still debatable, and needs prospective cohort studies to obtain more conclusive data.

Comparison of phylogenetic analysis in both NS5B and 5′-UTR regions showed differences of HCV genotypes in three samples, implying the possible recombination between different genotypes. Analysis of those samples was confirmed by repeating the sequencing experiment and data analysis. It is hypothesized that recombination might have occurred between genotype 1 and 2a, which results in 1/2a (for 07.22.133 and P.X01.27) or between subtype 3k and 2a, which results in 3k/2a (for 06.10.009). Further sequence analysis of full-length genome is needed to prove that a recombination event has occurred. Nevertheless, prior investigators reported the natural recombination of HCV from samples in St. Petersburg (2k/1b), Peru (1b/1a), the Philippines (2b/1b), Vietnam (2/6), Ireland (2k/1b), and France (2/5) [26–31]. Since there is no prevalence of recombination in certain stage of liver disease, it is suggested that virus recombination event could naturally occur during virus replication. The HCV recombination is a cause of genetic diversity of the virus, which may have an important implication for the pathogenesis, laboratory diagnosis, and treatment of HCV infection. However, so far there is no direct evidence on the clinical implication of the recombination, including response to certain therapy. In summary, our study demonstrated that HCV subtype 1b was prevalent in HCV-associated liver disease in the order of CH < LC < HCC. By comparing NS5B and 5′-UTR regions, putative recombinant of HCV was found in three samples.

Acknowledgments

The authors thank Mardiana Radjuni, Tities Anggraeni Indra, Marcella Harlan for sample collection. The authors also thank Drs. John D. Groopman (Johns Hopkins Bloomberg School of Public Health) and Ivet Suriapranata (Mochtar Riady Institute for Nanotechnology) for critical reading of the manuscript. This work was supported by MRIN Funding (Budget No. cc042/2007).

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