RESEARCH

Cloning and Molecular Analysis of Hepatitis C Virus (HCV) 1a NS5B from HCV Isolated in Surakarta Indonesia

Afiono Agung Prasetyo a,b,c
 aDepartment of Microbiology, Faculty of Medicine, Sebelas Maret University, Jl. Ir. Sutami 36A, Surakarta 57126, Indonesia
 bBiomedical Laboratory, Faculty of Medicine, Sebelas Maret University, Jl. Ir. Sutami 36A, Surakarta 57126, Indonesia
 cCenter of Biotechnology and Biodiversity Research and Development, Sebelas Maret University, Jl. Ir. Sutami 36A, Surakarta 57126, Indonesia

Received: 20 December 2010
Accepted: 25 February 2011

Abstract
The hepatitis C virus (HCV) NS5B protein is known essential for virus replication and suggested play important role in therapy resistance mechanism. To predict and more understanding the molecular pattern of HCV 1a NS5B we cloned the NS5B gene of HCV 1a isolated in Central of Java Indonesia using standard cloning procedure. The sequence results were then aligned with all of HCV 1a NS5B complete coding sequences reported in GenBank. In total, 397 sequences were retrieved and aligned by ClustalW. We revealed the amino acid sequence consensus of HCV NS5A gene. Overall, variations were found at aa 5, 11, 15, 33, 36, 37, 46, 47, 50, 55, 57, 59, 62, 63, 65, 66, 68, 71, 73, 77, 81, 82, 84-86, 90, 98, 101, 110, 113, 116, 117, 120, 124, 130, 134, 135, 144, 147-149, 151, 156, 162, 166, 173, 177-180, 184, 189, 198, 202, 206, 207, 209, 210, 212, 213, 231, 235, 238, 248, 251, 252, 254, 262, 267, 270, 273, 300, 307, 309, 310, 327, 330, 333, 334, 355, 374, 376, 377, 379, 389, 392, 400, 401, 402, 405, 412, 415, 421, 423-427, 431, 432, 434, 435, 437, 440, 451, 452, 454, 455, 459, 461, 464, 480, 487, 499, 506, 510, 512, 513, 517, 520, 523, 531, 535, 540, 543, 544, 549, 552, 556, 564-566, 572, 574, 580, 581, 585, and 588. Amino acid variations may have relevant changes of physicochemical properties so that influence the replication efficiency, therefore, the amino acid variations found in the present report need further study.

Key words: HCV 1a, NS5B

INTRODUCTION

Hepatitis C virus (HCV) is a positive-strand RNA virus that frequently causes persistent infections. Chronic infection by hepatitis C virus (HCV) can lead to severe hepatitis and cirrhosis and is closely associated with hepatocellular carcinoma. The HCV nonstructural protein 5B (NS5B) forms a complex with the retinoblastoma tumor suppressor protein (pRb), targeting it for degradation, activating E2F-responsive promoters, and stimulating cellular proliferation. The disruption of pRb/E2F regulatory pathways in cells infected with HCV is likely to promote hepatocellular proliferation and chromosomal instability, factors important for the development of liver cancer 

HCV-induced cytopathogenicity is associated with the level of intracellular viral replication and is determined by certain amino acid substitutions in HCV-NS5B regions.

The replication cycle of HCV is likely to involve interaction with host factors, HCV replicates its genome in replication complexes

Correspondence Author:
Afiono Agung Prasetyo
Department of Microbiology, Faculty of Medicine, Sebelas Maret University, Jl. Ir. Sutami 36 A, Surakarta 57126
Tel. +62-271-632489
E-mail: afieagp@yahoo.com; afie.agp.la@gmail.com
(RC) associated to endoplasmic reticulum (ER)-derived micro-vesicles. One key protein in these complexes is NS5B, HCV enzyme that shows the RNA binding and RNA-dependent RNA polymerase (RdRp) activities. The HCV NS5B RNA polymerase facilitates the RNA synthesis step during the HCV replication cycle. Nucleoside analogs targeting the NS5B provide an attractive approach to treating HCV infections because of their high barrier to resistance and pan-genotype activity. For this reason, NS5B protein has become one of the most important targets for designing new antiviral therapy compounds \(^{(4, 5)}\). To predict and more understanding the molecular pattern of HCV NS5B gene, we cloned the HCV NS5B gene from HCV 1a isolated in Central of Java Indonesia and performed bioinformatics study of all HCV 1a NS5B complete genes deposited in GenBank. Here we present our current analysis results of the NS5B gene of HCV 1a.

**MATERIALS AND METHODS**

**Isolate Selection for Cloning**

Previously, all plasma aliquot with anti-HCV positive from previous study (Prasetyo, et al., submitted for publication) were performed for nucleic acid extraction followed with nested RT-PCR addressed part of the HCV E1-E2 and HCV NS5B region. The positive PCR products were sequenced and phylogenetic analyzed to role out the predominant HCV in Central of Java Indonesia. All samples having the same genotype with the predominant genotype were further analyzed for its co-infection with another human blood borne viruses (HIV, HBV, HDV, HTLV-1/2, and TTV), HCV RNA titer, and sequencing results.

**Cloning of HCV 1a NS5B Gene from HCV 1a Isolated in Central of Java Indonesia**

The aliquot plasma from "the best candidate for cloning"-isolate were performed for RNA extraction followed with cDNA synthesis. The HCV 1a NS5B gene was cloned using HCV 1a NS5B-F: 5’-ATG TCR ATG TCY TAY WCY TGG ACA GCC-3’ and HCV 1a NS5B-R: 5’-TAA TCG RTT GGG RHR GAG RTA RAT RCC TAC C-3’ primers. The PCR cloning was performed using Accuprime Pfx Polymerase (Invitrogen, Carlsbad, CA) according its protocol. The PCR conditions for cloning was performed with early denaturation for 2 minutes of 95 °C, 16 cycles of 95 °C 15 seconds of denaturation followed with 55 °C 30 seconds of annealing and 68 °C 1 minute of elongation, respectively. The final elongation period was 72 °C 10 minutes. The PCR products were purified, analyzed, and subcloned into pETBlue-1 (Novagen, Darmstadt, Germany). The recombinant plasmids were then transformed into competent cells. The competent cells were propagated, harvested, and the plasmids were extracted and sequenced. The sequencing results were analyzed using CLC Sequence Viewer 6 and MEGA4.

**Complete HCV 1a NS5B Gene Sequences**

First, all HCV 1a NS5B gene sequences deposited in GenBank were downloaded. In total, 397 sequences with complete coding sequence were retrieved from GenBank. The sequences then were aligned by ClustalW, for both of nucleotide and amino acid sequences, using CLC Sequence Viewer 6 and MEGA4. From this alignment we revealed the amino acid consensus sequences. The tested sequences were aligned by ClustalW with subsequent inspection and manual modification.

**RESULTS AND DISCUSSION**

Prior of cloning the NS5B gene of HCV 1a, we performed phylogenetic analysis of all HCV isolated in Central of Java Indonesia from the previous study. In total, 140 out of 518 plasma samples were positive for anti-HCV. From all positive plasma samples, 32 isolates were successfully amplified with nested RT-PCR addressed for part of HCV E1-E2 and HCV NS5B region. The phylogenetic analysis retrieved that 43.72% (14/32) isolates were HCV 1a (Prasetyo, et al., submitted for publication).

To find the best isolate for cloning, all HCV 1a isolates were further analyzed. After checked the co-infection status with other human blood borne viruses, measured the quantity of the HCV RNA, and based on the prior HCV E1-E2 and HCV NS5B sequencing
results, 09IDSKAC-20 was chosen for cloning. The HCV 1a NS5B gene was cloned from 09IDSKAC-20 RNA using standard cloning procedure. Briefly, to design the primers for cloning, we performed alignment of all complete open reading frames for HCV 1a NS5B gene. The motif sequences of the 5’end and the 3’end regions were retrieved and subjected for primer design using FastPCR software. The initiation and stop codon were added into the forward and backward primer, respectively, to ensure the expression of HCV 1a NS5B in the pETBlue-1 plasmid. The PCR products was then subcloned into pETBlue-1, and transformed into competent cells. The recombinant plasmids were extracted and sequenced. The sequences were aligned with all HCV 1a NS5B genes retrieved from GenBank.

Different hepatitis C virus (HCV) proteins have been associated with different response to therapy, including that of the drugs resistance. However, the exact mechanisms of virus-mediated resistance are not completely understood. The importance of amino acid (aa) variations within the HCV 1a NS5B proteins for replication efficiency and viral decline during the therapy is unknown. To solve this problem we performed bioinformatics study by analyzing the all of HCV 1a NS5B gene complete coding sequences deposited in GenBank.

The molecular pattern of HCV 1a NS5B was found as follows:

```
0 191YGFQYSPG/A/R/KQR200
201VE/DFLVQ/R/KAV/TW/K/S/NS/A/R/H210
211KR/K/GT/V/A/N/SPMGFSYD220
221TRCFDSTVT230
231/S/N/G/HDIRT/MEA/T/EIY240
241QCCDDLDPQ/EAR250
251V/I/MA/VIK/RSLTERL260
261Y/V/IGGPLT/INSR/K270
271GEN/SCGYRCCR280 281ASGVLTTSCG290
291NTLTCYIKAR/Q/K300
301AACRAAG/RLQ/RD/N/S/A310
311CTMLVCDDL320
321VVICEA/Q/E/VGYQ/P/R330
331EDA/VAS/N/A/C/R/GLRAFT340
341EAMTRYAPP350
351GDPPQ/R/HPEYDL360
361ELITSCSSNV370
371SVH/A/QDG/D/S/NA/T/D/NGK/RR380
381VYLYLTRDPT/AT390
391PL/FARAAWETA/S/V400
401R/KH/RTPV/INSWLG410
411NJ/VIMF/YAPTLW420
421A/VRM/I/VL/MM/L/T/PHFF430
431S/GV/IIL/I/M/LA/TRD/EQLE/D440
441QALDCEIYGA450
451C/H/Y/H/SI/VE/Q/KPLDL/IP460
461P/L/Q/SIQ/EQLHS470
471AFSLHSYSPG/S480
481EINRVAA/TCLR490
491KLGVPPPLRA/TW500
501HRARN/S/T/GVRA/K510
511LL/MS/ARGR/K/GAAI/T/V/M520
521CGK/RYLFNWAY530
531R/KTKKL/RLTPIA/P540
541AAG/A/D/NQ/RDSLGS/SW550
551FT/K/RAGYS/GGDDIS60
561YHSV/M/PR/HARPR570
571WF/L/I/Y/A/F/ICLLLTA/T/G580
581A/TGVIHYVLL/F/YPN R591

549, 552, 556, 564-566, 572, 574, 580, 581, 585, and 588.

DISCUSSION

The hepatitis C virus (HCV) encodes an RNA-dependent RNA polymerase (NS5B), which is indispensable for the viral genome replication. NS5B is a tail-anchored protein with a highly conserved C-terminal transmembrane domain (TMD) that is required for the assembly of a functional replication complex (6). Different structural elements of NS5B have been reported to participate in RNA synthesis, especially a so-called "β-flap" and a C-terminal segment (designated "linker") that connects the catalytic core of NS5B to a transmembrane anchor. The transition from the first dinucleotide primer state to processive RNA synthesis requires removal of the linker and of the β-flap with which it is shown to strongly interact in crystal structures of HCV NS5B (7). Replication of the plus-stranded RNA genome of HCV occurs in a membrane-bound replication complex consisting of viral and cellular proteins and viral RNA. NS5B is anchored to the membranes via a C-terminal 20-amino-acid-long hydrophobic domain, which is flanked on each side by a highly conserved positively charged arginine. Anchoring NS5B to membranes is necessary but that the amino acid sequence of the anchor per se does not require HCV origin. This suggests that specific interactions between the NS5B hydrophobic domain and other membrane-bound factors may not play a decisive role in HCV replication (8).

The 249-nt-long RNA segment in the C-terminal NS5B coding region (NS5BCR) is predicted to form four stable stem-loop structures (SL-IV to SL-VII). Two of the stem-loops (SL-V and SL-VI) are essential for replication of the HCV. Mutations in the loop and the top of the stem of these RNA elements abolished replicon RNA synthesis but had no effect on translation (9).

The crystal structure of HCV NS5B highlights the presence of a unique A1-loop, which extends from the fingers to the thumb domain (amino acids 12-46), providing many contact points for the proposed "closed" conformation of the enzyme. The polymerase also possesses a tunnel, which starts at the active site and terminates on the back surface of the enzyme. The replacement of leucine 30 located in the Lambda 1-loop is detrimental to the NS5B activity. Amino acid substitutions in Arg-222 and Lys-151 within the putative NTP tunnel indicates that Arg-222 was critical in delivering NTPs to the active site, whereas Lys-151 was dispensable (10). The leucine-20 and arginine-222 were completely conserved; however, lysine 151 was not completely conserved in HCV 1a NS5B indicates that this residue is not essential for NS5B activity.

Several consensus sequence motifs have been identified in NS5B. A unique beta-hairpin structure located between amino acids 443 and 454 in the thumb subdomain has also been shown to play an important role in ensuring terminal initiation of RNA synthesis in vitro. Furthermore, a conservative substitution in motif D, from an arginine residue (AMTR(345)), which is conserved among all HCV isolates, to a lysine residue, resulted in significant improvements in both transient RNA replication and colony formation efficiencies (11). Interestingly, amino acid variations were found in 451, 452, and 454. These variations role in RNA synthesis needs further study. Mutations on Q377R, A450S, S455N, R517K, and Y561F in the NS5B region resulted in up-regulation of NS5B polymerase activity in vitro (12). However, aa 377, 455, and 517 have variations in HCV 1a. Amino acids 220 and 288, were critical, and amino acids 213 and 231, were important for efficient HCV replication (13). The aa 220 and 288 were fully conserved in HCV 1a while 213 and 231 were not.

Hepatitis C virus (HCV) replication and infection depend on the lipid components of the cell, and replication is inhibited by inhibitors of sphingomyelin biosynthesis. The sphingomyelin binding domain (SBD) of HCV NS5B was mapped to the helix-turn-helix structure (residues 231 to 260), which was essential for sphingomyelin binding and activation. Helix structures (residues 231 to 241 and 247 to 260) are important for NS5B activation, and 238S and 248E are important for maintaining the helix structures for
template binding and NS5B activation by sphingomyelin. 241Q in helix 1 and the negatively charged 244D at the apex of the turn are important for sphingomyelin binding \(^{(14)}\). These data were consistent with the alignment results since 241Q and 244D were conserved in HCV 1a NS5B. However, variations were found in amino acid 238 and 248.

It has been suggested that cellular proteins are involved in hepatitis C virus (HCV) RNA replication. Domains NS5B (84-95), NS5B (466-478), and alpha-actinin (621-733) are responsible for the interaction \(^{(15)}\). However, variations were found at aa 84-86, and 90 indicate that domains NS5B (84-95) maybe not essential for the interaction especially for that of genotype 1a. No amino acid variation was found in domains NS5B (466-478).

Hepatitis C Virus (HCV) non-structural proteins are major components of replication complex that is modulated by several host factors. Nucleolin, a representative nucleolar marker, interacts with the NS5B through two separated sequences, amino acids (aa) 208-214 and 500-506, and that W208 in the former stretch is essential for both nucleolin-binding and HCV replication \(^{(16)}\). Arginine-glycine-glycine (RGG) repeat in the Glycine arginine rich (GAR) domain were defined to be indispensable for NS5B-binding \(^{(17)}\). However, amino acid variations were found at aa 209, 210, 212, 213, and 506 of NS5B HCV 1a. W208 was fully conserved in HCV 1a.

The hepatitis C virus (HCV) NS5B RNA-dependent RNA polymerase (RdRp) plays a central role in virus replication. NS5B has no functional equivalent in mammalian cells and, as a consequence, is an attractive target for inhibition \(^{(18)}\). siRNAs targeting against HCV NS5B efficiently inhibit HCV replication \(^{(19)}\). Nucleoside analogs targeting the NS5B provide an attractive approach to treating HCV infections because of their high barrier to resistance and pan-genotype activity. Ribavirin (RBV), a nucleoside analogue, is used in the treatment of hepatitis C virus (HCV) infection in combination with interferons. Y2471H in NS5B region plays a role in the reduced susceptibility to RBV and leads to decrease in replication fitness of genotype 2a \(^{(20)}\). Mutation in Ser272Pro, Met312Thr, and Arg221Lys correlated with end-of-treatment response but relapsed treatment after 3 months due to anemia, and end-of-treatment-response but relapsed conditions, respectively \(^{(21)}\). NS5B F415Y mutation represents an RBV-resistant variant \(^{(22)}\). PSI-7851 is a highly active nucleotide analog inhibitor of HCV but less active against the S282T mutant \(^{(5)}\). The nucleotide analogue 2’-C-methyl CTP (2’-C-Me-CTP) is the active metabolite of NM283, a drug currently in clinical phase II trials. Resistance to 2’-C-Methyl-Cytidine was mapped to amino acid substitution S282T in the NS5B coding region \(^{(23, 24, 25)}\).

Multiple nonnucleoside inhibitor binding sites have been identified within the hepatitis C virus (HCV) polymerase, including in the palm and thumb domains. Hepatitis C virus (HCV) non-nucleoside inhibitors (NNIs) target the viral RNA-dependent RNA polymerase encoded by the NS5B gene. Several NNIs share a similar allosteric binding site, and their antiviral efficacy is attenuated by a cysteine-to-tyrosine mutation at amino acid 316 (C316Y) \(^{(26)}\). HCV-796 is a nonnucleoside inhibitor of the HCV NS5B polymerase. Mutations known to confer resistance to HCV-796 (NS5B C316Y) was present in the resistant replicons \(^{(27)}\). Mutations were observed at Leu314, Cys316, Ile363, Ser365, and Met414 of NS5B, which directly interact with HCV-796. Reduced binding affinity with HCV-796 was demonstrated in an enzyme harboring the C316Y mutation \(^{(28)}\).

Compounds A-782759 (an N-1-aza-4-hydroxyquinolone benzothiadiazine) is specific inhibit the HCV NS5B. Single substitutions in the NS5B polymerase gene (H95Q, N411S, M414L, M414T, or Y448H) resulted in substantial decreases in susceptibility to A-782759 \(^{(29)}\). A-837093 is a potent and specific nonnucleoside inhibitor of the HCV NS5B. It also exhibited an excellent metabolic profile and achieved high plasma and liver concentrations in animals. Several mutations were associated with resistance to A-837093, including S368A, Y448H, G554D, D559G, C316Y, G554D, Y555C, and D559G \(^{(30, 31)}\). Thiophene-based non-nucleoside
inhibitors (NNIs) bound non-covalently to NS5B. It has been reported that Pro495, Pro496, Val499 and Arg503 are part of the guanosine triphosphate (GTP) specific allosteric binding site (32). After a single treatment with a thumb site inhibitor (thiophene-2-carboxylic acid NNI-1), resistant HCV replicon variants emerged that contained mutations at residues Leu419, Met423, and Ile482 in the polymerase thumb domain. The mechanism of resistance is through the reduced binding of the inhibitor to the mutant enzymes (33).

Isothiazole analogs were discovered as a novel class of active-site inhibitors of HCV NS5B polymerase, covalently linked with Cys 366 of the 'primer-grip' (34). A series of benzimidazole 5-carboxamide compounds inhibit the cellular RNA replication of a HCV subgenomic replicon. A binding site has been localized for these inhibitors at the junction of the thumb domain and the N-terminal finger loop. Resistant mutations that emerged with the benzimidazole 5-carboxamide and related compounds were found at three amino acid positions in the thumb domain: Pro(495) with substitutions to Ser, Leu, Ala, or Thr; Pro(496) substitutions to Ser or Ala; and a V499A substitution (35).

Pyranoindoles target the NS5B polymerase through interactions at the thumb domain. This inhibition is independent of GTP concentrations and is likely mediated by an allosteric blockade introduced by the inhibitor during the transition to RNA elongation after the formation of an initiation complex. Mutations in the NS5B polymerase gene corresponding to the drug-binding pocket on the surface of the thumb domain identified by X-ray crystallography. An additional cluster of mutations present in part of a unique beta-hairpin loop was also identified. A single mutation (L419M or M423V), located at the pyranoindole-binding site, resulted in an 8- to 10-fold more resistant replicon, while a combination mutant (T19P, M71V, A338V, M423V, A442T) showed a 17-fold increase in drug resistance (36).

Triggering and propagating an intracellular innate immune response is essential for control of HCV infections. RNase L is a host endoribonuclease and a pivotal component of innate immunity that cleaves viral and cellular RNA within single-stranded loops releasing small structured RNAs with 5'-hydroxyl (5'-OH) and 3'-monophosphoryl (3'-p) groups. The NS5B region of HCV RNA was cleaved by RNase L to release an svRNA that bound to RIG-I, displacing its repressor domain and stimulating its ATPase activity while signaling to the IFN-β gene in intact cells. All three of these RIG-I functions were dependent on the presence in svRNA of the 3'-p. Furthermore, svRNA suppressed HCV replication in vitro through a mechanism involving IFN production and triggered a RIG-I-dependent hepatic innate immune response in mice. RNase L and OAS (required for its activation) were both expressed in hepatocytes from HCV-infected patients, raising the possibility that the OAS/RNase L pathway might suppress HCV replication in vivo (37).

Circulating auto-antibodies targeting conformational antigens on cytochrome P4502E1 (CYP2E1) are detectable in patients with chronic hepatitis C (CHC). The CYP2E1 is associated with more severe necro-inflammation and might contribute to hepatic injury. The CYP2E1(324-346) peptide showed good homology with two sequences (NS5B(438-449) and NS5B(456-465)) within the NS5B protein of hepatitis C virus (HCV) (38). However, in HCV 1a, variations were found at aa 440, 459, 461, and 464.

The epitopes of eight mAbs are localized in the middle region (amino acid 240-263) of NS5B protein. On the other hand, the epitopes of two mAbs are mapped to amino acids 67-88 at the N-terminus of NS5B protein. NS5B contains a B-cell epitope located between amino acid residues 67 and 88. Binding of this epitope with an antibody interferes with the enzymatic function of NS5B (39). Variations were found at aa 68, 248, 251, 252, 254, and 262 in HCV 1a NS5B.

The nucleotide sequence diversity present among hepatitis C virus (HCV) isolates allows rapid adjustment to external forces including host immunity and drug therapy. This viral response reflects a combination of a high rate of replication together with an error-
prone RNA-dependent RNA polymerase, providing for the selection and proliferation of the viruses with the highest fitness. Signatures of HCV 1a NS5B were found abundant. The amino acid variations found in the present report need further study. In addition, it is possible that the establishment of a new scoring system consisting of molecular information may be a useful marker to predict therapy sensitivity for HCV.

ACKNOWLEDGMENTS

This study was supported by DIPA Sebelas Maret University No. 2881/H27/KU/2010 and DIPA Faculty of Medicine Sebelas Maret University 2010.

REFERENCES


