

Cloning and Molecular Pattern Of HCV 1a NS5a Complete Coding Sequence as Predicting Factor for Resistance Therapy

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ABSTRACT

The hepatitis C virus (HCV) NS5A 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 NS5A we cloned the NS5A 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 NS5A complete coding sequences reported in GenBank. In total, 441 sequences were retrieved and aligned by ClustalW. We revealed the nucleotide and amino acid sequence consensus of HCV NS5A gene. Overall, only aa 5, 9-11, 13-15, 18-19, 22-23, 25-27, 29, 32-33, 35, 38-43, 45, 47, 50-51, 55, 57, 59-60, 63, 65-67, 70, 72, 76, 80, 82, 84, 86, 88-91, 94-97, 100, 102, 104-106, 109-113, 115-116, 118-120, 125, 128-129, 132, 134, 136, 140-143, 145, 147, 149-152, 154-157, 159, 162-163, 165, 167-168, 170, 173, 175, 178-179, 184-187, 189-193, 195, 201, 203, 207-208, 210, 216-218, 220, 222-225, 227-235, 238, 243, 249, 252, 257, 268, 270, 271, 274, 278, 283, 291-292, 298, 300, 306, 309-311, 313-314, 318-321, 324, 326, 329, 331, 336, 338-339, 342, 348, 350, 353, 356, 360, 363, 368-369, 377, 405-406, 409, 411-414, 416-419, 421-424, 427, 430-432, and 443 were found have no variation. 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, NS5A

Introduction

The hepatitis C virus (HCV) nonstructural protein NS5A is critical for viral genome replication and is thought to interact directly with the RNA-dependent RNA polymerase, NS5B, and viral RNA. NS5A consists of three domains which have roles in viral replication and assembly. A specific interaction between domains I and II of NS5A and the 3' UTR RNA was identified, suggesting that these are the RNA binding domains of NS5A. Domain III showed low in vitro RNA binding capacity. The preference of NS5A, in contrast to NS5B, for the polypyrimidine tract highlights an aspect of 3' UTR RNA recognition by NS5A which may play a role in the control or enhancement of HCV genome replication (Foster *et al.*, 2010). The N-terminal amphipathic alpha-

helix of the hepatitis C viral protein NS5A can localize dsRed and viperin to lipid droplets indicate that the amphipathic alpha-helices of viperin and NS5A are lipid droplet-targeting domains and suggest that viperin inhibits HCV by localizing to lipid droplets using a domain and mechanism similar to that used by HCV itself (Hinson and Cresswell, 2009). The NS5A membrane-active region might have an essential role in the membrane replication and/or assembly of the viral particle through the modulation of the replication complex, and consequently, directly implicated in the HCV life cycle (Palomares-Jerez, *et al.*, 2010).

NS5A, independent of other HCV viral proteins, may play an important role in the development of hepatic pathologies, including steatosis and hepatocellular carcinoma (Wang,

et.al., 2009). NS5A does not cause spontaneous liver disease but NS5A could impair both the innate and the adaptive immune response to promote chronic HCV infection (Kriegs, *et.al.*, 2009). HCV often establishes a persistent infection leading to chronic liver disease that most likely involves complex host-virus interplay. The NS5A protein has been implicated in this process as it modulates a variety of intracellular signaling pathways that control cell survival and proliferation. NS5A may perturb trafficking pathways of the epidermal growth factor receptor (EGFR) to maintain an optimal environment for HCV persistence. Also, HCV NS5A activates the mTOR pathway to inhibit apoptosis through impairing the interaction between mTOR and FKBP38, which may represent a pivotal mechanism for HCV persistence and pathogenesis (Mankouri, *et.al.*, 2008; Peng, *et.al.*, 2010). However, the role of HCV NS5A in HCV-associated pathogenesis is still enigmatic.

At present NS5A is also believed as the most important factor to determine the virological kinetics during peginterferon and ribavirin therapy, however, have not been elucidated yet. To predict and more understanding the molecular pattern of HCV NS5A gene, we cloned the HCV NS5A gene from HCV 1a isolated in Central of Java Indonesia and performed bioinformatics study of all HCV 1a NS5A complete genes deposited in GenBank. Here we present our current analysis results of the NS5A gene of HCV 1a.

Methods

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.

The aliquot plasma from “the best candidate for cloning”-isolate were performed for RNA extraction followed with cDNA synthesis. The HCV 1a NS5A gene was cloned using HCV 1a NS5A-F: 5'-ATG TSY GRY

WSC TGG YTR AGR G-3' and HCV 1a NS5A-R: 5'-TTA RCA RCA CAC GAC RYY YTC YRH G-3' primers. 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. 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 Sequence Viewer and MEGA4.

All HCV 1a NS5A gene sequences deposited in GenBank were downloaded. In total, 441 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 and MEGA4. From this alignment we revealed the nucleotide and amino acid consensus sequences. The tested sequences were aligned by ClustalW with subsequent inspection and manual modification (Thompson, *et.al.*, 1994; Tamura, *et.al.*, 2007).

Results and Discussion

Prior of cloning the NS5A 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, only 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 checked for its purity and RNA titer. 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 NS5A 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 NS5A 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 NS5A in the pETBlue-1 plasmid. The PCR cloning was performed using Accuprime Pfx Polymerase

according its protocol. 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 NS5A 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 NS5A proteins for replication efficiency and viral decline during the therapy is unknown. To solve this problem we performed a bioinformatics study by analyzing the all of HCV 1a NS5A gene complete coding sequences deposited in GenBank. The amino acids pattern of HCV 1a NS5A was as follow: 1XXXXLXXXWD 11WXCEVXXDFX 21XWLXAKLXPX 31XPGXPXXSCQ 41RGYXGXWXXD 51GXXXTXCXCG 61XXIXGHVXXG 71XMXXXGXXXC 81XNXWGXFPFI 91NXXTTGPXXP 101XPXPNYXXAL 111WRVXAEXYVE 121XXXXGXXHYX 131XGXTXDXXXC 141PCQXPXPXFF 151TEXDGVRRHX 161XAPXCXPLXR 171XXVFXFXGLX 181XXXVGSQXPC 191EPEXDXXXXX 201SXLXXXSHXT 211XXXXRRLLXR 221XSPPSXASSS 231ASQLSXXXSX 241XXCXXXXDX 251XLXXXLXXX 261XXXXXXXXRXE 271SXXKXXXLXX 281XXPXXXXXXXX 291REXXXXXEXL 301XXXXXFXAL 311PXWAXXXYNP 321PXXEXWXXPX 331YXXXXVXGXC 341XPXXXXXPX 351XXRXXRXXXL 361XXSXXXXALX 371XXXXXXXXFXXX 381XXXXXXXXXXX 391XXXXXXXXXXX 401XXXXSDXXXS 411SSMPXLEGEX 421GDPDXDXDXW 431STXXXXXXXXX 441XXVXX. Only aa 5, 9-11, 13-15, 18-19, 22-23, 25-27, 29, 32-33, 35, 38-43, 45, 47, 50-51, 55, 57, 59-60, 63, 65-67, 70, 72, 76, 80, 82, 84, 86, 88-91, 94-97, 100, 102, 104-106, 109-113, 115-116, 118-120, 125, 128-129, 132, 134, 136, 140-143, 145, 147, 149-152, 154-157, 159, 162-163, 165, 167-168, 170, 173, 175, 178-179, 184-187, 189-193, 195, 201, 203, 207-208, 210, 216-218, 220, 222-225, 227-235, 238, 243, 249, 252, 257, 268, 270, 271, 274, 278, 283, 291-292, 298, 300, 306, 309-311, 313-314, 318-321, 324, 326, 329, 331, 336, 338-339, 342, 348, 350, 353, 356, 360, 363, 368-369, 377, 405-406, 409, 411-414, 416-419, 421-424, 427, 430-432, and 443 were found have no variation.

Comparative analyses of consensus dominant quasispecies variants revealed that most mutations, occurring at the time of breakthrough, involved three functional viral genes, E2, NS2, and NS5a. These three genes had the highest values of average amino acid complexity at the HCV 1a population level. Viral breakthrough might be attributed to the selection of minor quasispecies variants at the baseline with or without additional mutations during antiviral therapy. The pattern for mutation clustering indicated potential mutation linkage among E2, NS2, and NS5a due to structural or functional relatedness in HCV replication (Xu, *et.al.*, 2008).

Within the HCV genome, sequence diversity of the viral nonstructural 5A protein-coding region (NS5A) has been linked to interferon responsiveness, especially that of within the interferon sensitivity-determining region (ISDR, aa 2209-2248), the PKR-binding domain (PKR-BD, aa 2209-2274), the variable region 3 (V3), and the interferon/ribavirin resistance-determining region (IRRDR, aa 2334-

2379) (Enomoto and Maekawa, 2010; Fukuhara, *et.al.*, 2010; Jenke, *et.al.*, 2009; Yuan, *et.al.*, 2010). For genotype 1b, L31F/V, P32L, and Y93H/N were identified as primary resistance mutations. L23F, R30Q, and P58S acted as secondary resistance substitutions, enhancing the resistance of primary mutations but themselves not conferring resistance. For genotype 1a, more sites of resistance were identified, and substitutions at these sites (M28T, Q30E/H/R, L31M/V, P32L, and Y93C/H/N) conferred higher levels of resistance. For both subtypes, combining two resistance mutations markedly decreased inhibitor susceptibility. As single mutations, Q30E and Y93N in genotype 1a conferred the highest levels of resistance (Fridell, *et.al.*, 2010). Mutation D320E mediated most of the resistance conferred by NS5A in all HCV genotypes (Puyang, *et.al.*, 2010).

The replication of the 2a-NS5A-containing replicons was more sensitive to IFN treatment than that of the 1b-NS5A-containing replicons. Deletion of the interferon sensitivity-determining region/protein kinase R-binding

domain (PKR-BD), the V3 domain, or the C-terminus region of NS5A significantly abrogated its anti-IFN activity. Domain swapping between 1b-NS5A and 2a-NS5A in the V3 domain and/or the C-terminus region resulted in a transfer of their anti-IFN activity. 1b-NS5As exert higher magnitudes of IFN antagonism than do 2a-NS5As. The V3 and the C-terminus regions are responsible for the differential anti-IFN effects. This phenomenon may partly explain the genotype-linked differences in the response of HCV to IFN treatment (Tsai *et.al.*, 2008).

The amino acid substitutions within the NS5A coding region also may reflect a host response that drives selective pressure for viral adaptation. BT (breakthrough) patients resembled SVR (sustained virologic responders) patients in having a higher number of amino acid substitutions at week 12 than NR (non-responders) patients; however, the number of amino acid substitutions in this group decreased at and after BT in peg-interferon and ribavirin therapy. The high number of substitutions in NS5A in both BT and SVR groups suggests that selective pressure is associated with viral response to therapy (Yuan, *et.al.*, 2010). Independent predictive factors for SVR to both PEG-IFN and RBV were no aa substitution at core aa 70, two or more aa mutations in the ISDR, low viral load, high values of platelet count, mild liver fibrosis and male gender (Okanoue *et.al.*, 2009).

The NS5A proteins of HCV genotype 1 were reported to inhibit the double-stranded (ds) RNA-dependent protein kinase (PKR), which is involved in the cellular antiviral response induced by interferon (IFN). The response to IFN therapy is quite different between genotypes, with response rates among patients infected with types 2 and 3 that are two-three-fold higher than in patients infected with type 1. Interestingly, a significant percentage of HCV genotype 3-infected patients do not respond to treatment at all. The ISDR domain is not predictive of treatment success in patients infected with HCV genotype 3a (Malta *et.al.*, 2010).

Two closely spaced polyproline motifs, with the consensus sequence Pro-X-X-Pro-X-Lys/Arg, located between residues 343 to 356 of NS5A are mediated interactions with cellular SH3 domains. The N-terminal motif (termed PP2.1) is only conserved in genotype 1 isolates, whereas the C-terminal motif (PP2.2) is conserved throughout all hepatitis C virus (HCV) isolates, although this motif was shown to be

dispensable for replication of the genotype 1b subgenomic replicon. The PP2.2 motif is dispensable for RNA replication of all subgenomic replicons and is not required for virus production. The PP2.1 motif is only required for genotype 1b RNA replication. Mutation of proline 346 within PP2.1 to alanine dramatically attenuated genotype 1b replicon replication in three distinct genetic backgrounds, but the corresponding proline 342 was not required for replication of the JFH-1 subgenomic replicon. However, the P342A mutation resulted in both a delay to virus release and a modest reduction in virus production (Hughes *et.al.*, 2009; Shelton & Harris, 2008).

NS5A is a key factor for the assembly of infectious HCV particles. NS5A is a three-domain protein and the domains I and II are required for RNA replication. NS5A domain III is not required for RNA replication; however, domain III of NS5A is important regulator of the RNA replication and virion assembly of HCV. A single serine residue within a casein kinase II consensus motif mimic phosphorylation in domain III has been found. The phosphorylation at this position regulates the production of infectious virus. NS5A requires casein kinase II phosphorylation at this position for virion production (Tellinghuisen, *et.al.*, 2008). The C-terminal domain III is the primary determinant in NS5A for particle formation. Both core and NS5A colocalize on the surface of lipid droplets, a proposed site for HCV particle assembly. Since domain III of NS5A is one of the most variable regions in the HCV genome, viral isolates may differ in their level of virion production and thus in their level of fitness and pathogenesis (Appel *et.al.*, 2008).

NS5A plays a key role in regulating the early phase of HCV particle formation by interacting with core protein and that its C-terminal serine cluster is a determinant of the NS5A-core interaction. Alanine substitutions for the C-terminal serine cluster in domain III of NS5A (amino acids 2428, 2430, and 2433) impaired NS5A basal phosphorylation, leading to a marked decrease in NS5A-core interaction, disturbance of the subcellular localization of NS5A, and disruption of virion production. Phosphorylation of these serine residues is important for virion production (Masaki *et.al.*, 2008).

A single amino acid residue of Val or Ile at position 121, which is well conserved among all genotypes of HCV, is critical for the specific

interaction with FKBP8. Substitution of the Val(121) to Ala drastically impaired the replication of HCV replicon cells, and the drug-resistant replicon cells emerging after drug selection were shown to have reverted to the original arrangement by replacing Ala(121) with Val. FKBP8 is partially colocalized with NS5A in the cytoplasmic structure known as the membranous web. The specific interaction of NS5A with FKBP8 in the cytoplasmic compartment plays a crucial role in the replication of HCV (Okamoto, *et.al.*, 2008).

Conclusions

The amino acid variations may have relevant changes of physicochemical properties so that influence the replication efficiency and or viral pathogenesis and therapy. Signatures of HCV 1a NS5A gene conversion were found abundant. The gene conversion signatures coincide with a striking lack of diagnostically informative sites between subtypes and a large number of shared mutations. 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.

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