REVIEW

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Hepatitis C Virus and Antiviral Drug Resistance

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Since its discovery in 1989, hepatitis C virus (HCV) has been intensively investigated to understand its biology and develop effective antiviral therapies. The efforts of the previous 25 years have resulted in a better understanding of the virus, and this was facilitated by the development of *in vitro* cell culture systems for HCV replication. Antiviral treatments and sustained virological responses have also improved from the early interferon monotherapy to the current all-oral regimens using direct-acting antivirals. However, antiviral resistance has become a critical issue in the treatment of chronic hepatitis C, similar to other chronic viral infections, and retreatment options following treatment failure have become important questions. Despite the clinical challenges in the management of chronic hepatitis C, substantial progress has been made in understanding HCV, which may facilitate the investigation of other closely related flaviviruses and lead to the development of antiviral agents against these human pathogens. **(Gut Liver 2016;10:890-895)**

Key Words: Hepatitis C virus; Direct-acting antiviral; Drug resistance

INTRODUCTION

Hepatitis C virus (HCV) infects approximately 130 to 170 million people worldwide and the infected patients suffer from chronic hepatitis, cirrhosis, and hepatocellular carcinoma. Most of HCV infection (70% to 80%) becomes chronic and has been a tremendous burden on the public health. The HCV infection was first known as non-A, non-B hepatitis (NANBH)[1,2] and the causative agent of this NANBH was identified as hepatitis C virus in 1989.[3] Since then, substantial progresses in the knowledge of HCV virology, development of research tools, and drug discovery have been made and now new antiviral therapies based on all-oral regimens are beginning to be used. A few notable accomplishments in HCV virology include the development of replicon system[4] and JFH1 cell culture-infectious clone,[5] which enabled screening of small molecule libraries for drug discovery as well as investigating all the steps of HCV life cycle including virus assembly and secretion.

VIROLOGY

HCV is an enveloped, positive-strand RNA virus and belongs to the genus *Hepacivirus* within the family *Flaviviridae* (for a comprehensive review of HCV virology, see Lemon *et al*.[6]). The RNA genome of this virus is approximately 9.6 kb long and encodes a single polyprotein by the internal ribosome entry site-dependent translation (Fig. 1). The polyprotein (~3,000 amino acids) is co- and post-translationally processed by both virus and host proteases to generate a total of 10 viral proteins (N terminus-Core-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-C terminus). The three proteins (Core, E1, and E2) at the N terminus are the structural proteins that are directly involved in the formation of virions while the rest at the C terminus (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) are the nonstructural proteins. Briefly, the core protein is a component of viral capsid and E1, E2 proteins are envelope glycoproteins that mediate the hepatocyte-specific entry of HCV virions. Nonstructural proteins are involved in many aspects of HCV life cycle including viral genome replication. p7 protein is known as an ion channel protein (viroporin) and NS2 protein is a cysteine autoprotease, which catalyzes the cleavage between NS2 and NS3 protein. Both p7 and NS2 proteins are also known to be involved in virus assembly process. The proteins from NS3 to NS5B (NS3-NS4A-NS4B-NS5A-NS5B) are indispensable for viral RNA replication as the major components of the replication complex.[4] NS3 protein contains two enzymatic activities: serine protease

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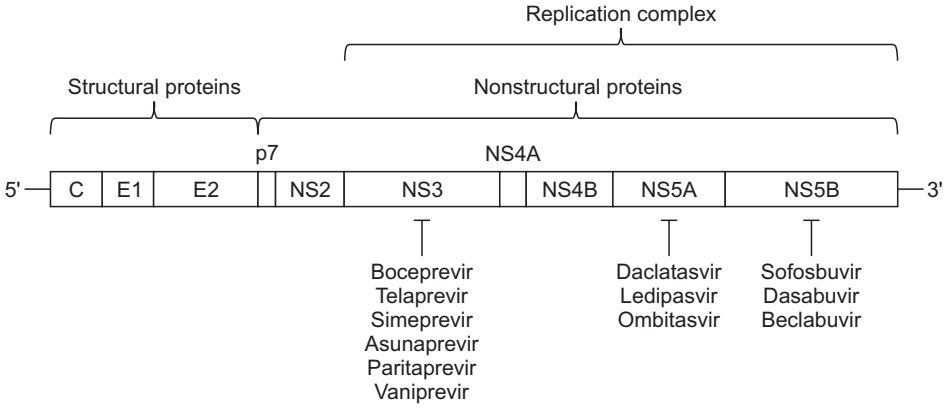

Fig. 1. Hepatitis C virus genome structure. Internal ribosome entry site-mediated translation of viral RNA generates a single polyprotein, which is co- and post-translationally processed by host and viral proteases to produce 10 viral proteins. Core (C), E1 and E2 envelope glycoproteins are the structural proteins, and the remaining proteins represent non-structural proteins. The replication complex comprises NS3 to NS5B proteins within the membranous web structure near the endoplasmic reticulum membrane.

and helicase. The protease activity of NS3 cleaves the viral proteins of the polyprotein from NS3 to NS5B, while the helicase activity is involved in viral assembly. NS4A protein is a cofactor of NS3 protein and helps NS3 protein associate with the cellular membrane. NS4B protein is known to generate membranous web structures near the ER membrane of the host cell, which are thought to be the place where RNA replication occurs. NS5A protein does not have any known enzymatic activity but is important for both viral RNA replication and infectious virus assembly. Finally, NS5B is an RNA-dependent RNA polymerase that synthesizes plus- and minus-strand of viral RNAs. Among these viral proteins, NS3, NS5A, and NS5B proteins are the major targets of direct-acting antivirals (DAAs) and currently several DAAs are being used for treatment of chronic hepatitis C (Fig. 1).

1. Virus life cycle

Overall, the life cycle of HCV is similar to those of other positive-strand RNA viruses. The virus enters the hepatocyte via the receptor-mediated endocytosis (for an updated, comprehensive review of HCV entry, see Ding *et al*.[7]). After fusion and uncoating of the virion within the cell, the viral genome is released into the cytoplasm. Since the viral genome is positive-sense, it is directly used as mRNA for translation of the viral polyprotein. Several viral proteins (NS3 to NS5B) after cleavage and processing of the viral polyprotein are recruited to the membranous web to make replication complexes. Within this replication complex, the viral genome replication occurs (for an updated, comprehensive review of HCV RNA replication and assembly, see Paul *et al*.[8]): a negative-strand RNA is synthesized first using the positive-strand viral RNA and then multiple copies of positive-strand RNA are synthesized using the negative-strand viral RNA. The structural proteins and the positive-strand viral RNA are then assembled to produce infectious virus particles near the lipid droplets[9] and the infectious virus particles are released out of the cell using the host VLDL-secretory pathway.[10,11]

2. Systems for studying HCV

Development of *in vitro* cell culture system for HCV replication is indispensable for HCV research and drug discovery. The first replicon system, that supports HCV genome replication when its RNA is transfected into the Huh7 hepatoma cells, was reported in 1999.[4] This replicon system used the genotype 1b Con1 isolate and currently the replicon systems are available for most of the HCV genotypes.[12-18] While the replicon system is useful for studying viral genome replication, it does not support the production of infectious virus particles. In 2003, the functional HCV pseudoparticle system (HCVpp) was described.[19] The virus that is produced by this system is in fact a retrovirus, but is coated with HCV envelope glycoproteins, E1 and E2, thus the infection by the HCVpp generally follows the steps of the specific HCV entry pathway. By incorporating the sequence of reporter genes such as green fluorescent protein or luciferase, the virus entry can be monitored either visually or quantitatively without further complications due to the viral genome replication. Finally, the first cell culture-infectious clone (HCVcc), which can recapitulate the entire viral life cycle of HCV in cell culture, was established in 2005 using the genotype 2a JFH1 isolate.[5] This virus was unique in that its RNA was able to replicate efficiently in Huh7 hepatoma cells without any cell culture-adaptive mutations, which were indispensable for other replicons to replicate in the *in vitro* cell culture system. When the JFH1 RNA was transfected into the Huh7 hepatoma cells, the transfected cells supported the production of infectious virus particles as well as the efficient RNA replication. The produced virus particles could then infect animal models such as chimpanzee and naïve hepatoma cells, thus completing an entire viral life cycle. This system can be used for investigation of all the steps of viral life cycle, specifically virus assembly and secretion steps. The HCVcc system is currently available for the genotypes 1a, 2a, and 3a.[5,20-23]

TREATMENT OF CHRONIC HEPATITIS C

Since the description of NANBH, treatment of chronic hepatitis C has evolved from the nonspecific antiviral treatment using interferon to the all-oral regimens using DAAs.[24] In the late 1990s, the interferon monotherapy for the treatment of chronic hepatitis C patients was modified by the addition of ribavirin, a nucleoside analog, which increased the sustained virological response (SVR) by 20% compared to the interferon monotherapy.[25] And later, pegylation of interferon (pegIFN) was demonstrated more effective than nonpegylated interferon by an increase of SVR of 10% to 15%.[26,27] Thus, until very recently, the administration of pegIFNα and ribavirin for 6 to 12 months had been the standard of care before using DAAs. During this evolution of treatment options, the SVR rates have increased from the 15% to 20%[28,29] by the IFNα monotherapy to over 90% by the DAA treatments.

The first DAAs, which were approved in 2011 for use in the treatment of chronic hepatitis C, were boceprevir (Merck) and telaprevir (Vertex), both NS3 protease inhibitors. These DAAs were not used by themselves, but used with pegIFNα and ribavirin as triple therapy due to the occurrence of resistant mutations. Despite some toxicity issues, an improved SVR of approximately 75% was achieved by this therapy for genotype 1 HCV-infected patients.[30,31] Since the approval of these NS3 protease inhibitors for the treatment of chronic hepatitis C, more DAAs have been approved and are still being tested in clinical trials. Currently, three different viral proteins (NS3, NS5A, and NS5B) are the major targets of DAAs and interferon-free, all-oral regimens are expected to be the standard of care in most of chronic hepatitis C treatments due to the severe side effects and poor tolerability of interferon treatment. However, antiviral drug resistance has become an issue again as was experienced in the treatment of other chronic viral diseases. From in vivo and *in vitro* data, the profiles of various resistant mutations against the DAA treatments have already been well documented (Fig. 2).[32] Currently, most of DAA therapies are dual or triple therapy combining DAAs from different classes with or without ribavirin in order to minimize the occurrence of resistant mutations.

1. NS3

NS3 protein has two known enzymatic activities: serine protease and helicase. Most of DAAs targeting this protein act as protease inhibitors. Thus, one main mechanism of action of this class of DAAs is inhibition of proteolytic cleavage of viral proteins from NS3 to NS5B, which eventually suppresses viral RNA replication. Among the currently available NS3-targeting DAAs, boceprevir and telaprevir were approved for clinical use for the first time, which were followed by simeprevir, asunaprevir, and paritaprevir. Several antiviral-resistant mutations are frequently observed, for example, at V36, V55, R155, and D168 of NS3 protein (Table 1, Fig. 2). Interestingly, when the viral fitness of the resistant mutations was assessed using the *in vitro* cell culture system, many resistant mutations showed defects in viral RNA replication while some (e.g., R155T) showed more severe defects in infectious virus production,[33] thus suggesting a potential role of NS3 protease in virus assembly.

2. NS5A

NS5A protein has no known enzymatic activity but is impor-

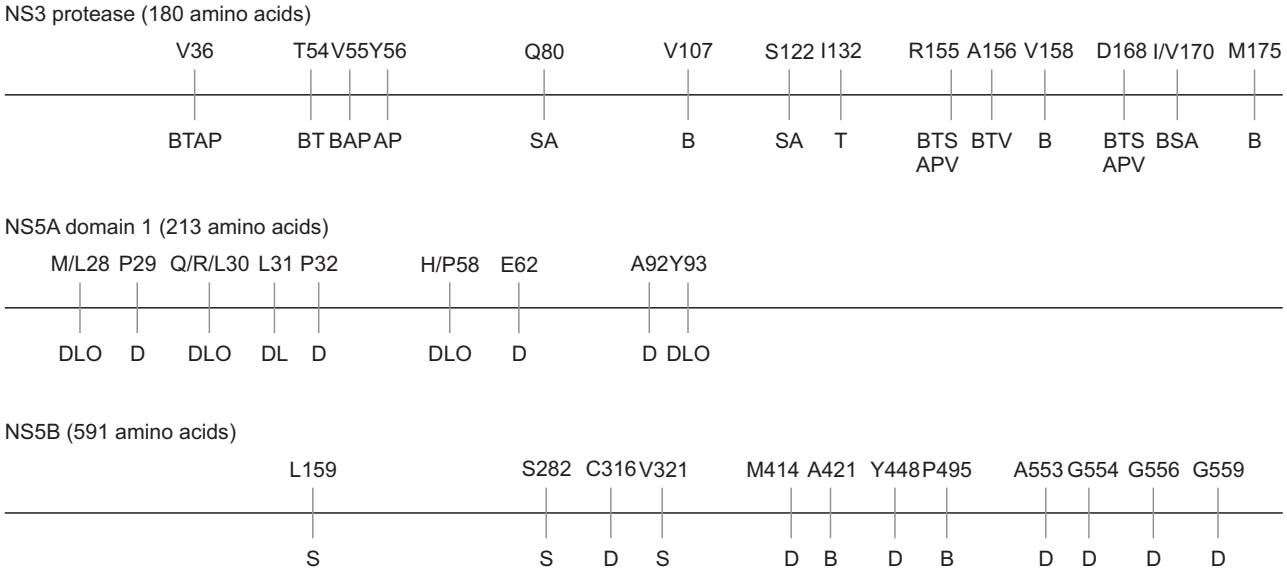

Fig. 2. Antiviral-resistant mutations of NS3 protease, NS5A and NS5B RNA-dependent RNA polymerase. The resistance-associated amino acids are located with the corresponding direct-acting antivirals that select these resistances: NS3 (B, boceprevir; T, telaprevir; S, simeprevir; A, asunaprevir; P, paritaprevir; and V, vaniprevir), NS5A (D, daclatasvir; L, ledipasvir; and O, ombitasvir) and NS5B (S, sofosbuvir; D, dasabuvir; and B, beclabuvir). Note that sofosbuvir is a nucleotide inhibitor, whereas dasabuvir and beclabuvir are nonnucleoside inhibitors.

Table 1. Most Commonly Observed NS3-Resistant Mutations[32]

Inhibitor	Genotype 1a	Genotype 1b
Boceprevir	V36M, T54S, R155K	T54A/S, V55A, A156S, V170A
Telaprevir	V36M, R155K	V36A, T54A, A156S
Simeprevir	R155K, D168E/V	Q80R, D168E/V
Asunaprevir	R155K, D168E	D168E/V/Y
Paritaprevir	D168A/V/Y	Y56H, D168V
Vaniprevir	R155K, D168T/V/Y	D168H/T/V

Table 2. Most Commonly Observed NS5A-Resistant Mutations[32]

Inhibitor	Genotype 1a	Genotype 1b
Daclatasvir	M28T, Q30E/H/R, L31M, H58D, Y93H/N	L31M/V, Y93H
Ledipasvir	Q30E/R, L31M, Y93C/H/N	Y93H
Ombitasvir	M28T, Q30R	Y93H

tant for both viral RNA replication and assembly of infectious virus particles. NS5A protein is composed of an N-terminal amphipathic α-helix that associates with the cellular membrane and three consecutive domains (domain 1 to 3). The domains 1 and 2 are important for viral RNA replication while the domain 3 is indispensable for virus assembly.[34-37] Daclatasvir (Bristol-Myers Squibb), the first NS5A-targeting DAA, was discovered during the small molecule library screening using the genotype 1b Con1 replicon cell line.[38] The inhibitory potency of daclatasvir was outstanding but this inhibitor also selected resistant mutations such as Y93H and L31V (Fig. 2). In fact, the presence of these resistant mutations is known as the key determinant of the success of daclatasvir + asunaprevir dual therapy.[39] In the presence of these mutations, the SVR for the daclatasvir + asunaprevir dual therapy was less than 50%.[39] So far, all known resistant mutations of NS5A were identified in the domain 1 (Table 2, Fig. 2). A recent kinetic analysis of DAAs from the different classes found that NS5A inhibitors restrict two distinct steps of HCV life cycle: replication complex formation and virus assembly.[40] Interestingly, NS5A protein exists as a dimer, which is also suggested by the symmetrical structure of daclatasvir.[41]

3. NS5B

NS5B protein is an RNA-dependent RNA polymerase, a key enzyme which directly catalyzes the synthesis of plus- and minus-strand of viral RNAs. This protein is a highly error-prone enzyme since it does not have the proofreading capability. Depending on the inhibition modes, NS5B inhibitors are classified into either nucleotide analogs (e.g., sofosbuvir) or nonnucleoside analogs (e.g., dasabuvir, beclabuvir), the latter of which act as allosteric inhibitors. Of these inhibitors, sofosbuvir (Gilead) is particularly interesting in that it showed excellent SVRs in numerous clinical trials and a very high genetic barrier to resistance development. This unique property of sofosbuvir has made it the first choice of DAA for several all-oral regimens for treatment of chronic hepatitis C. Despite its outstanding profiles as an antiviral agent, the cost is still an issue in many countries. Several resistance-associated mutations of NS5B are located in Fig. 2 and the most commonly observed NS5B resistant mutations are summarized in Table 3. Interestingly, no cross-resistance was observed between the nucleotide and nonnucleoside inhibitors due to the different mechanisms and sites of interaction with NS5B.

Table 3. Most Commonly Observed NS5B-Resistant Mutations[32]

Inhibitor	Genotype 1a	Genotype 1b
Dasabuvir	M414T, S556G	S556G
Baclabuvir	A421V, P495L/S	None

CONCLUSIONS

Substantial progresses in the development of antiviral therapies have increased the sustained virological response and now approximately 90% to 95% of chronic hepatitis C patients are expected to be cured, thus significantly reducing the number of more complicating liver diseases such as cirrhosis and hepatocellular carcinoma. Although this is a remarkable accomplishment and the first in the human history in that the chronic viral infection is curable, still the 5% to 10% of the chronic hepatitis C patients are suffering from this viral infection and awaiting better treatment options. In addition, more cases of antiviral drug resistance are being reported and the retreatment of the patients who have failed in the prior antiviral treatments is the issue that has to be immediately addressed.

In terms of virology, HCV was discovered relatively later than the other closely related viruses belonging to the family *Flaviviridae* (e.g., dengue virus, Japanese encephalitis virus,

West Nile virus, yellow fever virus, etc.). However, the significant global impact of HCV infection has far more advanced virology of this specific virus and the knowledge that was obtained from the study of HCV would accelerate a better understanding of the other clinically important flaviviruses. In addition, it would also lead to the development of new antiviral therapies to eradicate these deadly human pathogens.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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