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Hepatitis C Virus Molecular Biology, *In vivo*/*In vitro* Model Systems and Current Trends of Therapies: A Brief Review

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Abstract

Hepatitis C virus (HCV) infection is a global health concern affecting approximately 170 million people worldwide, and can lead to liver fibrosis and hepatocellular carcinoma. One of the top priorities in HCV infection should be development of effective therapies, by developing antiviral compounds for infected patients. Many small animal models have been used to understand the replication cycle of HCV, and to discover novel antiviral drugs against HCV infection. Nowadays, these animals are serving as powerful tools for better understanding of HCV infection. Recently, many *in vitro* models are being used to improve antiviral drugs against HCV, and have lead to the development of many novel antiviral compounds, thus decreasing the HCV cases worldwide. To date, there is no vaccine available due to high strain variation, and current approved treatment for HCV is pegylated interferon α (PEG-INF α), in combination with ribavirin and Boceprevir/Telaprevir. This review briefly summarizes HCV infection, its molecular biology, role of HCV viral proteins in HCV infection, HCV life cycle, *in vivo*/*in vitro* model systems and current trends of therapies against it.

Keywords: HCV infection; Genome; Model systems; Antiviral; Drugs; Therapies

Hepatitis C Virus (HCV) Infection

Hepatitis C virus (HCV) is a worldwide health problem affecting approximately 170 million people around the world, with the highest infection rates found in Asia and Africa [1]. Hepatitis C virus is a member of *Flaviviridae* family, that also includes other viruses like Dengue virus, West Nile virus and Yellow Fever [2], and has genome with a positive-single stranded RNA (ssRNA), having genome size of 9.6 kb. HCV genome encodes a single polyprotein precursor of 3010 amino acids, having an internal ribosome entry site at 5' untranslated region (UTR), vital for the translation. The length of 5`UTR is 324-341, and it posses an internal ribosome entry site (IRES), important for Cap-independent translation of viral RNA [3,4]. This polyprotein precursor is cleaved to generate at least 10 proteins in the order of NH(2)-Core-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS 5B-COOH (Figure 1). These viral proteins are responsible for viral replication and various cellular functions [5,6]. To date, significant progress has been made to understand the life cycle of HCV, especially the viral protein processing and the genome replication [7]. Interaction of the virion with various cell receptors results in HCV infection [8].

Viral initial attachment may involve glycosaminoglycans and the low-density lipoprotein receptor, followed by interaction with class B type I scavenger receptor, the tetraspanin CD81, tight junction protein Claudin-1, -6 or -9 and Niemann-Pick C1-like 1 (NPC1L1) (Figure 2) [9]. Once the virion enters cell through clathrin mediated endocytosis, its RNA is released into cytosol, which is eventually processed by cellular and viral proteases to generate 10 viral proteins [9,10]. After the cleavage of polyprotein by host endoplasmic reticulum (ER) Signal peptidase, HCV structural proteins (Core, E1, E2/p7) are released, and after the enzymatic cleavage of HCV proteases, NS2-3 and NS3-4A, non-structural proteins are released [11-16]. HCV causes acute and chronic hepatitis. The acute hepatitis is considered to be the first 6 months, and in this phase, spontaneous clearance is still possible [17]. Almost 70-80% of HCV infected patients become chronic carriers, of which 20% develop cirrhosis, 6% will decompensate to end-stage liver disease (ESLD), and 4% will develop hepatocellular carcinoma (HCC) [17,18]. Acute to chronic progression is rapid in people who are older, HIV coinfected, consume >50 grams of alcohol daily, or required organ transplantation and immunosuppression [1]. HCV is classified into six genotypes and a series of subtypes [19], and now a new sequence has been assigned as subtype 7a [20]. Among all genotypes, prevalence of 3a is most likely concerned with steatosis, leading to liver fibrosis [21,22].

HCV Structural Proteins

The HCV core protein is involved in forming viral capsid, and is a highly conserved basic protein [23]. The HCV core protein is released as a 191 amino acids precursor, and is divided in six domains on the basis of hydrophobicity [24]. Domain 1 (amino acids 1-117) contains mainly basic residues with two short hydrophobic regions. Domain 2 (amino acids 118-174) is less basic and more hydrophobic, and its C-terminus is at the end of p21. Domain 3 (amino acids 175-191) is highly hydrophobic and acts as a signal sequence for E1 envelope protein [25]. Viral core protein directly interacts with a number of cellular proteins and pathways involved in viral life cycle [26]. HCV has two envelope glycoproteins, namely E1 and E2, with molecular weights of 33-35 and 70-72 kDa, respectively. These envelop proteins are necessary for viral entry [27-29]. E1 and E2 are highly glycosylated. E1 contains 4 or 5 N-linked glycosylation sites and E2 contains up to 11, with most of the sites being well conserved. In addition, E2 contains hypervariable regions, with amino acid sequences differing up to 80% between HCV genotypes and between subtypes of the same genotype [30,31]. There is not much knowledge available about E1, but it is

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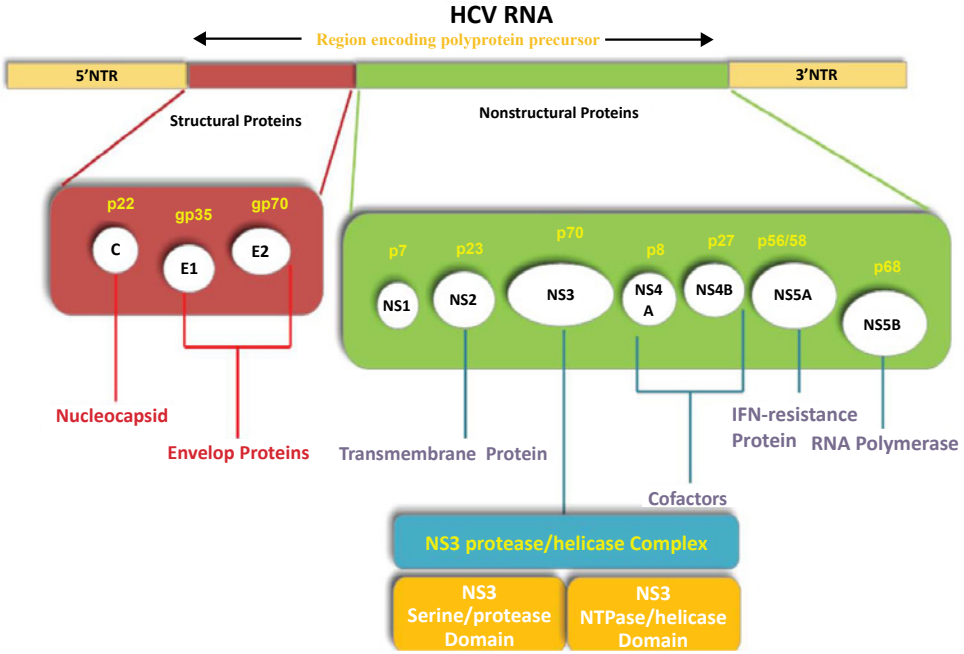

Figure 1: HCV genome. The HCV genome showing viral proteins in the order of NH(2)-Core-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS 5B-COOH.

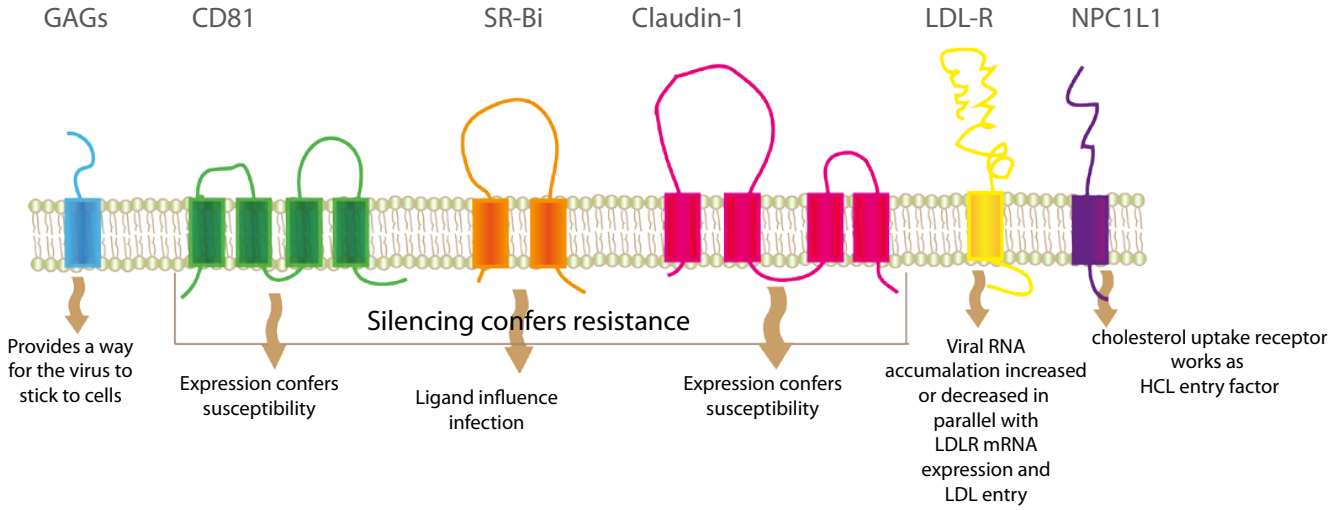

Figure 2: HCV cell entry receptors. Receptors involved in viral entry (GAGs, CD81, SR-BI, Caludin 1, LDL-R, NPC1L1).

thought to be involved in intra-cytoplasmic virus-membrane fusion, and truncated forms of E2 are known to interact with CD81, scavenger receptor type B class 1protein (SRB-1) and high density lipoprotein (HDL) binding molecule [32-34]. Mannose binding proteins (DC-SIGN and L-SIGN) have been suggested to have interactions with E2, but their function in viral entry is unclear [35].

HCV Nonstructural Proteins

HCV p7 protein is located in the endoplasmic reticulum, and is a 63-amino acids polypeptide. It has two transmembrane domains (TMDs), connected by a cytoplasmic loop; the amino- and carboxyl-terminal tails are oriented toward the endoplasmic reticulum lumen [36]. These proteins form ion channels that play an essential role in virus infection [37]. It might be a relevant target for future drug development [36]. p7 protein function at an early stage of virion morphogenesis, prior to the assembly of infectious virus [38]. NS2 protein is a 21-23 kDa transmembrane protein. NS2 protein is essential for completion of the viral replication cycle *in vitro* and *in vivo* [39,40]. NS2 protein is a hydrophobic protein [41]. The amino-terminal portion of HCV NS2 protein inhibits the expression of reporter genes driven by different promoters or enhancer elements. The inhibitory effect of HCV NS2 on liver and non-liver-specific promoters and enhancer elements might be relevant for the pathogenesis of chronic HCV infection [42]. NS2 is a short-lived protein, that loses its protease activity after self-cleavage from NS3, and is degraded by the proteasome in a phosphorylation-dependent manner by means of protein kinase casein kinase 2 [43]. The

NS3 is multifunctional protein having 67 kDa weight. NS3 N-terminal has serine protease activity and C terminal has NTPase/helicase activity [44]. NS3 protein is also involved in RNA binding activity [45]. The mature NS3 protein comprises 5 domains: the N-terminal 2 domains form the serine protease, along with the NS4A cofactor, and the C-terminal 3 domains form the helicase. The helicase portion of NS3 can be separated from the protease portion by cleaving a linker [46]. Most work has focused on the protease, rather than the helicase activities of the enzyme [47]. The target of NS3 is the mitochondrial antiviral signaling protein, MAVS, that activates NF-κB and IFN regulatory factor 3 to induce type-I interferons [48]. Therefore, NS3 is an important drug target in the effort to combat HCV [47]. NS4A protein acts as a cofactor for NS3 protein. The N-terminus of NS4A is highly hydrophobic, and is involved in targeting NS3 to the ER membrane [49]. Recently reported crystal structures demonstrated that NS4A forms an integral part of the NS3 serine proteinase [50]. Isoleucine-29 of NS4A is important for the interaction between NS4A and the NS4B5A substrate.

In addition, two more hydrophobic residues in the NS4A central region (valine-23 and isoleucine-25) are also essential for the cofactor activity, and for the interaction with either the NS3 proteinase or the NS4B5A polyprotein substrate [50]. NS4A is also required for the phosphorylation of NS5A and can directly interact with NS5A [51]. Since NS4A is required for processing at certain serine proteinase-dependent cleavage sites, this interaction may represent a new target for development of antiviral compounds [52]. NS4B is a small hydrophobic protein that induces a specialized membrane structure, which may serve as the replication platform for HCV RNA replication. It plays an important role for recruitment of other viral proteins [53,54]. The protein is localized in the endoplasmic reticulum (ER) [55]. NS4B is found to be a negative regulator of the NS3-NS5B replication complex. Overall, it is revealed that NS3, NS4B, and NS5B can interact to form a regulatory complex, that could feature in the process of HCV replication [56]. NS5a is associated with a variety of cellular signalling proteins [57]. NS5A causes the disturbance of intracellular calcium [58]. NS5A impairs TNF-mediated apoptosis by interfering upstream of the signal transduction pathway, and may play a role in HCV-mediated pathogenesis [59]. The NS5B is a 65 kDa protein that act as RNA dependent RNA polymerase, and has a significant role in the synthesis of the new RNA genome [60]. NS3-4A serine protease is therapeutically important and many antiviral compounds have been tested against it, from which some have entered clinical trials and two compounds have been approved as an HCV standard of care. NS5B is being targeted to develop novel antiviral drugs against HCV and hopefully in future, NS5B inhibitors will be useful in anti-HCV therapies. Thus, both NS3-4A and NS5B have emerged as most popular drug targets [61].

In vivo Animal Models

The lack of small animal models has been a hindrance in *in vivo* screening of HCV NS3 protease inhibitors [62]. Many small animal models have been used to study life cycle and replication of HCV genome. These animal models not only helped our understanding of HCV genome, but also helped in identifying many antiviral compounds to stop HCV infection. Chimpanzee is susceptible to hepatitis viral infection, but its use is not widespread in HCV research due to its endangered status and financial consideration limits [63]. Chimpanzee model also has a number of limitations, including their large size, expense, and limited availability [64]. The development of transgenic mice as in-vivo model has not only helped in understanding the pathogenesis of HCV infection, but also helped in development of new antiviral compounds against HCV [65]. GB virus B (GBV-B) marmoset model has been used to provide the first demonstration of the *in vivo* potency of a small-molecule inhibitor of HCV [66]. Recently, zebrafish has successfully been used as a model organism for HCV replication. HCV core protein expressed in the transgenic zebrafish accelerates the HCC development. Therefore, zebrafish model can serve as a powerful preclinical platform to study the molecular events of HCV, and to develop new therapeutic strategies [67]. These models are challenging, but once optimized, they can be extremely useful for HCV and drug development studies.

In vitro Model Systems

Recently, many *in vitro* models have been developed to improve antiviral drugs against HCV, and to eradicate the need of using *in vivo* small animals as models. These models have increased the understanding of HCV life cycle; viral attachment, entry, fusion, viral RNA translation, post translational processing and HCV replication [68], and have lead to the development of many novel antiviral compounds, thus decreasing the HCV cases worldwide.

In vitro transcription/translation (IVTT) assay systems

In the past, due to the absence of an efficient *in vitro* cultivation system for HCV, the proteolytic processing of the NS3 serine proteases has been studied using *in vitro* transcription-translation (IVTT) assay systems [52]. *In vitro* transcription-translation was used to examine the inhibitory effect of NS3 protease inhibitors. The availability of this method for the expression and purification of the NS3 protease will ease the development of antiviral drugs against HCV (73).

HCV sub-genomic replicons

HCV replicon systems are a good option to screen antivirals as all the viral enzymes that are thought to be prime targets for antiviral, are encoded by them. Another advantage of replicon is easy measurement of selectable sub-genomic RNAs [69]. Among the HCV replication models developed, the HCV RNA replicon model and the newly discovered infectious cell culture systems have enabled quantitative evaluation of the antiviral potency of HCV inhibitors [70,71]. Recently, hetrologous cDNA expression system and sub-genomic replicons are used to study viral life cycle and to examine novel antiviral therapies. Also, among the surrogate animal models that have been developed are mouse liver repopulated with human hepatocytes and transgenic mice expressing hepatitis antigens [63]. The replicating HCV RNA of genotype 2a JFH1 strain in Huh7 cells having a replicon system has helped in evaluation of antiviral compounds against viral factors involved in HCV replication [72].

HCV replication in Cell culture/Cell lines

Allowing HCV replication in cells is the hallmark for construction of its *in vitro* replication model. A stable cell culture model for HCV using HCV patient sera showed the susceptibility of hepatoma cell line 7721 for HCV. The transcription and translation stages of HCV life cycle were confirmed through RT-PCR, *in-situ* hybridization and immune-histo chemistry techniques. Intracellular expression of viral antigens and the presence of HCV RNA in cells were also demonstrated [73]. Infecting the appropriately growing primary hapatocyte culture and peripheral blood mononuclear cells with HCV containing sera resulted in a sequential increase of RNA titers in culture supernatant [74-76].

Cell lines scuh as Human hepatoma Huh7, HepG2 and porcine non-hepatoma PK15, STE when infected with HCV chronic patient sera resulted in increased viral RNA, and thus, proved that these cell lines are susceptible to HCV infection [77].

HCV full length viral particles (HCVcc)

The recently developed HCV sub-genomic replicons were inadequate by the fact that the sequence encoding the structural proteins was missing. By using three cells culture-adaptive mutations that improve RNA replication synergistically, selectable full-length HCV genomes were generated that increase to high levels in the human hepatoma cell line (Huh-7). In it, the structural proteins including viral glycoproteins E1 and E2 were proficiently expressed [78]. Stable cell lines HCVcc virions can reinfect naive Huh-7 cells, and it can suppress viral replication by alpha interferon [79]. HCVcc replication produced approximately 10^5 infectious units per milliliter within 48 hours. The entry of the virus depends on the cellular expression of HCV receptor, CD81. The replication was slow down by interferon α and many other HCV targeting antiviral compounds. Therefore, HCVcc as an *in vitro* system can help in improving antiviral drugs against HCV [80].

These models are currently being used to investigate the HCV life cycle, and to develop new antiviral therapies that can decrease HCV infection cases, or eradicate this viral disease by developing new and improved drugs.

Current Therapies

Early clearance of HCV, to prevent the risk of developing cirrhosis and HCC and to decrease mortality, has been the aim of antiviral treatment in patients with HCV infection. To date, there is no vaccine available for HCV prevention due to the high degree of strain variation. The approved treatment for HCV infection is pegylated alpha interferon (IFN-α) alone, or in combination with ribavirin. This leads to clearance of HCV in 50% and 80% of the cases of HCV genotype 1 and 2 infection, respectively, but this treatment has certain side effects and slow response rate, especially in patients infected with HCV genotype 1a and 1b [81-83]. After the success of protease inhibitors in the treatment of human-immunodeficiency virus-1 (HIV) infection, it was thought that protease inhibitors can also serve as important drug targets against HCV NS3 protease activity [84, 85]. NS3/4A protease inhibition interferes with the viral life cycle and restores pathways of innate immunity, therefore, it attracted the minds of researchers to block HCV infection [86]. To date, several novel NS3/4A protease inhibitors have been tested, of which some are effective in achieving constant virological response in patients with HCV genotype 1 [87]. Recently, two NS3 protease inhibitors, Boceprevir and Telaprevir, have been approved as standard of care for HCV genotype 1 patients, and can be used with triple therapy (PEG-IFN- α /ribavirin plus Boceprevir or Telaprevir) [88]. In recent years, NS3 helicase has attracted the mind of researchers to develop novel drugs, but there is a lack of knowledge available about HCV NS3 helicase mechanisms. Even so, swift progress is being made in the helicase field, and it will not be surprising if HCV helicase inhibitors someday enter clinical trials [89,90].

Conclusion

HCV causes one of the most prevalent chronic infectious diseases worldwide [91], but to date, there is no vaccination available to prevent it. HCV has 7 genotypes and more than 50 subtypes. Due to its high degree of variation, vaccine development against it has become a challenge. Currently, a combination therapy (interferon+ribavirin) is being used against it, but this therapy is costly and has potential complications. This therapy clears HCV in 50% of HCV genotype 1 and 80% in HCV genotype 2, but has slow response rate, especially in patients infected with HCV genotype 1a and 1b [81-83]. Recently, two NS3 protease inhibitors have been approved as a standard of care, and thus, are being used in combination with interferon and ribavirin. Several antiviral compounds have been tested using *in vivo* animal models and *in vitro* models against HCV viral proteins, especially NS3 and NS5, and some of them have shown significant inhibition of HCV infection, but still there is a need to develop cost effective and safe vaccine that can target all genotypes.

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