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TOPIC HIGHLIGHT

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Scotomas in molecular virology and epidemiology of hepatitis C virus

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Abstract

In the 1970s, scientists learned of a new pathogen causing non-A, non-B hepatitis. Classical approaches were used to isolate and characterize this new pathogen, but it could be transmitted experimentally only to chimpanzees and progress was slow until the pathogen was identified as hepatitis C virus (HCV) in 1989. Since then, research and treatment of HCV have expanded with the development of modern biological medicine: HCV genome organization and polyprotein processing were delineated in 1993; the first three-dimensional structure of HCV nonstructural protein (NS3 serine protease) was revealed in 1996; an infectious clone of HCV complementary DNA was first constructed in 1997; interferon and ribavirin combination therapy was established in 1998 and the therapeutic strategy gradually optimized; the HCV replicon system was produced in 1999; functional HCV pseudotyped viral particles were described in 2003; and recombinant infectious HCV in tissue culture was produced successfully in 2005. Recently, tremendous advances in HCV receptor discovery, understanding the HCV lifecycle, decryption of the HCV genome and proteins, as well as new anti-HCV compounds have been reported. Because HCV is difficult to isolate and culture, researchers have had to avail themselves to the best of modern biomedical technology; some of the major achievements in HCV research have not only advanced the understanding of HCV but also promoted knowledge of virology and cellular physiology. In this review, we summarize the advancements and remaining scotomas in the molecular virology and epidemiology of HCV.

Key words: Hepatitis C virus; Hepatitis C virus lifecycle; Molecular virology; Hepatitis C virus models; Epidemiology

Core tip: The review summarizes the advancements, as well as remaining scotomas, in the molecular virology of hepatitis C virus (HCV). We emphasize the contributions of HuH-7 hepatocellular carcinoma cell line to development of the HCV replicon, cell culture-derived HCV, and HCV pseudoparticles. In addition, we reiterate the importance of epidemiological issues because accurate assessment of HCV-related disease burden has been overlooked. This review provides a history of the fight against HCV, which has required scientists to avail themselves to the best of modern biomedical technology, which in turn has enriched our knowledge of virology and cellular physiology.

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INTRODUCTION

The hepatitis C virus (HCV) is an enveloped, single-

stranded, positive-sense RNA virus, classified as a *Hepacivirus* within the *Flaviviridae* family[1-3]. The 9.6-kb RNA genome contains one long open reading frame (ORF) flanked by 5' and 3' untranslated regions (UTR)[4-6]. The single ORF encodes an approximately 3000 amino acid (aa) polyprotein that undergoes co- and post-translational cleavage by host and viral proteases to yield 10 viral proteins, not including the F protein[7,8]. The structural proteins, nucleic acid-binding nucleocapsid core protein and envelope proteins (E1 and E2/P7) are encoded by 25% of the N-terminal portion of the genome[9]. The remaining 75% of the genome encodes the non-structural proteins, NS2, NS3, NS4A, NS4B, NS5A and NS5B[9].

Humans are the primary reservoir of HCV[10]. HCV transmission occurs primarily through exposure to infected blood and the majority of individuals with persistent infection develop chronic hepatitis, which can progress to cirrhosis or hepatocellular carcinoma[11-13]. Different from other viruses, such as influenza A viruses and human immunodeficiency viruses, HCV is difficult to isolate and culture[14-16]. Since HCV was identified in 1989[1], basic research on HCV has been being hindered by the absence of reliable, reproducible, and efficient culture systems[11]. Recently, tremendous advances in understanding the HCV replicon[15,16], the pseudo-typed HCV viral particle[17], cell based culture systems[18,19], receptors[20-24], life cycle[25,26], structural biology and HCV therapy strategy[27-29] have been gained. However, several scotomas in the molecular virology and epidemiology of HCV remain to be elucidated. This review summarizes the advancements and remaining scotomas in the molecular virology and epidemiology of HCV.

MAJOR PROGRESS IN FIGHTING HCV

Since HCV was identified in 1989[1], virological research has led to a great deal of progress in the pathogenesis, diagnosis, treatment, control and prevention of the disease[11,30]. Since virus elimination is the ultimate goal of viral disease therapy, here we emphasize two major recent achievements in hepatitis C treatment. The first achievement was the development of direct-acting antiviral (DAA) agents, which are inhibitors of the HCV protease[31-37]. Although peginterferon and ribavirin remain vital components of therapy, the emergence of DAA agents has led to an unprecedented improvement in sustained virologic response rates to approximately 94%[30,38]. This is indicative of two milestones in virology: a therapy with the highest documented antiviral effects and optimism that the virus could be eliminated by medications. The second achievement is the identification of several single-nucleotide polymorphisms associated with spontaneous and treatment-induced clearance of HCV infection[39,40]. This discovery is also a milestone because only the rare single nucleotide polymorphisms of rs12980275 and rs8099917, near the interleukin28B gene, have any reported biological effect[39,40].

CURRENT HCV MODELS

The *in vitro* and *in vivo* models for HCV have evolved significantly since the discovery of the virus. With any virus, cell-based culture systems and animal models are the essential tools for virological study, vaccine development, and antiviral drug discovery. Many viruses, such as the influenza virus, are easy to isolate and culture in cell lines[41,42]; however, HCV is difficult to isolate and propagate[15,16,18,19]. Before HCV was identified, many virologists had attempted to isolate and culture the pathogen of non-A, non-B hepatitis using traditional cell-based approaches[43]. After struggling for decades, it was determined that HCV could only survive in human or chimpanzee fetal liver cells and hepatocytes or human peripheral blood mononuclear cells[44-49]. These cells are inconvenient to obtain and have a finite lifespan in culture, so even though these early studies showed that HCV was selective with a narrow host range[50,51], these methods made little contribution to HCV research (Table 1).

Defeated by classical approaches to isolate HCV, virologists were forced to reproduce the HCV lifecycle using split models, which included the HCV genome RNA replication model (HCV replicon)[15,16], HCV structural proteins model (virus-like particle, VLP)[52] and HCV pseudotyped viral particles model[17] (Table 1). In 1999, Bartenschlager's group in Germany established a HCV replicon system[15], followed soon after in 2000 by Rice's group in the United States[16]. These models simulated the structure of the subgenomic selectable HCV replicons composed of the HCV 5'-UTR, the gene encoding the neomycin phosphotransferase or firefly luciferase, the encephalomyocarditis virus internal ribosome entry site, the region encoding HCV NS2-5B or NS3-5B, the authentic 3'-UTR, and the 12-16 5'-terminal codons of the core[15,16]. The replicon could replicate autonomously in hepatic cell cultures (*e.g.*, HuH7 cell line)[15,16], leading to a series of experiments that examined the function of 5'- and 3'-UTR and NS3 to NS5B in HCV genome replication, described the viral life cycle, and led to the development of antiviral drugs[27]. The HCV replicon was able to replicate itself within the cell; however, it was not capable of producing infectious viruses[27]. Furthermore, this replicon was not able to reproduce in HuH-7 cells with high efficiency and for an extended period of time[50]. Virologists attempted to improve the replication efficiency and modify the robust HCV replicon using several methods, including adaptive mutation hunting, to reduce the non-HCV genome and increase the HCV genome composition, by attempting to replace various wild HCV strains of different genotypes[27,50,53].

Pseudotyped viral particles are commonly known as lentiviral vectors. These vectors are composed primarily of three viral elements; the gag-pol, which forms the viral structure, recognizes the viral genome and is responsible for the genome lifecycle; the viral mimic genome, which provides the genome elements that will be recognized

Table 1 Summary of *in vitro* and *in vivo* models for hepatitis C virus

In vitro and *in vivo* models	Established year	Advantages	Deficiencies
In vitro			
Cultivation of HCV	1993-1999	Achieved cultivation of HCV in human foetal liver cells, human hepatocytes or PBMC. Illustrated HCV is quite species selective and has a narrow range of hosts	Requires specific cellular factors to support viral lifecycle. Primary human and chimpanzee hepatocytes or highly differentiated cells dependent. Most of them have yielded limited success. Poor reproducibility and low levels of HCV replication
HCV replicon	1995-2000	Provided a cell-based model for the study on HCV genome replication	
HCV VLP	1998-1999	Rare evidence to support that HCV structural proteins core, E1, and E2 could form VLP	
HCVpp	2003	Provided a convenient and feasible tool for studies on viral entry, HCV receptor, neutralizing antibody, *etc.*	
HCVcc	2005	A break through in production of infectious hepatitis C virus in tissue culture	
In vivo			
Chimpanzee	1979	The only recognized animal model for HCV study, played a critical role in HCV discovery and play an essential role in defining the natural history of HCV	Chimpanzees differ from humans in their course of infection, that chronic carriers do not develop cirrhosis or fibrosis, limited availability, cost performance, and public resistance
Tree shrew	1998	Might be a succedaneum for chimpanzees	Persistent HCV infection could not be established and only 25% of infected animals developed transient or intermittent viremia. Germ line was not available to a small animal model
Chimeric human liver mouse	2001	Exhibited prolonged infection with high viral titers following inoculation with HCV isolated from human serum. HCV can be transmitted horizontally. Drug evaluation	Since the mice were immunodeficient, they were not appropriate models to study HCV pathogenesis
Genetically humanized mouse	2011	Represents the first immunocompetent mice model for HCV study. Allows for the studies of HCV coreceptor biology *in vivo*	Operation is difficult

HCVpp: Hepatitis C virus (HCV) pseudotyped viral particles; VLP: Virus like particle; HCVcc: Cell culture derived HCV.

by gag-pol and ensures complete viral RNA metabolism; and the envelope proteins, which are presented onto the artificial viral particle[54-58]. Additionally, a lentiviral vector contains a reporter gene inserted into the artificial viral genome. Although the lentiviral vector was used widely in gene transduction, presenting an HCV envelope protein functionally in this viral particle was not considered. Virologists tried to generate the HCV VLP[52], because classic virological experience told us that VLP of a certain virus could be produced by cloning and expressing virus structural proteins, and Liang's group at the National Institutes of Health (Bethesda, MD) was successful in establishing the HCV-like particles using a baculovirus expression vector system[52]. In 2003, French virologist Bartosch *et al*[17] produced HCV pseudoparticles (HCVpp) using viral elements derived from murine leukemia virus. The HCVpp system led to advanced studies that identified a neutralizing antibody against HCV[59], explored HCV receptors and described the structure and function of the HCV envelope proteins[60-63].

In 2005, the Japanese virologist Wakita obtained a genotype 2a HCV strain (JFH-1) from a Japanese patient with a rare case of fulminant hepatitis C[18]. Based on the experience and methods accumulated in studying the HCV replicon, Wakita and his group rescued HCV in the HuH7 cell line, which was designated as HCVcc, for cell-culture-derived HCV[18]. HuH-7 cells infected with cloned and *in vitro* transcribed JFH-1 genomes produced viruses that were capable of infecting naïve HuH-7 cells[18]. In addition, the virus particles could be neutralized with a monoclonal antibody against the viral glycoprotein E2[18]. The study was the first *in vitro* experiment that showed the complete lifecycle of HCV. More importantly, virus obtained from the cell cultures was highly infectious in chimpanzees and immunodeficient mice with partial human livers[64].

As early as the 1970s, it was known that the etiological agent responsible for non-A and non-B hepatitis could be transmitted to chimpanzees[65], and chimpanzees were subsequently recognized as the only animal model of HCV[66] (Table 1). Chimpanzees played a critical role in defining the natural history of HCV[66] and since they are closely related to humans, any study of chimpanzees could reflect more closely what happens in humans than other animal models. However, chimpanzees that are chronic carriers of HCV do not develop cirrhosis or fibrosis[66,67], which are the most important consequences of HCV infection in humans.

Because chimpanzee studies are expensive and restricted by ethical responsibilities[67], scientists diverted their attention to other small animal models, such as the tree shrew and a chimeric human liver mouse. Xie *et al*[68] demonstrated that *Tupaia* could be infected by HCV when severely immunosuppressed; however, persistent

HCV infection could not be established and only 25% of infected animals developed transient or intermittent viremia[51]. By genetically manipulating the urokinase-type plasminogen activator transgenic mouse, Mercer *et al*[69] transplanted normal human hepatocytes into severe combined immunodeficient mice carrying a plasminogen activator transgene. The chimeric mice exhibited prolonged infection with high viral titers following inoculation with HCV isolated from human serum[69]. Since the mice were immunodeficient, they were not appropriate models for investigation of HCV pathogenesis, although they were useful in assessing the activity of antiviral drugs, as well as neutralizing antibodies[51] (Table 1).

Mouse models of HCV provided little information about the human hepatocellular factors required for HCV entry. Thus, Ploss *et al*[24] introduced human CD81, scavenger receptor type B class 1, claudin 1, and *OCLN* genes into mice using a recombinant adenovirus expression system. They found that mice expressing these human factors were sufficient for HCV infection[24]. This system allowed for the investigation of HCV co-receptor biology *in vivo* and evaluation of passive immunization strategies and, therefore, represented the first immunocompetent small animal model for HCV[70] (Table 1).

SCOTOMAS IN MOLECULAR VIROLOGY

Although much progress has been made in all aspects of HCV research in the last few decades, we are still far from achieving the ultimate goal of complete HCV control and prevention. Thus, a better understanding of the HCV life cycle is essential to optimize the antiviral strategy. As mentioned above, the major challenges to HCV research are that HCV is difficult to isolate and culture, and no vaccine is available[14-16,71]. However, rather than summarizing the many achievements in HCV research, we have chosen to enumerate the scotomas in HCV molecular virology.

Structural biology of the HCV particle

Since HCV was first proposed to be a distinct infectious pathogen, virologists of that era attempted to visualize this enigmatic microbe using electron microscopy[43]. Different from other hepatitis viruses, including hepatitis A virus, hepatitis B virus and hepatitis E virus and the other viruses within the *Flaviviridae* family[43], no clear electron microscope image of HCV was reported until Chisari' s and Rice's groups provided high-resolution images of highly enriched cell culture-derived HCV (HCVcc) particles in 2010 and 2013, respectively[72,73]. The reason for the difficulty in observing the crude HCV particle in HCV-harboring tissue remains unclear. The viral titer should not be an issue since viral copies in blood samples produced by HCV RNA are > 10^6/mL[74]. Serum-derived HCV particles are associated with the lipoprotein components apolipoprotein A-I (apoA-I), apoB-48, apoB-100, apoC-I and apoE[75]. The interaction between virus particles and serum lipoproteins suggests that HCV may form hybrid lipoviral particles[75] that facilitate virus entry into hepatocytes and protect the virus from the host immune response. In addition, the lipoprotein components might affect the morphological observation of crude HCV particles by electron microscopy. Our current knowledge of HCV morphology indicates that HCV particles are 40-80 nm in diameter, pleiomorphic, lack obvious symmetry or surface features and contain electron-dense cores[72,73]. The lack of details describing the overall architecture of HCV limits the ability of molecular biologists to study HCV structural biology and topology.

Molecular virology of HCV structural proteins

A quarter of the N-terminal region of the HCV polyprotein encodes the core structural protein and glycoproteins E1 and E2, which are believed to be incorporated into the HCV particle[17]. Based on general virological knowledge, the core protein should be a major component of the viral capsid, responsible for viral genome RNA recognition, binding and packaging[27]. Glycoproteins E1 and E2 located in the viral surface are also called envelope proteins; E1 and E2 are responsible for receptor recognition, receptor binding, endocytosis and membrane fusion[27]. The mature core protein contains a positively charged N-terminal RNA binding domain and a C-terminal domain that consists of two amphipathic helices and a palmitoylated cysteine residue to facilitate peripheral membrane binding[27]. Antibodies against core proteins are important for HCV serological detection[43]. Previous studies demonstrated that the core protein is involved in many pathogenic processes[43]. Furthermore, the core protein induces hepatocellular carcinoma in transgenic mice[76] and is a potent inhibitor of RNA silencing-based antiviral response[77]. However, the basic function of the core protein in capsid formation remains unknown. Although the region between amino acids 82 and 102 contains a tryptophan-rich sequence involved in homotypic core proteins interaction[78], the HCV core particle had not been successfully produced. *In vitro* nucleocapsid reconstitution experiments using the 1-124 or 1-179 core segments and structured RNA molecules have yielded irregular particles larger than those reported by the limited electron microscopy observations[79]. Scientists in China at Xiamen University successfully generated human papillomavirus VLP[80], hepatitis E virus VLP[81] and hepatitis B virus core capsids but failed to produce the HCV capsid (personal communication). In addition, the crystal structure of the HCV core protein is not yet available (Table 2).

E1 and E2 are type I transmembrane glycoproteins assumed to be class Ⅱ fusion proteins, with N-terminal ectodomains of 160 and 334 amino acids, respectively, and a short C-terminal transmembrane domain of approximately 30 amino acids[27,43]. Studies of HCVpp have indicated that 14 amino acids from the HCV core and 12 amino acids from the E1 C-terminus are required for E1 and E2 function[62]. The hemagglutinin and neuraminidase of influenza A viruses matches each other in a

Table 2 Summary of the properties of hepatitis C virus structural proteins

	Core	E1	E2	p7
Genome location	342-914	915-1490	1491-2579	2580-2769
Translation processing site		Rough ER		
Amino acid composition	191	192	363	63
Molecular weight (kDa)	21-23	33-35	70-72	7
Glycosylation	No	Yes	Yes	No
Cleavage		ER signal peptidase and SPP		
Crystal structure		Not available		Revealed
Functional unit	Dimer	Heterodimer?		Hexamer
Common function	Viral particle formation. Core, E1 and E2, together with p7 and NS2, are required for virus assembly (assembly module)			
Unique function	Capsid protein, viral particle formation, viral genome recognizing and packaging. Interacts with cLDs in early viral particle formation process. Counters host antiviral factors and involves pathogenesis	Envelope glycoproteins, interact with SRB1, CD81, CLDN1, OCLN, *etc.* to trigger viral entry. Promote fusion with the endosomal membrane. Counter host immune response *via* hypervariable regions		Viroporin. Has key roles in organizing the virus assembly complex. p7-NS2 complex interacts with the NS3-4A enzyme to retrieve core protein from cLDs to form viral particle
Major scotomas	How do the core form the viral capsid? The signals and processes that mediate RNA packaging are largely unknown. What impeded us to resolve the structure of the viral glycoproteins? What is the real process in HCV entry? How are these receptors and co-receptors temporally and spatially used to ensure the early infection processes?			

Start co-ordinates based on H77 (accession number, NC_004102). SRB1: Scavenger receptor class B member 1; CD81: Tetraspanin CD81; CLDN1: The tight junction protein claudin 1; OCLN: The tight junction protein occludin; cLDs: Cytosolic lipid droplets; ER: Endoplasmic reticulum; SPP: Signal peptidase and signal peptide peptidase; HCV: Hepatitis C virus. The molecular weights of E1 and E2 refer to the glycosylated forms.

relative slack manner regardless of gene homology[54-58], while the matching pattern of HCV E1 and E2 is relatively strict. We separated E1 and E2 of HCV genotypes 1a, 1b, and 2a into two individual expression plasmids and replaced the transmembrane domains of 1b and 2a E1 and E2 with that of genotype 1a. The complementation features of E1 and E2, as well as the contributions of both the ecto- and transmembrane domains to the formation of the E1E2 complex, were evaluated using the HCVpp system[63]. We found that 1aE2 could not only complement its native 1aE1 but also 1bE1; in genotype 1b, glycoprotein complex formation is dependent primarily on the overall biological characteristics of the intact native E1 and E2; in genotype 2a, although the interaction of intact native E1 and E2 is critical for the formation of the glycoprotein complex, the ectodomain made a greater contribution than did the transmembrane domain[63]. This study suggested that E1 and E2 formed a functional envelope protein complex dependent on E1 and E2 expression[63]. E1 and E2 are assembled as non-covalent heterodimers[82,83], although the number of E1 molecules necessary to aggregate with E2 for biological function has not been elucidated. We highlight this scotoma because the envelope proteins of many viruses do not function simply in a 1:1 ratio[54-58] and viral proteins are not translated in equal numbers[43]. A lack of understanding of this point will impede receptor discovery, regardless of how many receptors and co-receptors are identified[75]. The crystal structure of the dengue virus glycoprotein, which is another member of the *Flaviviridae* family, was revealed in 2004[84]. By contrast, virologists failed to produce the HCV E1 or E2 crystal, which limits our understanding of the biological characteristics of HCV envelope proteins. Since one of the most important biological functions of HCV envelope proteins is membrane fusion, E1 and E2 are assumed to be class Ⅱ fusion proteins[83]. This assumption has been challenged by recent studies suggesting that HCV and pestiviruses share an uncharacterized mechanism of membrane fusion[85]. These contradictory issues are common in HCV research and await further advances in our understanding of HCV virology (Table 2).

Molecular virology of non-structural HCV proteins

HCV proteins can be categorized into an assembly module (from core to NS2) and a replication module (from NS3 to NS5B) on the basis of viral essential functions[27,75]. Details on the function of structural and non-structural HCV proteins are lacking due to the limitations of *in vitro* models. There is also some controversy on topics such as whether the assembly module is necessary for viral particle formation or whether p7 is a structural or non-structural protein. Although the HCV replicon system provided solid evidence that non-structural proteins activate HCV RNA replication *in vitro*[15,16], some unresolved issues remain. These include why it is not possible to turn this system into a fully competent HCV cell culture model or why all replicons, except for the genotype 2a JFH-1 clone, contain cell-culture-adaptive mutations that when introduced back into viral genome, render it non-infectious in chimpanzees[67].

The p7 polypeptide is a small, 63-aa intrinsic membrane protein with a double-membrane-spanning topology in which its N- and C-terminal ends face the ER lumen[27]. Recent data indicate that p7 can mediate membrane ion permeability and form hexamers[86,87]. The three-dimensional structure of a hexameric p7 channel revealed a highly tilted, flower-shaped protein architecture with six protruding petals oriented toward the ER lumen[86,87]. These structural and membrane-permeability

Table 3 Summary of the properties of hepatitis C virus non-structural proteins

	NS2	NS3	NS4A	NS4B	NS5A	NS5B
Genome location	2769-3419	3420-5312	5313-5474	5475-6257	6258-7601	7602-9378
Translation processing site			Rough ER			
Amino acid composition	810-1026	1027-1657	1658-1711	1712-1972	1973-2420	2421-3012
Molecular weight (kDa)	21-23	70	8	27	56-58	65-68
Cleavage	Viral cysteine protease NS2-3 and the serine-type protease activity of the viral NS3-NS4A complex					
Crystal structure	C-terminal (aa904-1026) was solved	Revealed	Revealed	Not available	Revealed	Revealed
Functional unit	Homodimer	Monomer or oligomer	Monomer	Oligomer	Homodimer	Monomer
Common function	Replication module					
Unique function	A metal-dependent proteinase, many functions dependent on the interaction with P7 and NS3. Participation in proteolytic cleavage at the NS2-NS3 junction of the polyprotein. Both the TMDs and protease domain of NS2 are required for the production of virus particles	The DAA targeting protein. NS3 was anchored in ER membrane by cofactor NS4A. NS3-4A complex has serine-type protease activity and NTPase/RNA helicase activities. Nonspecific cleavage of two critical interferon induction proteins: MAVS and TRIF	The central portion of NS4A, residues 21-32, intercalates into NS3 and activates the protease activity by stabilizing this protease subdomain and contributing to the substrate recognition site. The C-terminal acidic portion of NS4A interacts with the NS3 helicase and other HCV proteins and contributes to RNA replication as well as assembly	A master organizer of replication complex formation. NTPase activity? RNA binding?	Produced as multiple phospho-variants. RNA-binding phosphoprotein involved in RNA replication. Phosphorylation of a specific serine residue within the C-terminus by CK Ⅱ α is essential for virus assembly. The interaction of NS5A with the cLD-bound core protein is the key steps in HCV assembly	RNA-dependent RNA polymerase
Major scotomas	How HCV particles are organized? What is the accurate duty of each nonstructural protein in viral lifecycle? How do the nonstructural proteins utilize host cellular factors for its own survival? Why HCV lifecycle is tightly associated with components of LDLs and VLDLs?					

Start co-ordinates based on H77 (accession number, NC_004102). Aa: Amino acid; TMD: Transmembrane domain; CK Ⅱ: Casein kinase Ⅱ; cLD: Cytoplasmic lipid droplet; LDL: Low-density lipoprotein; VLDL: Very-low-density lipoprotein; MAVS: Mitochondrial antiviral signaling protein; TRIF: TIR-domain-containing adaptor inducing interferon; HCV: Hepatitis C virus.

properties suggest that p7 belongs to the viroporin family and could play an important role in viral particle release and maturation[86,87]. However, the role of p7 in calcium and ion metabolism is unknown. Furthermore, HCVpp with or without p7 showed no changes in viral particle formation and pp infectivity[62,63]. A study of the closely related GB virus B, which infects tamarins and has an analogous but larger protein, p13, showed that p13 is processed into two components p6 and p7, and that p6 was dispensable while p7 was essential for infectivity[88].

NS2 is a metal-dependent proteinase, whose functions are dependent on the interaction with p7 and NS3[27]. Although the NS2 protease is dispensable for RNA replication, NS2 participates in proteolytic cleavage at the NS2-NS3 junction of the polyprotein[27]. The transmembrane and protease domains of NS2 are required for infectious virus assembly[89]. Why NS2 is critical for viral particle formation remains unknown and the interactions between NS2 and other structural and non-structural viral proteins to form an unknown viral particle formation network should be explored (Table 3).

NS3 is a 70-kDa multifunctional protein anchored by the cofactor NS4A[27,89]. NS2/NS3 junction cleavage is essential to liberate fully functional NS3 protein[27,89]. NS3-4A is a non-covalent complex, with a serine protease located in the N-terminus (aa 1-180) and an NTPase/RNA helicase in the C-terminus (aa 181-631)[27,89]. The substrate specificity of NS3-4A is low and causes non-specific cleavage of host proteins; *e.g.*, mitochondrial antiviral-signaling (MAVS) and TIR domain-containing adaptor inducing interferon β (TRIF), and thus might impact host IFN response[90]. The central portion of NS4A, residues 21-32, intercalates into NS3 and activates the protease activity by stabilizing this protease subdomain and contributing to the substrate recognition site[27]. The C-terminal acidic portion of NS4A interacts with the NS3 helicase and other HCV proteins and contributes to RNA replication, as well as assembly[27]. The DAAs telaprevir and boceprevir[31-37] are inhibitors targeting the NS3-4A protease that displayed promising effects in clinical trials, indicating that the NS3-4A protease is critical for viral life cycle (Table 3).

NS4B is a poorly characterized hydrophobic 27-kDa protein[27] comprised of a 66-aa N-terminal portion, a 120-aa central portion, and a 70-aa C-terminal portion[91]. Four transmembrane-spanning regions were predicted in the central portion, while the N-terminal portion plays an important role in assembly of a functional replication com-

plex[27,91]. Einav *et al*[92,93] and Thompson *et al*[94] demonstrated that NS4B harbors NTPase activity and has a role in viral assembly.

NS5A is a 447-aa membrane-associated protein that plays an important role in modulating HCV RNA replication and particle formation[91]. NS5A can be detected in basally phosphorylated and hyper-phosphorylated forms with molecular weights of 56- and 58-kDa, respectively[95,96]. NS5A is comprised of four domains: a N-terminal membrane anchor and three domains separated by two low complexity sequences[27,91]. The three separated domains are domain 1, aa 36-213; domain 2, aa 250-342 and domain 3, aa 356-447. Domains 1 and 2 are involved in RNA replication and domain 1 is involved in cellular lipid drop binding, domain 3 is essential for viral assembly and is involved in interaction with the core protein accumulated in cellular lipid drops[27,91]. Although studies showed that NS5A is critical for HCV RNA replication, deletions in D2 and D3 are tolerated in RNA replication[97], and viable replicons and viruses harboring GFP insertions displayed no change on HCV RNA replication[98]. Phosphorylation of a specific serine residue within C-terminal by casein kinase Ⅱα is essential for virus assembly[99]. The interaction of NS5A with the cytosolic lipid droplets-bound core protein is a key step in HCV assembly[97,100,101].

NS5B is an RNA-dependent RNA polymerase (RdRp). Its crystal structure was revealed in 1999[102]; the active site is highly conserved and located in the palm subdomain[91]. The low substrate specificity allows for the incorporation of ribavirin into nascent RNA. Thus, ribavirin remains a perfect RNA analog in HCV therapy[103]. Although recombinant NS5B is available and its crystal structure is known[104,105], its role in HCV RNA replication remains unclear (Table 3).

5'-non-translated regions and 3' non-translated regions

Viral non-translated regions (NTRs) and non-coding regions, harbor important biological functions, involving viral genome reorganization, replication, translation initiation, and viral assembly[106]. The HCV 5'NTR contains 341 bp (H77 strain, NCBI Reference Sequence: NC_004102.1)[107]. The predicted secondary structure of the HCV 5'NTR consists of four domains (domains Ⅰ-Ⅳ, numbered from 5' to 3'), and the largest domain Ⅲ was further categorized into sub-domains a-f[107]. The major functional unit in the HCV 5'NTR is an IRES, which includes three domains (Ⅱ-Ⅳ)[106-109]. Initiation of protein synthesis in host cells utilized by HCV is different from mRNA translation in eukaryotes because HCV initiates viral protein synthesis *via* its IRES, which is known as internal translation initiation. This process is a cap-independent mechanism of recruiting, positioning and activating the host cellular protein synthesis machinery driven by the HCV IRES[106-109], which is relatively weak in directing protein translation compared to the IRESs of other viruses, and may contribute to an insufficient host immune response[110]. Although structural and biochemical studies of the IRES found in HCV have provided the most detailed information thus far regarding the mechanism of IRES driven translation, unresolved issues remain. For example, it is unknown whether the HCV IRES acts as one determining factor for hepatotropism or how the HCV 5'NTR interacts with the 3'NTR to support HCV RNA replication and polyprotein translation. Additionally, the biological impact of the NTR to each hepatitis virus remains unclear since the HCV NTRs have a different structure compared to those of hepatitis A and E viruses. Finally, the interaction of miR-122 with the HCV 5'NTR to facilitate replication of viral RNA remains to be fully elucidated[111].

The 3' terminal of any genome is technically difficult to identify and the available complete sequence of the HCV 3'NTR is unusual. The 3' UTR is divided into three structurally distinct domains from 5' to 3', an upstream variable region of about 40 nucleotides, a long poly (U)-poly (U/UC) tract and a 98-nucleotide (3' X) sequence that forms three stem-loop structures[43,106]. The long poly (U)-poly (U/UC) tract was a major obstacle to obtaining an HCV genomic clone because no known DNA polymerase could amplify this region and the fidelity of a reverse transcriptase in this region was suspect. The function of the 3' UTR remains to be determined[43,106]. It may play an important role in minus intermediate RNA and genome RNA synthesis during HCV RNA replication[43,106] since variable region deletions of RNA replicons could replicate, albeit at a much lower level[112]. However, deletion of either the poly (U/UC) or the 3' X was not viable, which suggested that the poly (U/UC) and 3'X regions are critical for HCV RNA replication[107].

HuH7 cell line

HCV researchers should be familiar with the human hepatocellular carcinoma cell line HuH-7, also known as Huh7 or HuH7. This cell line is critical because the HCV replicon, HCVcc, and HCVpp are all dependent on this cell line or its derivatives, indicating that it harbors all critical factors for HCV replication, assembly, budding and entry[43,113-115]. HuH-7 is a well-differentiated hepatocyte-derived hepatocellular carcinoma cell line that originated from a liver tumor in a 57-year-old Japanese male in 1982 (http://huh7.com/). It was established by scientists at Okayama University of Japan in the 1980s (http://cellbank.nibio.go.jp/legacy/celldata/jcrb0403.htm). HuH-7 remains the only hepatocellular carcinoma cell line that can fully support the HCV life cycle. Improvements in hepatocellular carcinoma cell line isolation could provide more effective HCV-supporting cell lines; alternatively the advances in induced pluripotent stem cells could result in a breakthrough in HCV culture and isolation.

SCOTOMAS IN EPIDEMIOLOGY OF HCV

HCV carries a large disease burden in some countries and is the second most studied virus. Most HCV infections are subclinical with a long and insidious disease

Table 4 Epidemiological features of hepatitis C virus infection

Epidemiological index	Current consensus
Source of infection	Chronic HCV carriers
Route of transmission	HCV transmission occurs primarily through exposure to infected blood. Past: Receiving infected blood or organ transplantation, from accidental exposure to infected blood, and sexual transmission in persons with high risk behaviours. Present: HCV is usually spread by sharing infected needles with a chronic HCV carrier, and some people acquire the infection through nonparenteral means that have not been fully defined.
Susceptible population	General population
Incubation period	Average 6-10 wk
Prevalence and incidence	3% of the world's population have HCV
Rate of chronic infection	Up to more than 80%
Outcome of chronic infection	10%-20% of chronic HCV carriers may develop into cirrhosis and liver failure. 1%-5% of chronic HCV carriers are associated with the development of hepatocellular carcinoma
Molecular epidemiology	HCV is classified into eleven major genotypes (designated as 1-11), many subtypes (designated a, b, c, *etc.*), and about 100 different strains (numbered 1, 2, 3, *etc.*) based on the genomic sequence heterogeneity. Genotypes 1-3 have a worldwide distribution. Types 1a and 1b are the most common, accounting for about 60% of global infections. Type 2 is less frequently represented than type 1. Type 3 is endemic in southeast Asia and is variably distributed in different countries. Genotype 4 is principally found in the Middle East, Egypt, and central Africa. Type 5 is almost exclusively found in South Africa, and genotypes 6-11 are distributed in Asia.
Stability	HCV is inactivated by exposure to lipid solvents or detergents, heating at 60 ℃ for 10 h or 100 ℃ for 2 min in aqueous solution, formaldehyde (1:2000) at 37 ℃ for 72 h, β-propriolactone and UV irradiation.
Vaccine	Not available

HCV: Hepatitis C virus.

course. However, epidemiological surveillance of HCV is relatively weak compared to some acute respiratory transmission diseases (http://www.who.int/influenza/surveillance_monitoring/en/).

Chronic HCV carriers are the only reservoir of HCV since chimpanzees could be infected with HCV only experimentally[43,106]. HCV transmission occurs primarily through exposure to HCV-infected blood[43,106]. Blood transfusion, solid organ transplantation from an infected donor, and unsafe medical practices were the major transmission routes before HCV was identified in 1989[43,106]. Beginning in the early 1990s, strict screening of blood donors and precise control over the blood supply were implemented by national governments[43,106,116]. The majority of HCV infections are now limited to specific subpopulations, such as intravenous drug users and patients with certain hemopathies[117]. Although unsafe medical practices, occupational exposure to infected blood, maternal-fetal transmission, sex with an infected person and high-risk sexual practices are believed to be HCV transmission routes, the rate of acquisition of infection by these routes is low[118]. The average incubation period is 6-10 weeks and most virologists and hepatologists consider that up to 80% of HCV infected persons do not eliminate HCV spontaneously[43,106]. Cirrhosis and liver failure develop in 10%-20% of chronic HCV carriers; 1%-5% of chronic HCV carriers develop hepatocellular carcinoma[43,106] (Table 4).

The World Health Organization estimates that up to 3% of the global population is infected with HCV (http://www.who.int/csr/disease/hepatitis/Hepc.pdf), and the peak disease burden is expected around 2020[119]. However, these estimations lack sufficient evidence. Firstly, it is hard to track the origin of the data since most authors citing this statistic used inaccurate citations. Secondly, the HCV serological detection kit has undergone at least three iterations[117]. The first test developed in 1990 detected antibody to a single epitope within the core protein by enzyme linked immunosorbent assay and provided data on 170 million HCV carriers, even though it was plagued by poor sensitivity[43]. Third-generation enzyme immunoassays included antibodies against multiple antigens, which increased the sensitivity significantly[43], although no large-scale serological investigations have been performed. In China, a nationwide HCV serological survey performed in 2006 showed the prevalence of anti-HCV antibodies to be < 0.5% among more than 80000 Chinese subjects[116]. Furthermore, the rates of HCV were much lower than those of hepatitis B among clinical inpatient and outpatient populations, which was significantly different from a Japanese population[119,120]. Epidemiology is important because it will provide basic knowledge of disease and inaccurate epidemiological data will lead to inaccuracies in our knowledge of the disease burden, natural history and therapeutic efficacy.

The number of people that will become chronic carriers after HCV infection remains unknown. Scientists believe that as many as 40%-80% of HCV infections will develop into chronic infections[121-123] (Table 3). While these estimates are also likely inaccurate, how and when people are infected must be determined to ascertain a more precise figure. One recent cross-sectional study performed in intravenous drug users challenged the current assumptions regarding the rate of chronic infection; in that study as many as 77.8% of individuals cleared HCV infection without the need for anti-viral therapy[117].

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