Review Article

Of lives and livers: emerging responses to the hepatitis C virus

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Abstract

Hepatitis C is an infectious disease affecting the liver, caused by the hepatitis C virus (HCV). HCV is an etiological agent of acute and chronic liver disease that exists throughout the world. The high genetic variability of the HCV genome is reflected by six genotypes (1 to 6). Each genotype has a characteristic geographical distribution, which is important epidemiologically. HCV is a blood-borne virus that generally circulates in low titers in the serum of infected individuals. Epidemiologic studies show that the most efficient transmission of HCV is through the transfusion of blood or blood products, the transplantation of organs from infected donors, and the sharing of contaminated needles among injection-drug users. However, fewer than half of patients with acute hepatitis C report a history of such exposure. A small number of epidemiologic studies demonstrate that perinatal, sexual, household, and occupational transmission occurs, but our understanding of the risks of transmission in these settings has been limited. The therapy for chronic hepatitis C has evolved steadily since alpha interferon was first approved for use. At present, the optimal regimen appears to be a 24- or 48-week course of a combined pegylated alpha interferon and ribavirin regimen. Currently, the combination of RNAi (LV-shIRES) with IFN-α has been proposed to prevent therapeutic resistance, and to promote enhanced antiviral activity against HCV. However, any RNAi based therapy may be years away due to off-target effects.

Key words: HCV; pathogenicity; genomic islands; PCR; virulence factors


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Introduction

The liver and its function

The liver is the largest glandular organ of the human body and it is also the only organ that is able to regenerate to form new tissue. It is located in the upper right quadrant of the abdominal cavity just beneath the diaphragm and is comprised of four unequally sized lobes and two ligaments for support. Its highly vascular nature gives it a fleshy red coloring. The hepatic artery is a short vessel used to carry blood containing oxygen to the liver from the aorta. This vessel provides the liver with the blood needed to perform its numerous functions. The portal vein transfers blood from the small intestine of the digestive system into the liver for further metabolic processes and/or filtration. These two vessels continuously branch off in the liver to reach small hepatic capillaries. These capillaries then lead toward structures called lobules which are made up of hepatic cells, the basic metabolic cells of the liver.

The liver is responsible for numerous tasks that are necessary for the body to function properly, including: the detoxification of harmful chemicals, such as alcohol, from the blood; the storage of vitamins, such as vitamins A, D, K, and B12, and many minerals; the synthesis of carbohydrates; and the conversion of the foremost substance found in urine, ammonia, into urea. Without the liver a person would not be able to maintain the proper levels of glucose in the blood needed to produce the key amino acids required to make proteins, convert glucose to glycogen, and generate cholesterol.

Hepatitis

The word “hepatitis” means inflammation of the liver. Toxins, certain drugs, some diseases, heavy alcohol use, and bacterial and viral infections can all cause hepatitis. Hepatitis is also the name of a family of viral infections that affect the liver. Viruses that cause hepatitis are types A, B, C, D, E, and G. Most common in the United States are hepatitis A, hepatitis B, and hepatitis C (HCV) (Centers for Disease Control and Prevention; http://www.cdc.gov). The infection caused by HCV is often asymptomatic, but
once established, chronic infection can progress to scarring of the liver (fibrosis) and advanced scarring (cirrhosis), which is generally apparent only after many years. In some cases, individuals with cirrhosis go on to develop liver failure or other complications of this advanced scarring, including liver cancer.

In the mid-1970s, HCV was known as non-A non-B hepatitis because the inflammatory disease was not associated to either hepatitis A viral (HAV) or hepatitis B viral infections (HBV) [1]. Further studies showed that the non-A non-B hepatitis agent was less than 80 nm in size and was likely to be a virus [2]. In 1983, Bradley et al. [3] discovered that the agent in question was chloroform sensitive and thus was an enveloped virus. Then in 1987, under the direction of Dr. Daniel W. Bradley of the Centers for Disease Control (CDC; Atlanta, Georgia, USA) and Dr. Michael Houghton of Chiron Corporation (California, USA), researchers identified the hepatitis C virus using amino acid sequencing of the viral RNA [4].

HCV is the only known member of the Hepacivirus genus (of the family Flaviviridae) that causes hepatitis. Other members of this family include viruses that cause yellow fever, dengue, Japanese encephalitis, tick-borne encephalitis, and the GBV-C virus, which apparently is non-pathogenic.

**Epidemiology**

An estimated 170 million individuals have chronic HCV infection worldwide [5]. About 70% of infected individuals develop a chronic infection; for some, this includes fibrosis, cirrhosis, and hepatocellular carcinoma [6]. Approximately 10,000 deaths due to cirrhosis, and several thousand more deaths due to hepatocellular carcinoma, are attributed to HCV infection in the United States each year [7]. The World Health Organization (WHO) estimates that about 200 million people, or three percent of the world's population, are infected with HCV, and that between three and four million individuals are newly infected each year, which leaves a global total of 170 million chronic carriers at risk for developing liver cirrhosis and/or liver cancer [7]. Hence HCV infections account for a substantial proportion of all liver diseases worldwide.

**HCV mode of transmission**

Hepatitis C virus is transmitted through the transfusion of blood or blood products, through organ transplantation from infected donors, and through the sharing of contaminated needles among injection drug users [8]. HCV is less efficiently transmitted by a single small dose to percutaneous exposures (e.g., accidental needle sticks) [8], or by mucosal exposures to blood or serum-derived fluids (e.g., at birth from an infected mother or sex with an infected partner) [8]. Although the spread of HCV infection through a non-transfusion route is becoming increasingly common, injection drug use is now the identified risk factor in more than 60% of cases [9].

The transmission of HCV is relatively low through three possible routes: occupational, perinatal, and high-risk sexual exposures. Occupational transmission of HCV infection is mainly confined to health-care workers who have sustained contaminated needle stick injuries. The average incidence of anti-HCV seroconversion from an HCV-positive source is 1.8%, and transmission has been associated with hollow-bore needles and deep injuries [10]. Transmission rarely occurs from mucous membranes or from non-intact skin exposures to blood [11]. No transmission to health-care workers from intact skin exposures to blood has been reported.

Perinatal transmission rate of HCV is 4% to 7% per pregnancy and occurs only when HCV RNA is detectable in maternal serum at delivery. Transmission may be related to higher levels (above 10^6 copies per mL), although data have been inconsistent with regard to the effect of viral concentration [12]. Prolonged labor after membrane rupture, and internal fetal monitoring have been associated with perinatal infection [12,13]. No reports have linked HCV transmission to either vaginal or caesarian section delivery, or to breastfeeding.

Studies of sexual and household transmission of HCV have yielded conflicting results [9]. The strongest evidence for heterosexual activity as a risk factor for HCV infection has come from case-control studies of individuals with acute non-A non-B hepatitis (now known as hepatitis C) in the United States during the 1970s and 1980s, which identified sex with an infected partner or with multiple partners as independently associated with acquiring HCV infection [14]. Since then, 15% to 20% of acute hepatitis C cases have reported no other risk factor except one of these sexual exposures. In contrast, no association has been found with male homosexual activity. Moreover, cross-sectional studies conducted since 1990 of men who have sex with men (MSM), and of heterosexual individuals in long-term
monogamous relationships with a partner with chronic HCV, have found little evidence for sexual transmission of HCV [8].

Chronic haemodialysis is associated occasionally with sporadic outbreaks of HCV infection. The rate of anti-HCV in patients on haemodialysis ranges from 10% to 20% [15]. A correlation has been found between dialysis and anti-HCV positivity, suggesting that HCV is transmitted in dialysis units by inadequate infection control procedures [9].

There are other biologically plausible modes of transmission of HCV, including cosmetic procedures (e.g., tattooing, body piercing), intranasal drug use, and religious or cultural practices (e.g., ritual scarification, circumcision, acupuncture, and cupping). In most regions of the world, there are insufficient data to determine whether these risk factors make any measurable contribution to overall HCV transmission. In those countries where adequate studies have been conducted, none of these activities have been reported to be consistently associated with HCV transmission [16].

Structure and genomic organization

The hepatitis C virus is about 50-60 nm in size [17]. Besides having an outer coat (envelope), the virus also contains enzymes and proteins that allow the virus to replicate within the cells of the body, particularly within liver cells. The envelope is made of a lipid bilayer in which two envelope proteins, E1 and E2, are anchored. The envelope surrounds the nucleocapsid, composed of multiple copies of a small basic protein (core or C), and contains the RNA genome. The genome is approximately 9.6 kb in length, and is a single positive-sense strand [4] with a single open reading frame (ORF) encoding a polyprotein of about 3,000 amino acids. The ORF is flanked in 5' and 3' untranslated regions (UTRs) of 95-555 and 114-624 nucleotide in length, respectively. Both UTRs bear highly conserved RNA structures that are essential for both polyprotein translation and genome replication [18]. The HCV ORF contains 9,024 to 9,111 nt depending on the genotype. The ORF encodes at least 11 proteins, including three structural proteins (C or core, E1 and E2), a small protein (p7), six nonstructural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B), and the so-called “F” protein that results from a frameshift in the core coding region.

The polyprotein precursor is co-translationally and post-translationally processed by both cellular and viral proteases at the endoplasmic reticulum membrane to yield 10 mature proteins [19]. Included in the structural proteins are the core, which forms the viral nucleocapsid, and the envelope glycoproteins E1 and E2. These glycoproteins are released by host cell signal peptidases. The short membrane peptide p7, thought to be a viroporin, separates the structural proteins from the non-structural proteins. The non-structural (NS) proteins NS2 to NS5B are involved in polyprotein processing and viral replication. The proteolytic processing of NS polyproteins is complex, and requires two distinct proteinases: the NS2-NS3 zinc dependent metalloproteinases, and the NS3 serine proteinase located in the N-terminal region of NS3 [20]. The remaining NS proteins are released by the NS3 proteinase associated with the cofactor NS4A. The C terminal region of the NS3 protein includes RNA helicase and NTPase activities. NS5A is a polyphosphorylated protein of unknown function, and NS5B is the RNA dependent RNA polymerase (RdRp). The structural organization of the HCV genome is outlined in detail below.

5' untranslated region (UTR)

The HCV 5' UTR contains 341 nucleotides (nt) located upstream of the ORF translation initiation codon. It is the most conserved region of the genome [4]. The 5' UTR contains four highly structured domains, numbered I to IV, that contain numerous stem-loops, and a pseudoknot [21]. Domains II, III and IV, together with the first 12 to 30 nt of the core-coding region, constitute the internal ribosomal entry site (IRES) [22]. Structural characterization by electron microscopy (EM) indicates that domains II, III and IV form distinct regions within the molecule, with a flexible hinge between domains II and III [23]. The HCV IRES has the capacity to form a stable pre-initiation complex by directly binding the 40S ribosomal subunit without the need of canonical translation initiation factors, an event that likely constitutes the first step of HCV polyprotein translation [24].

3' untranslated region

The 3' UTR of HCV contains approximately 225 nt. It is organized in three regions, including, from 5' to 3', a variable region of approximately 30-40 nt, a long poly(U)-poly(U/UC) tract, and a highly conserved 3'-terminal stretch of 98 nt (3' X region) that includes three stem-loop structures (SL1, SL2 and SL3) [25]. The 3' UTR interacts with the NS5B RdRp, and with two of the four stable stem-loop
structures located at the 3’ end of the NS5B-coding sequence [26]. The 3’ X region and the 52 upstream nt of the poly(U/C) tract were found to be essential for RNA replication, whereas the remaining sequence of the 3’UTR appears to enhance viral replication [27].

Core protein

The HCV core protein is a highly basic, RNA-binding protein, which presumably forms the viral capsid. The HCV core protein is released as a 191 amino acid (aa) precursor of 23-kDa (P23). The core protein contains three distinct predicted domains: an N-terminal hydrophilic domain of 120 aa (domain D1), a C-terminal hydrophilic domain of about 50 aa (domain D2), and the last 20 or so aa that serve as a signal peptide for the downstream envelope protein E1 [28]. Domain D1 is involved in RNA binding and nuclear localization, as suggested by the presence of three predicted nuclear localization signals (NLS) [29]. Domain D2 is responsible for core protein association with endoplasmic reticulum (ER) membranes, outer mitochondrial membranes, and lipid droplets [29]. Besides its role in viral capsid formation, evidence suggests that the core protein directly interacts with a number of cellular proteins and pathways that may be important in the viral life cycle [30]. Remarkably versatile, the HCV core protein has pro- and anti-apoptotic functions [31], stimulates hepatocyte growth in the Huh-7 cell line by transcriptional up-regulation of growth-related genes [32], and has been implicated in tissue injury and fibrosis progression [33]. The core protein can also regulate the activity of cellular genes, including c-myc and c-fos, and alter the transcription of other viral promoters [34]. It induces both hepatocellular carcinoma, when expressed in transgenic mice [35], and lipid droplet formation. It may also play a direct role in steatosis formation [35].

E1 and E2 envelope glycoproteins

The two envelope glycoproteins, E1 and E2, are essential components of the HCV virion envelope, and are necessary for viral entry and fusion [36]. E1 and E2 have molecular weights of 33-35 and 70-72 kDa, respectively, and assemble as non-covalent heterodimers [37]. E1 and E2 are type I transmembrane glycoproteins, with N-terminal ectodomains of 160 and 334 aa, respectively, and a short C-terminal transmembrane domain of approximately 30 aa. The E1 and E2 transmembrane domains are composed of two stretches of hydrophobic aa separated by a short polar region containing fully conserved charged residues. They have numerous functions, including membrane anchoring, ER localization, and heterodimer assembly [38]. The ectodomains of E1 and E2 contain numerous proline and cysteine residues, but intramolecular disulfide bonds have not been observed [39]. E1 and E2 are highly glycosylated, containing up to 5 and 11 glycosylation sites, respectively. In addition, E2 contains hypervariable regions with aa sequences differing up to 80% between HCV genotypes and between subtypes of the same genotype [40]. Hypervariable region 1 (HVR1) contains 27 aa and is a major, but not the only, HCV neutralizing epitope [41]. Despite the HVR1 sequence variability, the physicochemical properties of the residues at each position and the overall conformation of HVR1 are highly conserved among all known HCV genotypes, suggesting an important role in the virus lifecycle [42]. E2 plays a crucial role in the early steps of infection. Viral attachment is thought to be initiated via E2 interaction with one or several components of the receptor complex [43]. Because HVR1 is a basic region with positively charged residues located at specific sequence positions, it can theoretically interact with negatively charged molecules at the cell surface. This interaction plays a role in host cell recognition and attachment, as well as in cell or tissue compartmentalization [44]. Less is known about E1, but it is thought to be involved in intra-cytoplasmic virus-membrane fusion [43].

Frameshift protein

The F (frameshift) protein or ARFP (alternate reading frame protein) is generated as a result of a -2/+1 ribosomal frameshift in the N-terminal core-encoding region of the HCV polyprotein. Antibodies to peptides from the F protein were detected in chronically infected patients, suggesting that the protein is produced during infection [45]. However, the exact translational mechanisms governing the frequency and yield of the F protein during the various phases of HCV infection are completely unknown. Thus the role of F protein in the HCV lifecycle remains enigmatic, but it has been postulated that the protein contributes to viral persistence [46].

Non-structural proteins

p7: A small (63 aa) polypeptide, p7 has been shown to be an integral membrane protein [47]. It
comprises two transmembrane domains that are organized in α-helices and connected by a cytoplasmic loop. A study has shown that p7 is essential because mutations or deletions in its cytoplasmic loop have suppressed infectivity of intra-liver transfection of HCV cDNA in chimpanzees [48]. In vitro studies suggest that p7 belongs to the viroporin family and acts as a calcium ion channel [49], but these results have yet to be confirmed in vivo.

**NS2**: NS2 is a non-glycosylated transmembrane protein of 21-23 kDa. It contains two internal signal sequences at aa positions 839-883 and 928-960, which are responsible for ER membrane association [50]. NS2, together with the amino-terminal domain of the NS3 protein, the NS2-3 protease, constitutes a zinc-dependent metalloprotease that cleaves the site between NS2 and NS3 [51]. 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 a protein kinase (PK) known as casein kinase 2 [52]. In addition to its protease activity, NS2 can interact with host cell proteins, such as the liver-specific pro-apoptotic cell death-inducing DFF45-like effector (CIDE-B), and affect reporter genes controlled by liver and non-liver-specific promoters and enhancers [53]. However, the consequences of such interactions within the context of the HCV life cycle are not clear.

**NS3-NS4A**: NS3 is a multi-functional viral protein containing a serine protease domain in its N-terminal third, and a helicase/NTPase domain in its C-terminal two-thirds. NS4A is a cofactor of NS3 protease activity. NS3-4A also interacts with host cell pathways and proteins that may be important in the lifecycle and pathogenesis of infection. Not surprisingly, the NS3-NS4A protease is one of the most popular viral targets for anti-HCV therapeutics [54].

**NS3-NS4A protease**: The NS3-NS4A protease is essential for the HCV lifecycle. It catalyzes HCV polyprotein cleavage at the NS3/NS4A, NS4A/NS4B, NS4B/NS5A, and NS5A/NS5B junctions. The 3D structure has been determined for the NS3 serine protease domain complexed with NS4A [55]. A catalytic triad is formed by residues His 57, Asp 81, and Ser 139 [56]. The central region of NS4A (aa 21-30) acts as a cofactor of NS3 serine protease activity, which facilitates its stabilization, its localization at the ER membrane, and its cleavage-dependent activation, particularly at the NS4B/NS5A junction [57].

HCV NS3-NS4A was shown *in vitro* to antagonize the dsRNA-dependent interferon regulatory factor 3 (IRF-3) pathway, an important mediator of interferon induction in response to a viral infection [58]. NS3-NS4A also appears to prevent dsRNA signaling via the toll-like receptor 3 upstream of IRF-3 [59]. One potential mechanism includes a blockade of the intracellular double-stranded RNA sensor protein (RIG-I) pathway by NS3-NS4A [60]. Thus HCV could utilize NS3-4A protease to circumvent the innate immune response at the early stages of infection. In addition, NS3 was also reported to induce malignant transformation of NIH3T3 cells (a murine embryonic fibroblastic cell line) [61], to suppress actinomycin D-induced apoptosis in murine cell lines [62], and to be involved in hepto-carcinogenesis events [63].

**NS3 helicase-NTPase**: The NS3 helicase-NTPase domain consists of the 442 C-terminal aa of the NS3 protein, and is a member of the helicase superfamily-2. The NS3 helicase-NTPase has several functions, including RNA-stimulated NTPase activity, RNA binding, and unwinding of RNA regions of extensive secondary structure by coupling unwinding and NTP hydrolysis [64]. It has been suggested that during RNA replication, the NS3 helicase translocates along the nucleic acid substrate by changing protein conformation and utilizing the energy of NTP hydrolysis. One study proposed that the helicase directional movement step is fueled by single-stranded RNA binding energy, while NTP binding allows for a brief period of random movement that prepares the helicase for the next cycle [65]. In addition, NS3 helicase activity appears to be modulated by the NS3 protease domain and the NS5B RdRp [66].

**NS4B**: NS4B is an integral membrane protein of 261 aa with an ER or ER-derived membrane localization [67]. It is predicted that NS4B harbors at least four transmembrane domains, and an N-terminal amphipathic helix, that are responsible for membrane association [68,67]. One of the functions of NS4B is to serve as a membrane anchor for the replication complex [68]. Additional putative properties include inhibition of cellular synthesis [69], modulation of HCV NS5B RdRp activity [70], transformation of NIH3T3 cell lines [71], and induction of interleukin 8 [72].

**NS5A**: NS5A is a 56-58 kDa phosphorylated zinc-metalloprotein that probably plays an important
role in virus replication and in the regulation of cellular pathways. The N-terminal region of NS5A (aa 1-30) contains an amphipathic α-helix that is necessary and sufficient for membrane localization in perinuclear membranes, and for assembly of the replication complex [73]. Downstream of this motif, the NS5A protein has been predicted to contain three domains, numbered I to III. Domain I, located at the N-terminus, contains an unconventional zinc-binding motif formed by four cysteine residues conserved among the hepacivirus and pestivirus genera [74]. HCV replicon RNA replication was inhibited by mutations in the NS5A sequence [73], and abolished by alterations of the zinc-binding site [74]. The 3-D structure of domain I suggests the presence of protein, RNA, and membrane interaction sites [75].

The mechanisms by which NS5A regulates HCV replication are not entirely clear. It is suggested that NS5A associates with lipid rafts derived from intracellular membranes through its binding to the C-terminal region of a vesicle membrane-associated protein of 33 kDa (hVAP-33) [76]. This interaction appears to be crucial for the formation of the HCV replication complex in connection with lipid rafts [77]. A study on the replicon system proposed a model in which NS5A hyperphosphorylation disrupts the interaction with hVAP-33 and negatively regulates viral RNA replication [78]. Another report has suggested that the level of NS5A phosphorylation plays an important role in the viral lifecycle by regulating a switch from replication to assembly, whereby hyperphosphorylated forms function to maintain the replication complex in an assembly-incompetent state [79]. Furthermore, NS5A can interact directly with NS5B, but the mechanism by which NS5A modulates the RdRp activity has not yet been elucidated [80]. In addition, NS5A has been reported to interact with a geranylgeranylated cellular protein [81]. This is potentially significant because assembly of the viral replication complex has been shown to require geranylgeranylation of one or more host cell proteins [82].

Multiple functions have been assigned to NS5A based on its interactions with cellular proteins [83]. For instance, NS5A plays a role in interferon resistance by binding to and inhibiting PKR, an antiviral effector of interferon-α [84]. NS5A also bears transcriptional activation functions [85], and appears to be involved in the regulation of cell growth and cellular signaling pathways [83].

**NS5B RNA dependent RNA polymerase (RdRp):** NS5B belongs to a class of membrane proteins termed tail-anchored proteins [86]. Its C-terminal region (21 residues) forms an α-helical transmembrane domain responsible for post-translational targeting to the cytosolic side of the ER, where the functional protein domain is exposed [84]. The crystal structure of NS5B revealed that the RdRp has a classical “fingers, palm and thumb” structure formed by its 530 N-terminal aa [87]. Interactions between the fingers and thumb subdomains result in a completely encircled catalytic site that ensures synthesis of positive- and negative-strand HCV RNAs [85]. The RdRp is another important target for the development of anti-HCV drugs [88,54].

Interactions between NS5B and cellular components have also been reported. The C-terminus of NS5B can interact with the N-terminus of hVAP-33, and this interaction plays an important role in the formation of the HCV replication complex [77,86]. NS5B has also been found to bind cyclophilin B, a cellular peptidyl-prolyl cis-trans isomerase that apparently regulates HCV replication through modulation of the RNA binding capacity of NS5B [89].

**HCV lifecycle**

**Attachment and cell entry**

The first step in any viral lifecycle is the attachment of the infectious particle to the host cell, and specific interactions between a receptor on the host cell surface and a viral attachment protein on the surface of the particle. CD81 was identified as a hepatitis C virus receptor because of its strong interaction with E2 and with viral particles in vitro [90]. Human CD81 (the target of antiproliferative antibody 1, TAPA-1) is a 25 kDa molecule that belongs to the tetraspanin or transmembrane 4 superfamily. It is found at the surface of numerous cell types, where it is thought to assemble as homodimers or heterodimers by means of a conserved hydrophobic interface. Apart from this route, HCV may enter the cell by the scavenger receptor class B type I (SR-BI) receptor [91] and the low-density lipoprotein (LDL) receptor [92]. E1 is involved in membrane fusion [93]. E2 acts as a chaperone for E1, which in the absence of E2 forms misfolded aggregates [94].

**Polyprotein translation and processing**

Once inside the cytoplasm, the genomic HCV RNA is directly translated. Translation of the viral RNA is not mediated by a cap dependent mechanism
but rather by internal ribosomal entry site (IRES) [95]. The activity of HCV IRES is influenced by several factors. First, the X-tail at the 3’ end of the HCV genome appears to enhance IRES-dependent translation [96]. Second, several cellular factors have been observed to bind to the HCV IRES to stimulate translation in the polypyrimidine-tract-binding protein [97], the La antigen [98], the heterogeneous nuclear ribonucleoprotein L [99], and other unidentified proteins. Directed by the IRES, the polyprotein is translated at the rough endoplasmic reticulum, and cleaved co- and post-translationally by host cell signalases and two viral proteinases. Hydrophobic sequences precede the cleavage sites and there is a dependence upon microsomal membranes. The C-NS2 region is processed by the host signal peptidases that cleave at the C/E1, E1/E2, E2/p7, and p7/NS2 junctions [51]. The processing between NS2 and NS3, via a rapid intramolecular reaction, is accomplished by the NS2-3 proteinase. The zinc that is bound by the NS3 domain plays an essential role in the catalysis [100]. The HCV proteins most likely form a stable higher-order complex associated with intracellular membranes. Although enzymatically active on its own, proteolytic activity of NS3 is greatly stimulated by NS4A [100].

**RNA replication**

NS5B RdRp catalyzes the synthesis of minus- and plus-strand RNA. Sequences at the 3’ end fold back intramolecularly and hybridize, which generates a 3’ end that can be used for elongation [101]. Using high concentrations of GTP or ATP, NS5B can synthesize RNA primers [102]. Initiation of RNA replication is triggered by an interaction between proteins of the replication complex, the 3’ X region of the 3’ UTR, and 5BSL3.2. The latter is a cis-acting replication element consisting of 50 bases, located in a large predicted cruciform structure at the 3’ end of the HCV NS5B-coding region [103], which forms a pseudoknot structure with a stem-loop in the 3’ UTR [103]. In addition, a phosphorylated form of polypyrimidine tract-binding protein (PTB) has been found in the replication complex, and shown to interact with two conserved stem-loop structures of the 3’ UTR, in an interaction thought to modulate RNA replication [104]. Importantly, inhibition of PTB expression by means of small interfering RNAs reduces the amount of HCV proteins and RNA in HCV replicon-harbor Huh7 cells [105].

**Virus assembly and release**

It has been suggested that particle formation may be initiated by the core proteins interacting with the RNA genome. Different variants of the HCV core protein, which can exist as dimeric, and probably multimeric forms as well, have been shown to be capable of self-assembly in yeast in the absence of viral RNA, and of generating virus-like particles with an average diameter of 35 nm [105]. Recent reports suggest that the N-terminal portion of the core protein is sufficient for capsid assembly, in particular the two clusters of basic residues [106]. In bacterial systems, researchers have observed that HCV core proteins efficiently self-assemble to yield nucleocapsid-like particles with a spherical morphology and a diameter of 60 nm, but the presence of a nucleic acid is required [107]. Hence, overall, particle formation is probably initiated by the interaction of the core protein with genomic RNA. The HCV core can indeed bind positive-strand RNA in vitro through stem-loop domains I and III, and nt 23-41 [108]. Virus-like particles were produced in mammalian cells by using a chimeric virus replicon that allowed high-level expression of HCV structural proteins in BHK-21 cell lines [109]. The budding of virus-like particles of 50 nm in diameter in the dilated ER lumen was observed [110]. Transfection of full-length HCV RNA in HeLa G and HepG2 cell lines led to the formation of virus-like particles with a diameter of 45 to 60 nm, which were synthesized and assembled in the cytoplasm and budded into the ER cisternae to form coated particles [111,112]. The HCV envelope glycoproteins E1 and E2 associate with ER membranes through their transmembrane domains [113], which implies that virus assembly occurs in the ER. The presence of N-glycan residues on the surface of HCV particles suggests that the HCV transits via the Golgi apparatus. Structural proteins have been detected both in the ER and the Golgi apparatus, which suggests that both compartments are involved in later maturation steps. A distinctive property of the HCV E proteins in their position in the ER compartment indicates that viral nucleocapsids acquire their envelopes by budding through ER membranes, and in this case suggests that the virus may be exported via the constitutive secretory pathway [114].
HCV Genotypes

Different isolates of HCV show substantial nucleotide sequence variation distributed throughout the genome. Regions encoding the envelope proteins are the most variable, whereas the 5′ non-coding region (NCR) is the most conserved [115]. Because it is the most conserved with minor heterogeneity, several researchers have considered the 5′ NCR to be the region of choice for virus detection by reverse transcription (RT)-PCR. Sequence analysis performed on isolates from different geographical areas around the world has revealed the presence of different genotypes, labeled 1 through 6 [115]. HCV genotypes display significant differences in their global distribution and prevalence, making genotyping a useful method to determine the source of HCV transmission in an infected localized population, as well as a helpful technique to assess virulence, and to assist with treatment [116].

Molecular epidemiological studies have shown marked differences in HCV genotype distribution by geographical distribution. Genotypes 1, 2, and 3 are widely distributed throughout the USA, Europe, Australia, and East Asia (Japan, Taiwan, Thailand, and China), whereas geographical distributions of other genotypes are more restricted [117-119]. Genotype 4 is largely confined to the Middle East, Egypt, and Central Africa. Genotypes 5 and 6 are found predominantly in South Africa and Southeast Asia, respectively [117,118,120].

Genotyping helps to inform those providing treatment because some viral genotypes respond better to therapy than others [116]. The genetic diversity of HCV is one reason that it has been difficult to develop an effective vaccine, since the vaccine must contain viral proteins from each genotype.

The rates of infection in the general population and the incidence of newly-acquired cases indicate an appreciable change in the epidemiology of HCV infection in recent years. Prior to the widespread screening of blood donations, infected blood and blood products represented a common source of infection. Currently, injecting drug users (IDUs) are considered to be the main risk group for HCV infection and act as a reservoir for this blood-borne virus. The seroprevalence of HCV varies between 31% to as high as 98% in different parts of the world [121]. Based on age-specific prevalence data, three distinct transmission patterns have been identified. In the first pattern, age-specific prevalence is low among individuals less than 20 years of age, but rises steadily through middle age, with most infections occurring among persons 30 to 49 years old and declines sharply among persons greater than 50 years old. This pattern is found in the United States and Australia, indicating that the risk for HCV infection occurred in the relatively recent past (10-30 years ago) and primarily affected young adults. In countries with the second pattern (e.g., Japan, Italy), age-specific prevalence is low in children and younger adults but increases sharply among older persons, who account for most infections, which is consistent with the risk for HCV infection having been greatest in the distant past (30-50 years ago). In countries with the third pattern (e.g., Egypt), the prevalence of HCV infection increases steadily with age and high rates of infection are observed in all age groups, indicating an ongoing high risk for acquiring HCV infection [122]. In countries with the first pattern, injection drug use has been the predominant risk factor for HCV infection, whereas in those with the second or third patterns, unsafe injections and contaminated equipment used in health-care-related procedures appear to have played a predominant role in transmission [122]. HCV genotypes are further divided into multiple epidemiologically distinct subtypes such as a, b, and c due to the difference in nucleotide sequence of the sub-genomic regions such as core/E1 and NS5B (non-structural 5B) [123].

Global patterns for the distribution of different HCV genotypes are as follows: 1a is mostly found in North and South America, but it is also common in Australia; 1b is mostly found in Europe and Asia; 2a is the most common genotype 2 in Japan and China; 2b is the most common genotype 2 in the US and north Europe; 2c is the most common genotype 2 in western and southern Europe; 3a is highly prevalent in Australia (40% of cases) and south Asia; 4a is highly prevalent in Egypt; 4c is highly prevalent in central Africa; 5a is highly prevalent only in South Africa; 6a is restricted to Hong Kong, Macau and Vietnam [124]. Hence these results suggest that different subtypes of HCV genotypes possibly could have been introduced to the different parts of the world by travellers.

Signs and symptoms

Relatively few patients seek medical care for acute hepatitis C, since most patients are asymptomatic or have only mild, flu-like symptoms. Of those who do present with acute hepatitis C, 70 to
80 percent have detectable anti-HCV antibodies at clinical presentation, and 90 percent have such antibodies by 12 weeks after onset [125]. Therefore, anti-HCV testing should be repeated if acute hepatitis C is suspected and the initial test result is negative. Most patients with acute hepatitis C remain chronically infected, and approximately two-thirds or more of patients with chronic infection have abnormal alanineaminotransferase (ALT) activity. Based on serial serum samples with normal values for ALT and negative results for HCV RNA, it is estimated that in 15% of HCV infected patients, the infection resolves and the patient recovers from hepatitis C [126].

**Acute HCV infection**

Although the incubation period for newly acquired (acute) HCV infection ranges from two weeks to six months, with an average incubation period of six to seven weeks [127], viral replication can be detected as early as one week after exposure. Of patients with acute HCV infection, between 60% and 70% have no discernible symptoms; 20% to 30% have jaundice; and 10% to 20% have nonspecific symptoms, such as loss of appetite, fatigue, and abdominal pain [128].

Most patients (about 80%) who seek medical care for symptoms related to acute hepatitis C have bilirubin levels of at least 3.0 mg per dL (51 µmol per L), average: 4.1 mg per dL (70 µmol per L); and alanineaminotransferase (ALT) levels greater than 600 IU per L, average: 1,410 IU per L (Hepatitis C Reference Manual – The Body; U.S. Centers for Disease Control and Prevention; [http://www.body.com](http://www.body.com)). Only 15% of patients require hospitalization, and fulminant disease is rare [129]. The course of acute hepatitis C is variable, although its most characteristic feature is some type of fluctuating polyphasic ALT pattern. Some patients have variations of several hundreds of U per L from week to week, and such variations are sometimes recurrent, with the magnitude of the ALT elevations diminishing over time. The normalization of ALT levels suggests full recovery, but this is frequently followed by ALT elevations, an indication of chronic liver disease [130]. This facet of hepatitis C necessitates prolonged follow-up to ensure appropriate diagnosis and management.

**Chronic HCV infection**

Most patients (85% or more) with acute HCV infection develop persistent infection [130], and chronic hepatitis develops in an average of 70% of infected patients [127,128,130,131]. In the United States, about 40% to 60% of cases of chronic liver disease are associated with HCV infection [5]. The progression of chronic liver disease is usually insidious: it is slow and without symptoms or physical signs in most patients during the first two decades after infection. Frequently, chronic hepatitis is not recognized until symptoms appear with the development of advanced liver disease.

**Diagnostic tests**

**Serologic assays**

Some patients have a mild, acute infection due to HCV that disappears without treatment. When the infection continues for six or more months, it is known as chronic hepatitis C, which can be marked by fatigue and liver function impairment. Those with chronic hepatitis C have an increased risk of later developing cirrhosis or liver cancer.

The diagnosis of HCV infection can be made by detecting either anti-HCV or HCV RNA. Enzyme immunoassay (EIA) is the main screening assay for the detection of HCV antibodies along with confirmatory recombinant immunoblot assay (RIBA) for all positive anti-HCV results of EIA.

Confirmation or exclusion of HCV infection in a person with indeterminate anti-HCV supplemental test results should be made on the basis of further laboratory testing, which might include repeating the anti-HCV test in two or more months, or testing for HCV RNA and determining the ALT level [8,132].

**Nucleic acid detection**

Although patients should be screened by EIA, measurement of serum HCV RNA by highly sensitive reverse transcriptase PCR may be required for detection of HCV in individuals with undetectable antibodies, and with other evidence of chronic liver disease [126]. HCV RNA can be detected within one to two weeks after exposure to the virus, weeks before the onset of ALT elevations or the appearance of anti-HCV antibodies [133].

HCV RNA may be detected intermittently during the course of infection, so a single negative PCR result is not conclusive. Because of assay variability, rigorous proficiency testing is recommended for clinical laboratories performing this assay, and results of PCR testing should be interpreted cautiously [8,134].

**MicroRNA (miRNA)**
MicroRNAs (miRNAs) are small RNAs that regulate eukaryotic gene activity at the post-transcriptional level [135,136]. Several hundred miRNAs have been characterized up to now, and each miRNA is assumed to regulate hundreds of target mRNAs [137]. MiRNAs are assumed to regulate the activity of mammalian mRNAs, and are apparently involved in all important aspects of cellular regulation, including growth, development, differentiation, and metabolism.

MiRNAs are processed in the nucleus from primary precursors, exported to the cytoplasm, and then further processed to ~22 bp miRNA duplexes with 3' overhangs. The protein complex containing this miRNA duplex then chooses one of the strands as a sequence-specific guide to find its target mRNA by base-pairing. Therefore, the duplex is unwound, and the guide strand is retained in the complex, while the opposite strand (the passenger) is discarded [138,139,140]. The final miRNA ribonucleoprotein (miRNP) complex contains an Argonaute (Ago) protein [141], and several other proteins [142].

Within the complex, the mature miRNA guide strand is positioned with its 3' end in a pocket of the Ago’s PAZ domain, with its 5' end between the MID and PIWI domains [143], thereby making the so-called seed region near the miRNA’s 5’ end accessible for base-pairing with the target sequence in the mRNA [144].

The effector function of this Ago-containing complex then depends on the extent of base-pairing between the small RNA (miRNA or siRNA), and its mRNA target [135,136]. When the small RNA matches its target perfectly, RNA induced silencing complex (RISC) forms, and Ago cleaves the target mRNA opposite to the guide strand. In contrast, when base-pairing between miRNA and its target mRNA is imperfect [137], the interaction of the miRNA-protein complex with the target mRNA usually results in translation repression.

Some viruses make use of miRNAs to increase the efficiency of their own replication in the infected cell. On one hand, viruses can express their own miRNAs to manipulate cellular gene expression [145]; on the other, viruses can employ cellular miRNAs to advance their own reproduction. A plus strand RNA virus, such as hepatitis C virus (HCV), is an example of the latter case since its RNA genome can directly bind cellular miRNAs.

The influence of two different naturally occurring miRNAs acting on the HCV 5' UTR, the liver-specific miR-122 and miR-199a*, has been clearly demonstrated. A significant initial contribution to the understanding of the role of miR-122 in the lifecycle of HCV was made by Jopling and co-workers in Peter Sarnow’s group [146]. The development of a subgenomic HCV RNA replicon capable of replication in the human hepatoma cell line, Huh-7, was a significant advance [145,147]. Recently, complete replication of HCV in cell culture has also been achieved [148].

Additionally, miR-122 is present preferentially in liver cells or in the hepatoma cell line Huh-7 [149], and can thus be suspected to contribute significantly to HCV liver tropism. In the non-coding regions of HCV, there are three sequences complementary to the seed sequence of miR-122. A 7-nucleotide sequence (ACACUCC) and a 6-nucleotide sequence (CACUCC) are located between stem-loops I and II of the highly conserved 5' UTR, and thus in a region that is involved in the regulation of both translation and replication; a third sequence (ACACUCC) is located in the 3' UTR.

Jopling and co-workers [146] found that the sequestration of the endogenous miR-122 contained in Huh-7 cells by antisense oligonucleotides resulted in a decrease of HCV RNA accumulation 48 hours after transfection, leading to their conclusion that miR-122 stimulates HCV replication. By using mutations in the HCV 5' UTR, and compensatory mutations in the miR-122 seed sequence, the authors clearly demonstrated a physical interaction of miR-122 with the first target site in the 5' UTR. In contrast, the miR-122 complementary site in the 3' UTR appears not to be involved in this stimulation. Later on, the same group extended this knowledge to include the second miR-122 target site in the 5' UTR in this process [150]. To complement their HCV replication analyses, the Jopling team used translation assays to differentiate between RNA synthesis and translation. From the results of these translation experiments, these authors concluded that miR-122 has no effect on HCV translation.

Recently it has been shown that miR-122 stimulates HCV translation [151] by accelerating and enhancing the association of the small ribosomal subunit with the HCV RNA. This translation stimulation was observed even with the non-structural 5B-polymerase defective HCV genome. Mutations in the seed, and in the target sequences, demonstrated a physical interaction of miR-122 with the 5' UTR of the HCV RNA; miR-122 was detected in the 48S translation initiation complexes with the HCV RNA. Moreover, the interaction between HCV
RNAs with a mutated miR-122 target site, and the corresponding mutated miR-122, led to a strong increase in ribosome association in living HeLa cells [152]. In light of these results, it is clear that the interaction of miR-122 with the HCV 5' UTR stimulates translation.

MicroRNA 122 (miR-122) is specifically expressed and highly abundant in the human liver [149]. Directly downstream of the second miR-122 binding site in the HCV 5' UTR, is a binding site for another miRNA, miR-199a*, in stem-loop II. This miR-199a* binding site includes seed matches, as well as some additional matches, that extend down to the base of stem-loop II [153]. It can be expected that the base-pairing of miR-199a* to this region would at least partially invade and destroy the secondary structure of stem-loop II. Since stem-loop II is involved in both translation and replication, it is not surprising that miR-199a* reduces HCV replication efficiency [153]. A possible effect of miR-199a* on HCV translation was not analyzed.

When considering a possible contribution of miR-199a* to the tissue tropism (cells and tissues of a host support growth of a particular virus) of HCV in the infected body, it is interesting to look at the available data on the tissue-specific expression of miR-199a*. It appears to be expressed at moderate levels in several human tissues [154], but the expression in the human liver is rather low [153,154]. Therefore, it had been speculated [155] that a possible suppression of HCV replication in tissues other than the liver by miR-199a* contributes to HCV liver tropism. Depletion of Huh7 hepatoma of the liver-specific miRNA miR-122 (that directly interacts with the 5' end of the HCV RNA genome), and low levels of miR-199a*, could be associated with inhibition of HCV replication and infectious viral production. These findings suggest that HCV takes advantage of the presence of miR-122 in hepatocytes.

Most current and emerging anti-HCV therapies target viral genes as well as liver-specific expression of miR-122 [156] and miR-199a*[153], and are, therefore, prone to the emergence of resistance. Hence studies should be directed to specific miRNAs which are homologous to HCV genes.

**Treatment**

*Alpha interferon and ribavirin*

Unfortunately, no vaccine against hepatitis C is available, but the current standard therapy, pegylated interferon-alpha (IFN-α) in combination with ribavirin, has achieved substantial success [157]. At the present time, the optimal regimen appears to be a 24- or 48-week treatment period. Nonetheless, half the patients still fail to develop a sustained virologic response after this therapy. To improve treatment outcomes, novel monotherapies, or alternative combinations of IFN-α based therapies, are being investigated.

**RNA interference (RNAi)**

RNA interference (RNAi) has emerged as a novel therapy for viral infections. Since the HCV genome is a single-stranded RNA that functions as both a template for transcription, and a template for a negative strand replication intermediate, it is a prime candidate for RNAi [158]. Instead of a 5' cap, the internal ribosome entry site (IRES), located at the 5' noncoding region of the viral genome, plays an essential role in initiating translation [159]. Because it is the most conserved sequence within the viral genome, IRES seems an ideal target for RNAi, and several promising studies have demonstrated inhibition of HCV replication by targeting this region [160]. HCV replication is mediated by NS5B, an RNA-dependent RNA-polymerase that lacks proofreading abilities. As a result of extremely high mutation (10^3 per nucleotide per generation) and replication (10^{12} virions per day) rates in patients, HCV quasispecies are generated [161]. The NS5B region has been shown to be very effective for RNAi-induced suppression of HCV replication [162].

The success of RNAi in therapeutic applications also depends on an efficient delivery system, which can support long-term siRNA production and continuous gene silencing. Integrating self-inactivating lentiviral vectors (LV) can achieve these criteria by encoding small hairpin RNA (shRNA), a precursor of siRNA that is cleaved into biologically active siRNA by Dicer, the host cell enzyme [163].

Previous studies have shown that simultaneously targeting both viral and host cell elements by RNAi could increase the potency of antiviral therapies [164]. IFN-α possesses indirect antiviral activity by stimulating genes that can lead to a non-virus-specific antiviral response, possibly inducing therapeutic resistance in treating infection, whereas RNAi can directly interfere with viral entry and replication through targeting viral RNA genome or mRNA of cellular factors. Based on RNAi complementary antiviral mechanisms, it has been proposed that combining RNAi (LV-shIRES) with IFN-α may prevent therapeutic resistance and promote enhanced
antiviral activity [165]. Moreover, the additional combination of ribavirin to RNAi and IFN-α may further improve the therapeutic aspects of treatment for chronic hepatitis C.

Conclusion
Liver transplantation is the only effective treatment for liver failure but is greatly limited by the shortage of donor organs. Due to the shortage of donor livers and other organs, tissue engineering and regenerative medicine have emerged. Interferon alpha (IFN) is the drug of choice for the treatment of recurrent hepatitis C (HCV) in liver transplant recipients, but one of its potential adverse effects is acute and chronic rejection.

The capability of the liver to fully regenerate after injury is a unique phenomenon which is essential for the maintenance of its important biological functions in the control of metabolism and detoxification. Genes that orchestrate liver regeneration have been only partially characterized. Of particular interest are cytokines and growth factors, which control different phases of liver regeneration. Functions of growth factors, cytokines and their downstream signalling targets in liver regeneration are being studied in genetically modified mice.

MicroRNAs are capable of specific, highly stable, and functional silencing of gene expression in a variety of cell types. Hence they may also provide new approaches for gene therapy, and this application of miRNA may be a target for novel approaches in the treatment of HCV infection.

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