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TOPIC HIGHLIGHT

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Understanding the interaction of hepatitis C virus with host DEAD-box RNA helicases

Megha Haridas Upadya, Jude Juventus Aweya, Yee-Joo Tan

Megha Haridas Upadya, Jude Juventus Aweya, Yee-Joo Tan, Department of Microbiology, Yong Loo Lin School of Medicine, National University Health System (NUHS), National University of Singapore, Singapore 117597, Singapore
Yee-Joo Tan, Institute of Molecular and Cell Biology, Agency for Science, Technology and Research (A*STAR), Singapore 138673, Singapore
Author contributions: Tan YJ designed the research; Upadya MH performed the research; Upadya MH, Aweya JJ and Tan YJ co-wrote the manuscript; all authors read and approved the final version of the manuscript.
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Correspondence to: Yee-Joo Tan, Associate Professor, Department of Microbiology, Yong Loo Lin School of Medicine, National University Health System (NUHS), National University of Singapore, MD4A, 5 Science Drive 2, Singapore 117597, Singapore. yee_joo_tan@nuhs.edu.sg
Telephone: +65-651-63692 Fax: +65-677-66872
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Abstract

The current therapeutic regimen to combat chronic hepatitis C is not optimal due to substantial side effects and the failure of a significant proportion of patients to achieve a sustained virological response. Recently developed direct-acting antivirals targeting hepatitis C virus (HCV) enzymes reportedly increase the virologic response to therapy but may lead to a selection of drug-resistant variants. Besides direct-acting antivirals, another promising class of HCV drugs in development include host targeting agents that are responsible for interfering with the host factors crucial for the viral life cycle. A family of host proteins known as DEAD-box RNA helicases, characterized by nine conserved motifs, is known to play an important role in RNA metabolism. Several members of this family such as DDX3, DDX5 and DDX6 have been shown to play a role in HCV replication and this review will summarize our current knowledge on their interaction with HCV. As chronic hepatitis C is one of the leading causes of hepatocellular carcinoma, the involvement of DEAD-box RNA helicases in the development of HCC will also be highlighted. Continuing research on the interaction of host DEAD-box proteins with HCV and the contribution to viral replication and pathogenesis could be the panacea for the development of novel therapeutics against HCV.

Key words: Hepatitis C virus; Chronic hepatitis C; Hepatitis C virus therapy; DEAD-box helicases; Host factors; Hepatocellular carcinoma

Core tip: Alternative treatments to combat chronic hepatitis C include direct-acting antivirals targeting hepatitis C virus (HCV) enzymes and host targeting agents that interfere with host factors essential for the viral life cycle. Several members of a family of host proteins known as DEAD-box RNA helicases have been shown to modulate HCV replication. This review highlights their interaction with HCV and their role in viral replication. Since hepatocellular carcinoma (HCC) is a major cause of death among patients with chronic hepatitis C, the involvement of these DEAD-box RNA helicases in the development of HCC will also be discussed.

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INTRODUCTION

Hepatitis C is a contagious liver disease that results from infection with hepatitis C virus (HCV), a positive-stranded RNA virus of the family *Flaviviridae*. HCV was discovered in 1988 during the course of investigating the cause of non-A, non-B, transfusion-associated hepatitis. Chronic hepatitis C can lead to liver damage which includes fibrosis, cirrhosis and eventually hepatocellular carcinoma (HCC). Consequently, these complications are a common indication for liver transplantations around the world, especially in developed countries. Chronic hepatitis C affects about 170 million people (or 3% of the world's population) and approximately 350000 deaths are reported from hepatitis C-related diseases every year[1]. Unfortunately, as of today, there is no protective vaccine for hepatitis C and the most practical way of preventing the occurrence and transmission of this disease is by raising public awareness, adopting universal precautions in medical settings and conducting thorough screening of blood and blood products.

As obligate cellular parasites, viruses can only replicate by entering a host cell with the subsequent manipulation of cellular functions. Processes such as viral entry, translation, processing and modification of viral proteins, maturation and release of viral particles from host cells involve intimate interactions between viral and host proteins. Contrary to humans with a large genome size of approximately 24000 genes, the HCV genome is very small. Hence, HCV interacts extensively with the cellular machineries in the human host and uses them to its advantage[2]. This review summarizes the literature on the interaction of HCV with a family of host proteins, namely the DEAD-box RNA helicases. Several members of this family have been shown to interact with HCV proteins and/or regulate HCV replication. Recently, some DEAD-box RNA helicases have been shown to be involved in the progression of several types of tumours including HCC. Thus, their roles in the development of HCC will be highlighted and the potential link to HCV pathogenesis will be explored.

HCV GENOME AND VIRAL PROTEINS

HCV has a single stranded RNA genome containing a single, large open reading frame that encodes a large polyprotein precursor of approximately 3000 amino acid residues. This polyprotein is cleaved by a combination of host and viral proteases into at least 10 viral proteins. The N-terminus encodes three structural proteins namely core, envelope glycoprotein 1 and 2 (E1 and E2) while the C-terminal two-thirds of the polyprotein comprises of six non-structural proteins namely non-structural protein 2 (NS2), NS3, NS4A, NS4B, NS5A and NS5B[3]. These proteins encode enzymes or accessory factors that catalyze and regulate the replication of the HCV genome[4]. Another protein called p7 lies at the junction between the structural and non-structural regions of the virus polyprotein. Cleavages from the N-terminus through p7 are mediated by host-encoded proteases, cleavage between NS2 and NS3 is mediated by the autoprotease NS2/NS3, and the remaining cleavages in the C-terminal part of the polyprotein are mediated by the NS3/NS4A serine protease. HCV is characterized by substantial genome heterogeneity with at least 6 major genotypes and > 100 subtypes defined on the basis of nucleotide and amino acid sequences of conserved and non-conserved genome regions[5]. The HCV genotypes exhibit different geographic and regional distributions with genotypes 1 and 3 being the most prevalent worldwide[5].

CURRENT THERAPY FOR HEPATITIS C

HCV replication occurs in hepatocytes and mononuclear cells. Both viral replication and host immune responses are believed to contribute to liver pathology. Despite that, hepatitis C infection does not always require treatment. In acute HCV infected patients, the virus is cleared spontaneously in about 30% of the cases[6]. However, in the majority of cases, the infection becomes chronic and necessitates treatment. The primary end point of therapy, the sustained virological response (SVR) defined as the absence of detectable serum HCV RNA, 24 wk after therapy may be considered equivalent to a clinical cure of HCV infection[7].

Combination antiviral therapy with pegylated interferon (Peg-IFN) and ribavirin (RBV) has been the mainstay of hepatitis C treatment across all genotypes so far. Regrettably, this mode of treatment is not optimal because a significant proportion of patients fail to achieve SVR. It has been reported that the failure rate is almost 50% for patients with genotype 1 HCV and approximately 20% for those with genotype 2 or 3 HCV[8-10]. Thus, there is evidence to show that this treatment regimen is effective only for a subset of patients with chronic HCV. Moreover, interferon is not widely available globally and not always well tolerated owing to the substantial side effects. Consequently, the development of new classes of antiviral compounds to combat this global health burden has been receiving widespread attention in recent years[11].

Scientific advances have led to the development of new direct-acting antiviral (DAA) agents for hepatitis C, which potentially could be more effective and well tolerated than the current treatment regimens. In 2011, the FDA approved the first two DAAs, Boceprevir (Victrelis) and Teleprevir (Incivek), which target the HCV protease[12]. A combination of these DAAs with Peg-IFN and RBV in triple combination therapy substantially increased the SVR rate in difficult to treat, treatment-naïve and treatment-experienced patients[13-18]. It has been reported that SVR rates for patients with genotype 1 HCV infection have increased to 70%-80%, and about two-thirds of patients are eligible for a shortened duration of therapy[16,17,19]. However, the efficacy of these new drugs is not genotype-wide and some infected individuals respond poorly. Moreover, these drugs do not appear to be suitable for monotherapy because the selective pressure

imposed by the drugs can lead to the rapid emergence of DAA-resistant viral mutants[20-22].

Another major target for anti-HCV drugs is NS5B, the inhibitors of which can be grouped into two categories, namely the nucleoside/nucleotide inhibitors which target the catalytic site of the enzyme and non-nucleoside inhibitors which target the allosteric sites of the RNA-dependent RNA polymerase[23]. Other drugs targeting the NS5A RNA-binding protein are also in the final stages of clinical development[24]. The mechanism of action of these compounds is however poorly understood due to the lack of a comprehensive insight into the specific function of NS5A. There are also studies to evaluate inhibitors of NS4B, another HCV-encoded RNA binding protein[25]. To minimize drug resistance and be effective across all genotypes, it is desirable to devise combination therapies with drugs acting through diverse modes of action[26]. However, it is still not clear as to how many classes of these aforementioned new drugs will need to be combined in order to replace the Peg-IFN/RBV treatment.

VIRAL-HOST INTERACTIONS AS POTENTIAL DRUG TARGETS

As discussed in the previous section, the prevailing therapeutic arsenal against HCV is directed towards the viral components, specifically the viral enzymes. Targeting viral components that are required for the replication cycle could reduce the likelihood of inducing undesirable side effects such as toxicity. However, a major limitation here is the small HCV genome which will limit the number of viral targets. In addition, HCV mutates rapidly and churns out many viral variants or quasispecies[27]. Alternatively, there are a class of new compounds termed as host-targeting agents (HTAs) and these target the host factors that are essential for HCV replication. Recent studies have suggested that these HTAs could potentially serve as a promising strategy to broaden the therapeutic repository against HCV. The mode of action of HTAs is conceptualized in two strategies. The first strategy involves interfering with the host factors that play an integral role in the various stages of the viral life cycle such as viral entry, replication, assembly and release. The second involves boosting the innate immunity of the host through the administration of agents such as IFN-λ[28], or agonists of Toll-like receptors[29,30].

HTAs have several advantages over DAAs including: (1) A wide-ranging pan-genotypic activity with reported inhibition of HCV infection caused by all major genotypes, coupled with highly variable quasispecies isolates and resistant escape variants[31-33]; (2) A higher genetic barrier to resistance development due to the fact that the genetic variability of the host is lower than that of the virus[31-33]; and (3) Complementary mechanisms of action to DAAs, paving the way for a potential synergistic therapeutic effect[32,34]. However, the side effects of HTAs are a prime concern in therapeutics when host factors instead of viral components are targets[35]. Nevertheless, targeted strategies against host factors such as CD81 and SR-BI that are involved in viral entry are currently under investigation[36], while miR-122 and inhibitors of cyclophilin A (alisporivir) targeting viral replication, with possible action on virion assembly and release are currently at various stages of clinical development[37]. Efforts are also underway to develop and evaluate several other HTAs with different host targets in order to improve and broaden the therapeutic net. Targeting host factors is an attractive option in the search for more potent HCV therapies with an additional advantage of minimizing the development of resistant mutant virus and ensuring an adept treatment of HCV across all genotypes.

In the past, the lack of a cell culture model for the study of HCV had severely hampered research on the role of host proteins in the replication cycle of HCV and the significance of virus-host interactions. However, with the development of the replicon system, as well as infectious cell culture system, studying viral replication and pathogenesis has been made much less cumbersome. In particular, the development of the HCV infectious clone and the chimeric forms which produce HCV robustly in cell culture, termed the HCVcc system[38,39]. The most widely used HCVcc system is based on the JFH-1 strain or the intragenotypic chimeric J6/JFH-1 strain (both are of genotype 2a) and allows the entire virus life cycle, including entry, replication, assembly and release, to be studied in hepatoma cell lines. As described further, this has allowed much progress in the characterization of the interaction of several DEAD-box RNA helicases with HCV proteins and has helped to define their roles in different steps of the virus life cycle.

DEAD-BOX RNA HELICASES

The DEAD-box RNA helicases represent the largest family of RNA helicases, comprising of 38 members in humans. DEAD-box helicases have been implicated in various cellular processes involving RNA such as splicing, mRNA export, transcriptional and translational regulation, RNA decay and ribosome biogenesis[40]. They contain a central region comprised of 2 conserved domains, termed as domain Ⅰ and Ⅱ, flanked by highly variable N and C-terminal sequences. A comparison of the known structures of domain Ⅰ and Ⅱ reveal that they have a fold belonging to the RecA super family, where 5 β-strands are surrounded by 5 α-helices[41-43]. The DEAD-box helicases are divided into several subgroups based on the DEAD-box motif such as DEAD, DEAH and DEXH subfamilies. These DEAD-box proteins are highly conserved in nine motifs but exhibit substantial sequence divergence outside these nine conserved motifs. The nine conserved motifs are the Q-motif, motif 1, motif 1a, motif 1b, motif Ⅱ, motif Ⅲ, motif Ⅳ, motif Ⅴ, and motif Ⅵ (Figure 1A). Motif Ⅱ is also known as the Walker B motif and contains the amino acid sequence D-E-A-D (asp-glu-ala-asp), which gave this family of proteins the

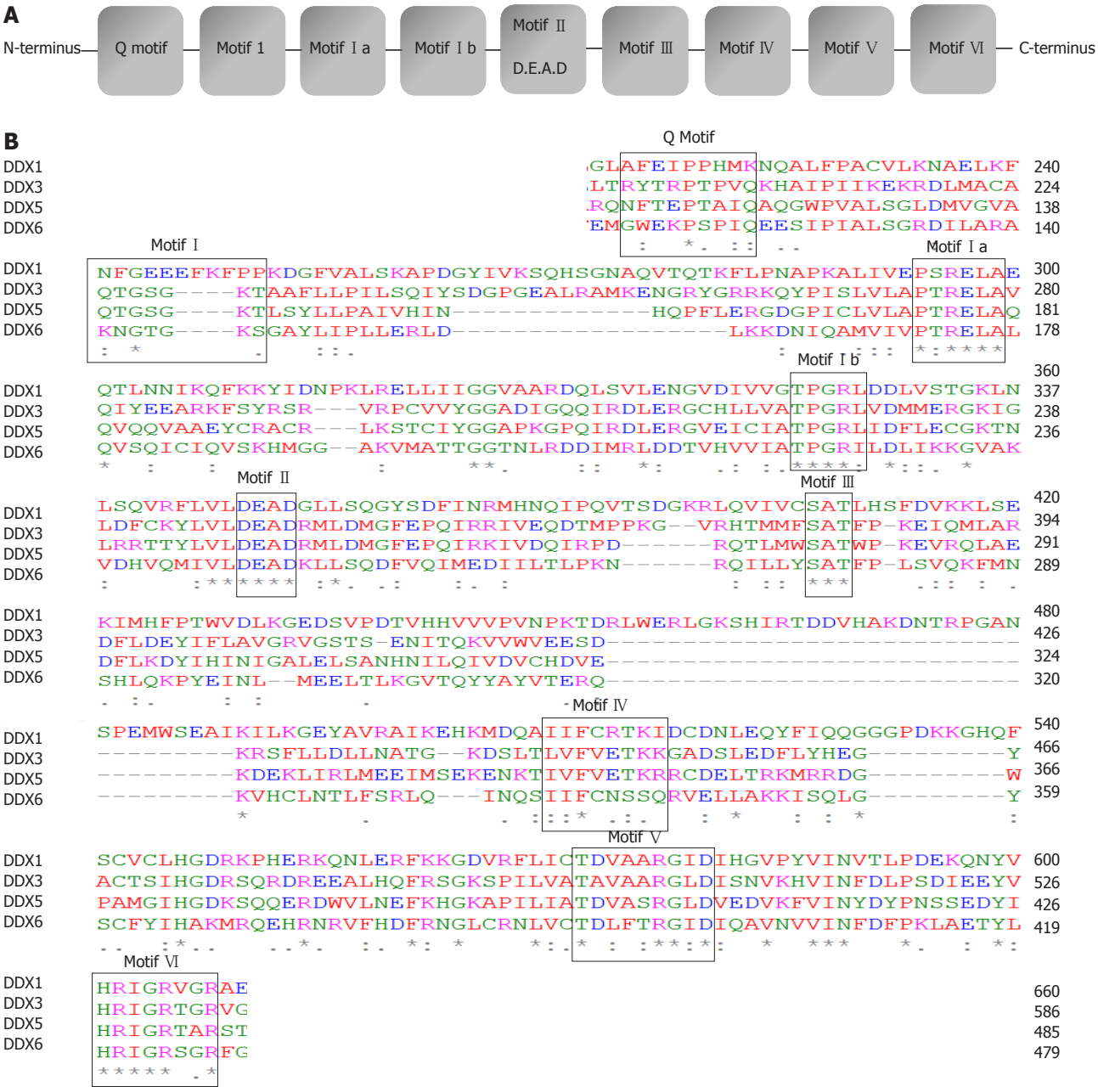

Figure 1 Schematic diagram of DEAD-box proteins and sequence alignment of selected members. A: Conserved motifs within the DEAD-box family; B: Sequences of the conserved motifs-Q motif, Ⅰ, Ⅰa, Ⅰb, Ⅱ, Ⅲ, Ⅳ, Ⅴ, Ⅵ from DDX1, DDX3, DDX5 and DDX6.

name "DEAD box". Motif 1, motif Ⅱ, the Q motif, and motif Ⅵ are all needed for ATP binding and hydrolysis, while motifs, 1a, 1b, Ⅲ, Ⅳ, and Ⅴ may be involved in intramolecular rearrangements and RNA interaction. A sequence alignment of 4 members of the DEAD-box group of proteins known to be involved in HCV replication shows that there is a high degree of conservation in these motifs (Figure 1B). Current knowledge of the interaction of these 4 DEAD-box proteins with HCV has been summarized below. In addition, several DEAD-box helicases, namely the retinoic acid inducible gene-I (RIG-I), melanoma associated differentiation factor-5 and laboratory of genetics and physiology, have been shown to be important factors in the RIG-I pathway, which is one of the major antiviral pathways activated during viral infection[44,45]. However, the intrinsic interaction between the RIG-I pathway and HCV is beyond the scope of this review and has been extensively covered in several recent publications[46,47].

DDX1 AND HCV

A study had previously identified an ATP-dependent helicase DDX1 (DBP-RB/UKVH5d) which can bind to HCV 3' (+) UTR as well as its reverse complementary 5' (-) UTR[48]. It was reported that siRNA-mediated knockdown of DDX1 caused a reduction in the replication of subgenomic replicon RNA. This suggested a possible

role of DDX1 in the initiation of HCV RNA replication. DDX1 has also been reported to be important for the human immunodeficiency virus type 1 (HIV-1) replication as it binds to and serves as a cofactor of the HIV-1 Rev protein, which induces nuclear export of viral RNA and the transition from the early to the late phase of viral gene expression[49,50]. In recent years, there has been a startling increase in the number of cases of HIV-1 and HCV co-infection, with about one-third of HIV-1 infected patients and about 90% of HIV-1 infected drug abusers in the United States and Europe being co-infected with HCV[51,52]. The large proportion of co-infection with HIV-1 and HCV suggests that these two viruses may use similar host machineries for their replication.

DDX3 AND HCV

DDX3 plays an important role in several cellular processes but the precise mechanism of action as well as the extent of involvement in these processes is poorly understood[53]. There is evidence to show that DDX3 contributes to antiviral innate immunity[54,55]. With regard to HCV, the host cells sense viral RNA particularly *via* the mitochondrial antiviral signalling (MAVS) pathway[56]. It is believed that MAVS signals TANK-binding kinase 1 and IkB kinase epsilon, to phosphorylate IFN regulatory factors IRF-3 and IFR-7, thereby inducing type 1 IFN[57]. It has been shown that DDX3 serves as a positive regulator for MAVS-mediated type 1 IFN induction[47,58]. A dichotomy exists in the role of DDX3 where viruses like HCV are able to manipulate DDX3 to support replication yet at the same time, DDX3 could suppress the antiviral activity of HCV by inducing IFN. An in-depth understanding of the functions of DDX3 is important because two viruses that pose a major global health threat, namely HIV and HCV exploit DDX3 and use it to their advantage[59-61]. There is a plethora of literature on DDX3 and its role as a target for viral manipulation has been summarized in other review articles[53,62]. Based on the evidence that DDX3 is required for viral replication, its suppression may serve as a novel strategy and means to widen the repertoire of treatment against these two viruses. Similarly, since HIV/HCV co-infection increases the likelihood of developing other life-threatening complications, it could potentially contribute towards a "killing two birds with one stone" approach to therapy. However, a detailed understanding of the interaction between DDX3 and the virus is required so as to determine if this interaction can serve as a therapeutic target without adversely affecting normal cellular function.

The first HCV protein that was reported to interact with DDX3 was the HCV core protein and this interaction was described by three independent publications[63-65]. However, these early studies were limited by the lack of a robust cell culture model for HCV infection at that time. Two of the above-mentioned publications[64,65] provided evidence to show that the HCV core binds to the C-terminus of DDX3 (amino acids 553-622) and the interaction is mediated by the N-terminus of HCV core (amino acids 1-59). Owsianka *et al*[64], demonstrated the direct interaction of HCV core with the RS-like domain of DDX3. RS domains are stretches of protein sequence very rich in alternate arginine and serine residues. By analyzing deletion mutants, they demonstrated that the N-terminal 59 amino acid residues of core and a C-terminal RS-like domain of DDX3 were the regions associated with this interaction. The presence of an RS-like domain near the C-terminus of DDX3 and the observed localization of DDX3 predominantly in nuclear speckles which was similar to splicing factors, suggest the possible involvement of DDX3 in RNA splicing. Interestingly, it was observed that in the presence of HCV core, the DDX3 protein was redistributed in distinct spots in the perinuclear region of the cytoplasm where it co-localized with HCV core. This change in the pattern of localization of DDX3 in the presence of core was suggested to be a result of the core protein targeting the cytoplasmic function of DDX3. You *et al*[65], designated a cellular RNA helicase that is a homologue of DDX3 as "CAP-Rf" and demonstrated the direct interaction between CAP-Rf and HCV core. This group reported that the N-terminal 40 amino acid residues of core and a C-terminal tail of CAP-Rf were the regions associated with this interaction. Furthermore, HCV core protein was found to enhance the nucleotide triphosphatase-deoxynucleoside triphosphatase activity of CAP-Rf as well as potentiate its trans-activation effects. Mamiya *et al*[63], showed that HCV core protein prevented DDX3 from rescuing *ded1*-deletion yeast and that it inhibited the translation of capped but not uncapped RNA in experimental assays which suggests that it may inhibit cellular mRNA translation in infected human cells. Thus, these three studies highlighted the multifunctional role of DDX3 with regard to exploitation by HCV with the interaction between HCV core and DDX3 proposed to manipulate splicing[64], transcriptional regulation[65] and translational regulation[63].

With the establishment of the HCVcc system, numerous studies have shown that DDX3 is essential for HCV RNA replication. A study in 2007 reported for the first time that DDX3 is required for HCV RNA replication[59]. When DDX3 was knocked-down in Huh7 derived cells, there was a decrease in the accumulation of both genome-length HCV RNA (HCV-O, genotype 1b) and its replicon RNA[59]. Subsequently, another study identified 26 human genes that modulate HCV replication and implicated the RNA interference (RNAi) pathway itself as a key regulator of HCV replication. It was reported that among the host proteins that played a role in HCV replication, DDX3 was one of them[60].

Although there have been studies detailing the interaction between DDX3 and HCV core, it is still unclear if the effect of DDX3 on HCV replication is dependent on this interaction. There is contradictory evidence to show the relationship between the interaction of DDX3 with core protein and its contribution towards HCV replica-

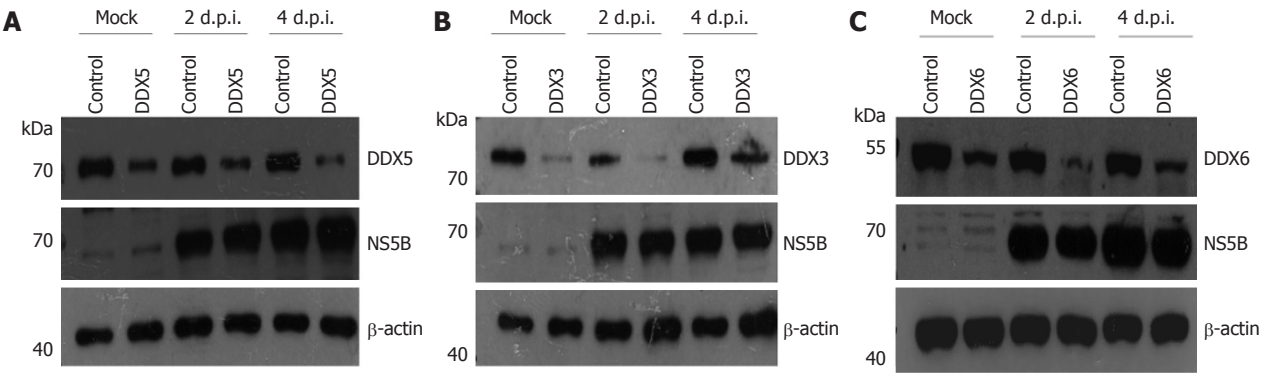

Figure 2 Analysis of protein expression following knockdown of endogenous DDX5, DDX3 and DDX6 in Huh7.5 cells. A: Cells transfected with 40 nmol/L of DDX5 short interfering RNA (siRNA) or a control siRNA; B: Cells transfected with 40 nmol/L of DDX3 siRNA or control siRNA; C: Cells transfected with 40 nmol/L of DDX6 siRNA or control siRNA. At 48 h, cells were infected with J6/JFH-1 (p47) hepatitis C virus (HCV) at multiplicity of infection of 2 as previously described[72]. Cell lysates were prepared 0, 2, 4 d post-infection (d.p.i.) and subjected to immunoblotting with the respective primary antibodies. Expression of NS5B was used to confirm HCV infection and β-actin served as a loading control. Three independent experiments were performed and one representative set of data is shown.

Table 1 Target sequences of short interfering RNA of a selected group of DEAD-box proteins used in this study

siRNA	Sequence	Ref.
Control	AAA ACG UUC UGA UGC CUU AAG	From SARS genome
DDX3	CGC UUG GAA CAG GAA CUC UUU	Chang *et al*[100]
DDX5	AAG UGG AAU CUU GAU GAG CUG	Goh *et al*[68]
DDX6	GCA GAA ACC CUA UGA GAU UUU	Chu and Rana[89]

siRNA: Short interfering RNA.

tion. One study demonstrated that HCV (of genotype 2a) having a core protein mutation can recruit DDX3 for its replication process despite the fact that the alanine substitution in core disrupted the interaction with DDX3[66]. On the other hand, it was reported that in a HCV genotype 1b replication system, the inhibitory effect of core-derived peptides was reversed by overexpressing DDX3, suggesting that the mechanism of action was by targeting DDX3[67]. Therefore, the relevance of DDX3-core interaction in HCV replication has not been thoroughly elucidated and warrants further investigation.

DDX5 AND HCV

DDX5 was first identified as an interacting partner of HCV NS5B through a yeast-two-hybrid screen[68]. Interestingly, the overexpression of NS5B caused the endogenous DDX5 in Hela cells to redistribute from the nucleus to the cytoplasm and the C-terminal of NS5B was found to be important for this interaction[68]. Consistently, another study reported that DDX5 underwent a similar translocation when Huh7 cells were infected with cell culture derived and JFH-1-based infectious HCV particles[69]. Further experiments revealed that there are two independent NS5B-binding sites in DDX5, one located at the N-terminus and another at the C-terminus[70]. The N-terminal regions (NTRs) of DEAD-box helicases are highly variable and a crystal structure of the N-terminal fragment of DDX5 reveals the first 51 residues are highly flexible while the remaining part of the NTR (residues 52-78) forms an extensive loop and an alpha-helix[70]. Binding studies revealed that the highly flexible first 51 residues of DDX5 could fold back to block one of the NS5B binding sites located between 61-305 residues in DDX5, suggesting that the interaction between DDX5 and NS5B in infected cells is likely to be highly dynamic.

Previously, we showed that the knockdown of endogenous DDX5 by RNAi reduced the synthesis of negative-strand HCV RNA in 293 cells transiently transfected with a genotype 1b full-length HCV expression construct (HCV-S1, Genebank accession number AF356827) developed in our laboratory[68]. As opposed to the HCVcc system which produces HCV robustly in cell culture[38], introducing the HCV-S1 genome into liver-derived cell lines produced a very low level of virus[71]. Hence, we repeated the knockdown of DDX5 in Huh7.5 cells and determined if the knockdown of DDX5 affects HCV replication in the HCVcc system. As shown in Figure 2A, the endogenous level of DDX5 was significantly reduced in the DDX5 siRNA treated cells at different days post infection (d.p.i.) when compared to cells treated with an irrelevant control siRNA. To achieve a better comparison, the experimentation was performed in parallel with siRNAs targeting DDX6 and DDX3 because these DEAD-box proteins have been shown to contribute to HCV replication (as described herein). The target sequences of the siRNAs used in this study have been listed in Table 1. As seen in the case of DDX5, the endogenous levels of DDX3 and DDX6 were reduced in the corresponding samples (Figure 2B, C, respectively). Upon infection with the J6/JFH-1 virus at multiplicity of infection (MOI) of 2, expression of the NS5B protein was detected at 2 and 4 d.p.i. However, no clear difference in NS5B expression was observed for the samples with reduced DDX3, DDX5 or DDX6 probably because western blot analysis does not provide a quantitative measure of HCV infection.

Hence, reverse transcription followed by real-time PCR was performed to determine the level of intracellu-

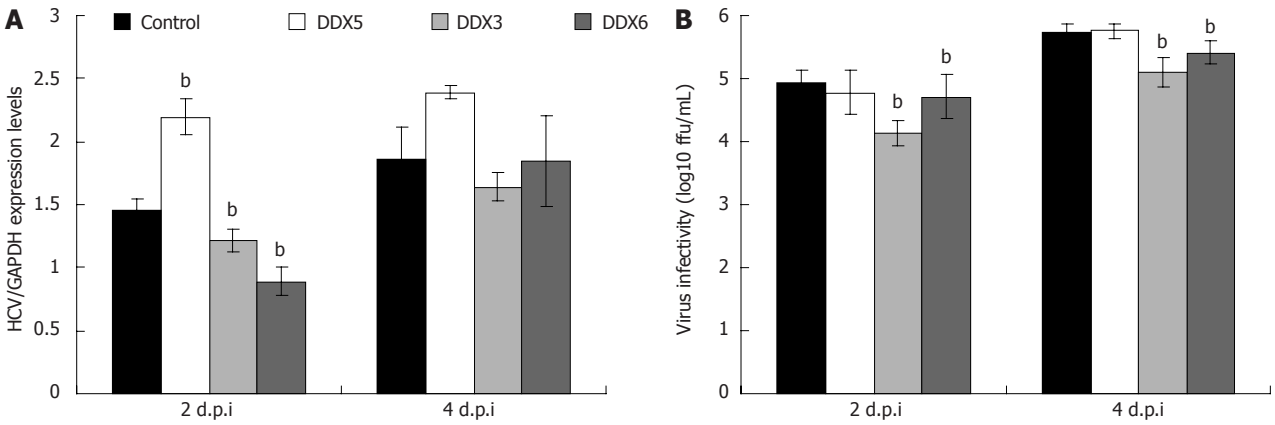

Figure 3 Analysis of viral replication and viral infectivity following knockdown of endogenous DDX5, DDX3 and DDX6 in Huh7.5 cells. Cells transfected with DDX5 short interfering RNA (siRNA) in parallel with a control siRNA, DDX3 siRNA and DDX6 siRNA. At 48 h, cells were infected with J6/JFH-1(p47) hepatitis C virus (HCV) at an multiplicity of infection of 2. A: At 2 and 4 d.p.i., the total RNA was isolated from the cells, reverse transcribed, and the resulting cDNA was subjected to quantitative polymerase chain reaction for detection of genomic HCV RNA and glyceraldehyde-3-phosphate dehydrogenase mRNA as previously described[73]; B: Virus supernatants collected 2 and 4 d.p.i. were titrated on naive Huh7.5 cells by using indirect immunofluorescence as previously described[74] and expressed as focus forming units per millilitre (mL). Statistical analysis was performed using the two-tailed Student's *t* test to determine if the differences between the gene specific siRNA-treated and control siRNA-treated cells were statistically significant. $^{b}P < 0.01$ *vs* control siRNA-treated cells. Data were obtained from three independent experiments and one representative set of data is shown. Each measurement was performed in triplicate, and the average (± SD) is presented.

lar HCV RNA as a quantitative measure of viral replication. As shown in Figure 3A, the level of HCV RNA in the DDX5 siRNA treated cells was higher than the cells treated with control siRNA at both 2 and 4 d.p.i., suggesting that DDX5 has the ability to inhibit HCV RNA replication. However, the change in the level of HCV RNA was statistically significant only at 2 d.p.i. Consistent with previous publications (as described herein), the levels of intracellular HCV RNA in both the DDX3 and DDX6 siRNA treated cells were significantly reduced at both time points (Figure 3A). Next, the production of cell-free infectious virus particles was compared (Figure 3B). Surprisingly, the DDX5 siRNA treated cells did not produce higher level of cell-free infectious virus particles than the control siRNA treated cells despite the higher level of intracellular HCV RNA. As would be expected, the production of cell-free infectious virus particles was significantly reduced in both the DDX3 and DDX6 siRNA treated cells.

In summary, DDX5 interacts with the HCV NS5B protein and the expression of NS5B in transfected or infected cells causes the redistribution of DDX5 from the nucleus to the cytoplasm. The interaction between DDX5 and NS5B in the cytoplasm seems to inhibit viral RNA multiplication because the knockdown of endogenous DDX5 resulted in a slight increase in the level of intracellular HCV RNA in J6/JFH-1 virus infected Huh7.5 cells. Curiously, the increase in HCV RNA in the DDX5 siRNA treated cells did not cause an increase in the production of cell-free infectious virus particles. This may indicate that DDX5 has another positive regulatory role at a later stage of the virus life cycle. In a recent study by Kuroki *et al*[75], it was reported that the knockdown of DDX5 reduces JFH-1 virus production in OR6c JRN/3-5B cells, which harbour the subgenomic HCV-JFH1 RNA with luciferase[76], infected at an MOI of 0.05. Despite the use of different cell lines and MOIs, both studies suggest that DDX5 may be important for a late stage of the HCV life cycle. Hence, the precise role of DDX5 in HCV replication needs to be further investigated and it should be determined if DDX5 can interact with other HCV proteins. Interestingly, single nucleotide polymorphisms in the *DDX5* gene have been shown to be significantly associated with increased risk of advanced fibrosis in HCV patients[77] and future studies can be directed to determine if these polymorphisms affect the interaction of DDX5 with one or more HCV proteins.

DDX6 AND HCV

DDX6 is normally found as a component of the large messenger ribonucleoprotein (mRNP) complex. It has been demonstrated that RNAi-mediated silencing of DDX6 and a few other related proteins impaired the replication of HCV which was thought to be due to impairment in HCV translation in the absence of expression[78]. Shortly afterwards, another study also demonstrated the role of DDX6 in HCV replication in which the knockdown of DDX6 was found to reduce the expression of HCV proteins, accumulation of intracellular viral RNA and infectious virus yields[79]. Overexpression studies showed that an abundance of DDX6 did not significantly affect the expression of viral proteins but increased the infectious virus yields. Furthermore, this study showed that DDX6 knockdown did not affect the abundance of miR-122 which is an amply expressed liver-specific miRNA. In addition, the supplementation of miR-122 reversed the defect in HCV replication observed after DDX6 knockdown, suggesting that these two host fac-

Table 2 Summary of selected members of the DEAD-box family that are involved in hepatitis C virus replication and/or have been implicated in the development of hepatocellular carcinoma

Dead-box family	Alternate names	Interaction with HCV	Effect on HCV replication	Ref.	Effect on HCC	Ref.
DDX1	DBP-RB UKVH5d	HCV 3′ (+) UTR and 5′ (-) UTR	Unknown	NA	Unknown	NA
DDX3	DDX3X DBX DDX14 HLP2	HCV core	Required for HCV replication	Ariumi *et al*[59] Randall *et al*[60] Angus *et al*[66]	Overexpressed in HCC Downregulated in HCC	Huang *et al*[97] Li *et al*[98] Liu *et al*[99] Chang *et al*[100] Chao *et al*[101]
DDX5	p68 G17P1 HLR1 HUMP68	HCV NS5B	Inhibit an early step and promote a late step in viral replication	Data presented herein Kuroki *et al*[75]	Downregulated in HCC	Kitagawa *et al*[86]
DDX6	Rck P54 HLR2	HCV core	Required for HCV replication	Scheller *et al*[78] Jangra *et al*[79] Pager *et al*[80] Huys *et al*[81]	Overexpressed in HCC	Miyaji *et al*[88]
DDX17	P72 RP3-434P1.1 RH70	Unknown	Unknown	NA	Downregulated in HCC	Kitagawa *et al*[86]
DDX20	Gemin3 DP103	Unknown	Unknown	NA	Downregulated in HCC	Takata *et al*[94]

HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; NA: Not applicable.

tors promote HCV replication by independent mechanisms. By conducting co-immunoprecipitation experiments, it was found that DDX6 forms an intracellular complex that involves the HCV core protein and that the C-terminal domain Ⅱ of DDX6 was essential for this. However, the significance of this interaction is uncertain because the facilitation of HCV replication by DDX6 seems to occur independently of core expression. The findings of this study[79] demonstrated that DDX6 expression is required for HCV replication but in contrast to the findings by Scheller *et al*[78], that it is not required for translation. While the overexpression of wild-type DDX6 enhanced HCV replication, a helicase-defective mutant suppressed it instead, thus suggesting that the helicase activity of DDX6 is required for HCV replication. The reduction in HCV replication following knockdown of DDX6 has also been described by two other recent publications[80,81].

DEAD-BOX PROTEINS AND LIVER CANCER

Approximately 70% of HCV infected individuals become chronic carriers and 50% of them will develop chronic liver diseases. Some of them will subsequently progress to cirrhosis, which is a major risk factor for the development of HCC[82]. Similar to other types of cancer, the development of HCC involves many steps including genetic and epigenetic alterations, activation of oncogenes and inactivation of tumor suppressor genes in the infected hepatocytes. Indeed, several HCV proteins have been implicated in disrupting cellular functions and contributing to malignant transformation[69,83,84]. Consequently, the dysregulation of multiple cellular pathways contributes to tumour progression and several members of the DEAD-box family have been shown to be involved in this process[85]. Here, we will highlight those studies involving HCC (Table 2) and discuss the potential contribution of the interactions between DEAD-box helicases and HCV.

The expression of two closely related members, namely DDX5 and DDX17, was found to be significantly downregulated in primary HCC when compared to non-cancerous liver tissue[86]. Notably, DDX5 and DDX17 are components of the miRNA biogenesis pathway and the same study reported that another 5 important genes in this pathway were also downregulated. Thus, it appears that the dysregulation of the miRNA biogenesis pathway is associated with the development of HCC, which is consistent with the results of studies performed in mice[87]. However, it seems that the downregulation of DDX5 in HCC may not be related to its ability to interact with HCV proteins because DDX5 was found to be downregulated in HBV-positive and non-HBV, non-HCV HCC but not in HCV-positive HCC.

Immunohistochemistry and Western blot analysis showed that DDX6 was over-expressed in 26 out of 29 HCV-positive liver tissues taken from patients with chronic hepatitis[88]. In addition, another 8 HCC samples all showed overexpression of DDX6. In contrast, DDX6 was not overexpressed in 3 control samples taken from the non-tumor portion of the liver of 3 patients who had non-HCV, non-HBV liver tumor. Interestingly, the level of DDX6 was found to be lower after treatment with interferon-α in 2 out of 3 patients whose levels of HCV RNA decreased after treatment. Hence, the replication of HCV may be responsible for upregulating DDX6 expression. Given that DDX6 is required for efficient HCV replication in Huh7.5 cells[78,79], it is probable that a positive feedback loop contributes to the development of HCC because HCV upregulates DDX6 expression

and the higher level of DDX6 further enhances HCV production. Coincidentally, DDX6, like DDX5, is also involved in the miRNA biogenesis pathway as it is part of the miRNA-induced silencing complex[89].

Similarly, DDX20 is also a component of the miRNA-containing ribonucleoprotein complexes[90,91]. It was first linked to cancer when an oncogenomic-based *in vivo* RNAi screen identified it as a novel tumor suppressor in murine liver cancers[92]. Subsequent studies suggested that DDX20 functions to suppress NF-κB activity by preferentially regulating the function of the NF-κB-suppressing miRNA-140[93]. Consistent with its role as a tumor suppressor, the knockdown of DDX20 resulted in NF-κB activation in hepatoma cell lines[93,94] and it is known that such activation is frequently observed in hepatitis-related HCC[95,96]. Indeed, up to 67% (47/70) of HCC samples were found to have reduced levels of DDX20 when compared to paired samples taken from the surrounding noncancerous liver, suggesting that the downregulation of DDX20 may contribute to the development of HCC[94]. Up until now, it has not been determined if DDX20 is involved in HCV replication or if it interacts with HCV proteins.

By comparing liver cancer cell lines with high and low transformation capabilities, DDX3 was identified as a potential oncogene and the overexpression of DDX3 increased the transformation capability of Tong cells in an anchorage-independent growth assay[97]. Quantitative RT-PCR further showed that the mRNA level of DDX3 was higher in 29 out of 45 (64%) HCC samples when compared to the surrounding normal tissues[97]. Consistent with this, two independent studies reported that the levels of auto-antibodies against DDX3 were significantly higher in serum taken from patients with HCC when compared to patients with chronic hepatitis or healthy volunteers[98,99].

While the above studies suggest that DDX3 is overexpressed in HCC, another study reported that DDX3 was significantly downregulated in HCCs from HBV^+ but not HCV^+ patients[100]. The reason for these contradictory observations is not clear but some possibilities could be the use of different types of assays, the demographic or clinicopathologic characteristics of the study populations. Curiously, a higher prevalence of DDX3 downregulation was observed in HCC from male patients compared to female patients[100]. Similarly, another study reported that the mRNA level of DDX3 was lower in 26 out of 45 HCC samples when compared to surrounding normal tissues[101]. Moreover, most of these 26 samples also showed lower mRNA levels of $p21^{waf1/cip1}$ and there was significant correlation between the expression of DDX3 and $p21^{waf1/cip1}$. Consistently, *in vitro* studies showed the overexpression of DDX3 in the liver cancer cell line Huh7 inhibited colony formation and upregulated the $p21^{waf1/cip1}$ by transactivating its promoter[101]. Conversely, knockdown of DDX3 by siRNA led to the downregulation of $p21^{waf1/cip1}$ and upregulation of cyclin D1, resulting in deregulation of cell cycle control in NIH-3T3 cells[100].

In summary, several members of the DEAD-box family have been found to be deregulated in HCC and they may contribute to the development of HCC. As the deregulation of miRNA expression is now recognized as being important in many cancers[102,103], it is intriguing to note that the DEAD-box proteins found to be up or downregulated in HCC are all involved in some aspects of the miRNA biogenesis pathway. Unfortunately, most of these studies have grouped together HCC samples from HCV^+, HBV^+ and non-HCV, non-HBV patients rather than analyzed each of the groups separately. Hence, further studies are needed to address whether the involvement of DEAD-box proteins in HCV replication is linked to the development of HCC in HCV patients. There is also a gap in the knowledge on the role of DEAD-box proteins in other forms of HCV-induced pathogenesis like fibrosis and cirrhosis.

CONCLUSION

The standard of care for anti-HCV therapy with Peg-IFN plus RBV is not optimal because a significant proportion of patients fail to achieve SVR and many patients are unable to tolerate the substantial side effects of treatment. Moreover, the treatment options for immunocompromised patients, patients with advanced liver disease and co-infection cases with HIV or HBV remain unsatisfactory. Limitations of the existing HCV treatment have led to extensive interest in the development of DAAs that target specific HCV proteins. Such anti-HCV agents are currently being evaluated in clinical trials and a couple of them have already been approved for clinical use in conjugation with Peg-IFN/RBV. To minimize the drug resistance, properly tailored combination therapies with drugs acting by different mechanisms are the need of the hour. It has become increasingly important to identify novel drug targets and widen the repertoire of anti-HCV agents. Since viral-host interactions are essential for HCV replication, they can potentially serve as important drug targets. HTAs have shown to possess certain merits over DAAs including a broader pan-genotypic activity, a higher genetic barrier to resistance development and a potential synergistic therapeutic effect when combined with DAAs.

As highlighted in this review, several members of the DEAD-box family are involved in HCV replication (Table 2). As these DEAD-box proteins share nine conserved motifs (Figure 1), it is intriguing that multiple DEAD-box RNA helicases are involved in the regulation of HCV replication and further studies are warranted to determine if they are involved in the same step(s) of the viral life cycle. It will also be important to determine if they act independently of each other or in parallel. For instance, Choi and Lee[104], revealed a novel protein-protein interaction between two DEAD-box RNA helicases, DDX3 and DDX5, which is affected by the phosphorylation states of both proteins and the cell cycle. Such interaction between members of the DEAD-box proteins may result in co-operativity or redundancy of their roles in relation to HCV replication. It is also possible that these host proteins are involved in more than one viral-host

interaction, for example, DDX3 not only binds to the HCV core protein, but is probably also associated with a HCV non-structural protein or HCV RNA itself[59,64,75]. A comprehensive understanding of the relative importance and specificity of the interaction between HCV and DEAD-box proteins may raise the possibility of targeting such a viral-host interaction for the development of therapeutics. However a detailed understanding of each viral-host interaction is required to evaluate if the interaction can be targeted without affecting the normal cellular homoeostasis.

While several DEAD-box helicases have been implicated in the development of HCC (Table 2), there is currently insufficient data to determine if their deregulation in HCC is related to their interaction with HCV. Furthermore, the dearth of small animal models that can mimic the HCV infection and liver pathogenesis that is observed in humans has significantly hampered studies to define the role of viral-host interactions in HCV-induced pathogenesis. However, with the recent advancement in the development of fully immunocompetent mouse models for the study of HCV[105,106], we hope to employ this model so as to further characterize the roles of DEAD-box proteins in HCV infection. For instance, we can leverage on the novel humanized mouse model developed by Washburn *et al*[107], which consists of a humanized mouse engineered by engraftment of both human hepatocytes and human immune cells. These humanized mice were shown to harbour both human hepatocytes and T cells, hence, they could support HCV infection and induce HCV-specific human immune response, liver inflammation, hepatitis, and fibrosis[107]. Similarly, we could use the recently developed genetically humanized inbred mouse model with blunted antiviral immunity, which allows the entire HCV life cycle to be completed[108]. This inbred mouse model is reported to overcome most of the inherent challenges of previous models, and thus can be manipulated and used in various stages of HCV research. With these humanized mice, we should be able to study *in vivo* the interaction of DEAD-box proteins with HCV and how this interaction could affect viral replication as well as contribute to disease pathogenesis. Moreover, investigations can be performed to determine if certain DEAD-box proteins play important roles in acute HCV infection while others are more involved in chronic HCV infection. It will also be crucial to delineate the contribution of individual DEAD-box protein to different steps in the virus life cycle or different viral-induced liver diseases like fibrosis and HCC. Knowledge gained from such studies would enhance our basic understanding of HCV replication and probably aid future work towards the development of more efficacious drugs and vaccine.

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