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Regulation of Vascular Endothelial Growth Factor (VEGF) Splicing from Pro-angiogenic to Anti-angiogenic Isoforms

*A NOVEL THERAPEUTIC STRATEGY FOR ANGIOGENESIS**

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Vascular endothelial growth factor (VEGF) is produced either as a pro-angiogenic or anti-angiogenic protein depending upon splice site choice in the terminal, eighth exon. Proximal splice site selection (PSS) in exon 8 generates pro-angiogenic isoforms such as $VEGF_{165}$, and distal splice site selection (DSS) results in anti-angiogenic isoforms such as $VEGF_{165}b$. Cellular decisions on splice site selection depend upon the activity of RNA-binding splice factors, such as ASF/SF2, which have previously been shown to regulate VEGF splice site choice. To determine the mechanism by which the pro-angiogenic splice site choice is mediated, we investigated the effect of inhibition of ASF/SF2 phosphorylation by SR protein kinases (SRPK1/2) on splice site choice in epithelial cells and in *in vivo* angiogenesis models. Epithelial cells treated with insulin-like growth factor-1 (IGF-1) increased PSS and produced more $VEGF_{165}$ and less $VEGF_{165}b$. This down-regulation of DSS and increased PSS was blocked by protein kinase C inhibition and SRPK1/2 inhibition. IGF-1 treatment resulted in nuclear localization of ASF/SF2, which was blocked by SPRK1/2 inhibition. Pull-down assay and RNA immunoprecipitation using VEGF mRNA sequences identified an 11-nucleotide sequence required for ASF/SF2 binding. Injection of an SRPK1/2 inhibitor reduced angiogenesis in a mouse model of retinal neovascularization, suggesting that regulation of alternative splicing could be a potential therapeutic strategy in angiogenic pathologies.

Vascular endothelial growth factor (VEGF-A, hereafter referred to as VEGF)[5] is a key regulatory component in physiological and pathological angiogenesis. Inhibition of VEGF has shown to be effective in cancer (1) and ocular angiogenesis (2), and it is up-regulated by a number of growth factors also implicated in these conditions, including insulin-like growth factor-1 (IGF-1) (3). VEGF is generated as multiple isoforms by alternative splicing (4). There are two principal families of VEGF isoforms, the pro-angiogenic $VEGF_{xxx}$ isoforms, generated by proximal splice site selection in the terminal exon, exon 8a (5), and the anti-angiogenic $VEGF_{xxx}b$ isoforms (6), generated by use of a distal splice site 66 bp further into exon 8, generating mRNA isoforms that contain exon 8b. As the stop codon for the protein is encoded in exon 8, these two isoforms contain alternate six amino acids at the C terminus (Fig. 1*A*). The pro-angiogenic isoforms such as $VEGF_{165}$ encode a terminal six amino acid sequence of CDKPRR, and the anti-angiogenic isoforms such as $VEGF_{165}b$ encode SLTRKD (7). Many normal tissues, including the eye generate both isoforms (8), and previous studies have shown that the anti-angiogenic isoforms dominate in non-angiogenic tissues such as the normal colon (9) and the vitreous (8). However, there is a splicing switch in angiogenic conditions such as proliferative diabetic retinopathy (8), colon (9), prostate (10), renal (7), and skin cancers (11), and in Denys Drash Syndrome (12). In contrast, in non-angiogenic conditions where VEGF is up-regulated, such as glaucoma and rhegmatogenous retinal detachment associated with proliferative vitreoretinopathy (13) or glaucoma (14), the anti-angiogenic isoforms are up-regulated. We have previously shown that IGF-1 can switch splicing in cultured epithelial cells from anti-angiogenic to pro-angiogenic isoforms (15). As IGF-1 has been implicated in a number of angiogenic conditions including diabetic retinopathy and colon cancer, we hypothesized that the mechanism through which IGF-1 mediates this change in splicing may be a potential therapeutic target to prevent angiogenesis. To this end, we have investigated the signaling pathways, the splicing factors involved, and the possibility of therapeutic intervention in the pathway in an animal model of diabetic retinopathy.

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[5] The abbreviations used are: VEGF, vascular endothelial growth factor; IGF-1, insulin-like growth factor; nt, nucleotide; PBS, phosphate-buffered saline; PKC, protein kinase C; ELISA, enzyme-linked immunosorbent assay; MBP, maltose-binding protein; PSS, proximal splice site selection; OIR, oxygen-induced retinopathy; UTR, untranslated region; ITS, insulin transferrin selenium; PMA, phorbol myristate acetate; HEK, human embryonic kidney.

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ARTICLE

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Development of a novel selective inhibitor of the Down syndrome-related kinase Dyrk1A

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Dyrk1A (dual-specificity tyrosine-(Y)-phosphorylation-regulated kinase 1A) is a serine/threonine kinase essential for brain development and function, and its excessive activity is considered a pathogenic factor in Down syndrome. The development of potent, selective inhibitors of Dyrk1A would help to elucidate the molecular mechanisms of normal and diseased brains, and may provide a new lead compound for molecular-targeted drug discovery. Here, we report a novel Dyrk1A inhibitor, INDY, a benzothiazole derivative showing a potent ATP-competitive inhibitory effect with IC_{50} and K_i values of 0.24 and 0.18 μM, respectively. X-ray crystallography of the Dyrk1A/INDY complex revealed the binding of INDY in the ATP pocket of the enzyme. INDY effectively reversed the aberrant tau-phosphorylation and rescued the repressed NFAT (nuclear factor of activated T cell) signalling induced by Dyrk1A overexpression. Importantly, proINDY, a prodrug of INDY, effectively recovered *Xenopus* embryos from head malformation induced by Dyrk1A overexpression, resulting in normally developed embryos and demonstrating the utility of proINDY *in vivo*.

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Hepatitis C Virus Core Protein Abrogates the DDX3 Function That Enhances IPS-1-Mediated IFN-Beta Induction

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Abstract

The DEAD box helicase DDX3 assembles IPS-1 (also called Cardif, MAVS, or VISA) in non-infected human cells where minimal amounts of the RIG-I-like receptor (RLR) protein are expressed. DDX3 C-terminal regions directly bind the IPS-1 CARD-like domain as well as the N-terminal hepatitis C virus (HCV) core protein. DDX3 physically binds viral RNA to form IPS-1-containing spots, that are visible by confocal microscopy. HCV polyU/UC induced IPS-1-mediated interferon (IFN)-beta promoter activation, which was augmented by co-transfected DDX3. DDX3 spots localized near the lipid droplets (LDs) where HCV particles were generated. Here, we report that HCV core protein interferes with DDX3-enhanced IPS-1 signaling in HEK293 cells and in hepatocyte Oc cells. Unlike the DEAD box helicases RIG-I and MDA5, DDX3 was constitutively expressed and colocalized with IPS-1 around mitochondria. In hepatocytes (O cells) with the HCV replicon, however, DDX3/IPS-1-enhanced IFN-beta-induction was largely abrogated even when DDX3 was co-expressed. DDX3 spots barely merged with IPS-1, and partly assembled in the HCV core protein located near the LD in O cells, though in some O cells IPS-1 was diminished or disseminated apart from mitochondria. Expression of DDX3 in replicon-negative or core-less replicon-positive cells failed to cause complex formation or LD association. HCV core protein and DDX3 partially colocalized only in replicon-expressing cells. Since the HCV core protein has been reported to promote HCV replication through binding to DDX3, the core protein appears to switch DDX3 from an IFN-inducing mode to an HCV-replication mode. The results enable us to conclude that HCV infection is promoted by modulating the dual function of DDX3.

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Introduction

The retinoic acid inducible gene-I (RIG-I) and the melanoma differentiation-associated gene 5 (MDA5) encode cytoplasmic RNA helicases [1–3] that signal the presence of viral RNA through the adaptor, IPS-1/Mitochondrial antiviral signaling protein (MAVS)/Caspase recruitment domain (CARD) adaptor inducing interferon (IFN)-beta (Cardif)/Virus-induced signaling adaptor (VISA) to produce IFN-beta [4–7]. IPS-1 is localized to the mitochondrial outer membrane through its C-terminus [6]. Increasing evidence suggests that the DEAD-box RNA helicase DDX3, which is on the X chromosome, participates in the regulation of type I IFN induction by the RIG-I pathway.

DDX3 acts on the IFN-inducing pathway by a complex mechanism. Early studies reported that DDX3 up-regulates IFN-beta induction by interacting with IKKepsilon [8] or TBK1 [9] in a kinase complex. Both TBK1 and IKKepsilon are IRF-3-activating kinases with NF-kappaB- and IFN-inducible properties. DDX3 has been proposed to bind IKKepsilon, and IKKepsilon is generated after NF-kappaB activation [10]. Yeast two-hybrid studies demonstrated that DDX3 binds IPS-1, and both are constitutively present prior to infection (Fig. 1). Ultimately, DDX3 forms a complex with the DEAD-box RNA helicases RIG-I and MDA5 [11], which are present at only low amounts in resting cells, and are up-regulated during virus infection. Previously we used gene silencing and disruption, to show that the main function of DDX3 is to interact with viral RNA and enhance RIG-I signaling upstream of NAP1/TBK1/IKKepsilon [11]. Hence, DDX3 is involved in multiple pathways of RNA sensing and signaling during viral infection.

DDX3 resides in both the nucleus and the cytoplasm [12], and has been implicated in a variety of processes in gene expression regulation, including transcription, splicing, mRNA export, and translation [13]. A recent report suggested that the N-terminus of hepatitis C virus (HCV) core protein binds the C-terminus of DDX3 (Fig. S1) [14,15], and this interaction is required for HCV replication [16]. Although DDX3 promotes efficient HCV infection by accelerating HCV RNA replication, the processes

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Original Article

Deregulation of miR-92a expression is implicated in hepatocellular carcinoma development

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MicroRNAs (miRNAs) belong to a class of the endogenously expressed non-coding small RNAs which primarily function as gene regulators. Growing evidence suggests that miRNAs have a significant role in tumor development and may constitute robust biomarkers for cancer diagnosis and prognosis. The *miR-17-92* cluster especially is markedly overexpressed in several cancers, and is associated with the cancer development and progression. In this study, we have demonstrated that miR-92a is highly expressed in hepatocellular carcinoma (HCC). In addition, the proliferation of HCC-derived cell lines was enhanced by miR-92a and inhibited by the anti-miR-92a antagomir. On the other hand, we have found that the relative amount of miR-92a in the plasmas from HCC patients is decreased compared with that from the healthy donors. Interestingly, the amount of miR-92a was elevated after surgical treatment. Thus, although the physiological significance of the decrease of miR-92a in plasma is still unknown, deregulation of miR-92 expression in cells and plasma should be implicated in the development of HCC.

Key words: hepatocellular carcinoma, microRNA, miR-638, miR-92a, plasma

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MicroRNAs (miRNAs) are small endogenous non-coding RNAs that regulate gene expression and have a critical role in many biological and pathological processes.[1] Recent studies have shown that deregulation of miRNA expression contributes to the multistep processes of carcinogenesis, and have shown promise as tissue-based markers for cancer classification and prognostication.[2,3] However, biological roles of only a small fraction of known miRNAs have been elucidated to date.

The miR-17-92 cluster at 13q31.3 is consists of six miRNAs: miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92a-1, and plays an important role for development of lung cancer,[4] B-cell lymphomas,[5] chronic myeloid leukemia,[6] medulloblastomas,[7] colon cancer[8] and hepatocellular carcinoma (HCC).[9] In addition, mice deficient in the miR-17-92 cluster died shortly after birth with lung hypoplasia, and B-cell development was impaired in the mice.[10] It has been reported, however, that miR-92a increases cell proliferation by negative regulation of an isoform of the cell-cycle regulator p63.[11] Furthermore, miR-92a regulates angiogenesis.[12] Thus, it is clear that the miR-92a has some oncogenic characteristics. However, the specific biological role of miR-92a in the processes of human cancer development has remained unclear.

Here, we have revealed that miR-92a is implicated in human HCC development. Furthermore, we have demonstrated that miR-92a in human blood has the potential to be a noninvasive molecular marker for diagnosis of human HCC.

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ORIGINAL ARTICLE

Establishment of an indicator cell system for hepatitis C virus

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ABSTRACT

Although a cell culture system for HCV JFH-1 strain has been developed, no robust cell culture system for serum-derived HCV is available. In this study, we have established systems capable of monitoring infection with JFH-1 virus based on specific reporter gene expression through proteolysis of chimeric transcription factors by HCV NS3/4A protease. We utilized a transcriptional factor Gal4-TBP that synergistically enhances transcription of the GAL4UAS and HIV-1 LTR tandem promoter with the Tat protein. We constructed chimeric Tat and Gal4-TBP transcription factors containing the HCV NS3/4A cleavage sequence of a mitochondria-resident IPS-1, but not those of the HCV polyprotein, and manipulated them to localize in the ER. Upon infection with JFH-1 virus, the transcription factors were efficiently cleaved by HCV protease, migrated into the nucleus and activated the reporter gene under the tandem promoter. Upon infection with JFH-1 virus, the Huh7OK1/TG-Luc cell line carrying the transcription factors and a luciferase gene under the promoter expressed luciferase in a dose-dependent manner in close correlation with HCV RNA replication. Huh7OK1/TG-LNGFR cells carrying the transcription factors and a cDNA of human low affinity nerve growth factor receptor under the promoter were selectively concentrated by immunomagnetic cell sorting upon infection with JFH-1 virus. These results indicate that the chimeric constructs bearing the ER-resident IPS-1 sequence are specifically recognized and efficiently cleaved by HCV protease and are harnessed for detection of HCV replication and for recovery of HCV-infected cells. This strategy may be applicable for the establishment of cell culture systems for the isolation of serum-derived HCV.

Key words HCV, indicator, IPS-1, NS3/4A, reporter.

HCV infects more than 170 million people worldwide, and is a major cause of chronic liver disease, including hepatic steatosis, cirrhosis, and hepatocellular carcinoma (1). Combination therapy with pegylated IFN and ribavirin has achieved a sustained virological response in 50% of individuals infected with HCV genotype 1 (2). The establishment of cell culture systems using an HCV genotype 2a JFH-1 strain isolated from a patient with fulminant

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List of Abbreviations: cDNA, complementary DNA; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; FFU, focus-forming units; FITC, fluorescein isothiocyanate; Gal4 DB, Gal4 DNA-binding; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, hemagglutinin; HCV, hepatitis C virus; HCVrv, recombinant VSV enveloped HCV E1 and E2 proteins; HIV-1, human immunodeficiency virus type 1; HO-1, heme oxygenase-1; IFN, interferon; IPS-1, interferon-β promoter stimulator-1; JEV, Japanese encephalitis virus; LNGFR, low affinity nerve growth factor receptor; LTR, long terminal repeat; MAGI, multinuclear activation of a galactosidase indicator; Mβ-CD, methyl-β-cyclodextrin; MOI, multiplicity of infection; PBE buffer, PBS containing 0.5% BSA and 2 mM EDTA; PBS-FBS, PBS containing 2% FBS; P-TEFb, positive transcription elongation factor b; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; RLU, relative luciferase units; SEAP, secretory alkaline phosphatase; S/N, signal-to-noise; TAR, transactivator response region; TBP, TATA-box binding protein; UAS, upstream activating sequence; VSV, vesicular stomatitis virus.

Network based analysis of hepatitis C virus Core and NS4B protein interactions†

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Hepatitis C virus (HCV) is a major cause of chronic liver disease worldwide. Here we attempt to further our understanding of the biological context of protein interactions in HCV pathogenesis, by investigating interactions between HCV proteins Core and NS4B and human host proteins. Using the yeast two-hybrid (Y2H) membrane protein system, eleven human host proteins interacting with Core and 45 interacting with NS4B were identified, most of which are novel. These interactions were used to infer overall protein interaction maps linking the viral proteins with components of the host cellular networks. Core and NS4B proteins contribute to highly compact interaction networks that may enable the virus to respond rapidly to host physiological responses to HCV infection. Analysis of the interaction networks highlighted enriched biological pathways likely influenced in HCV infection. Inspection of individual interactions offered further insights into the possible mechanisms that permit HCV to evade the host immune response and appropriate host metabolic machinery. Follow-up cellular assays with cell lines infected with HCV genotype 1b and 2a strains validated Core interacting proteins ENO1 and SLC25A5 and host protein PXN as novel regulators of HCV replication and viral production. ENO1 siRNA knockdown was found to inhibit HCV replication in both the HCV genotypes and viral RNA release in genotype 2a. PXN siRNA inhibition was observed to inhibit replication specifically in genotype 1b but not in genotype 2a, while SLC25A5 siRNA facilitated a minor increase in the viral RNA release in genotype 2a. Thus, our analysis can provide potential targets for more effective anti-HCV therapeutic intervention.

1. Introduction

Hepatitis C virus (HCV) is the causative agent of chronic liver disease including liver steatosis, cirrhosis and hepatocellular carcinoma (HCC) and infects nearly 3% of the population worldwide. HCV is a positive single-stranded RNA virus with a single 9600 nucleotide ORF flanked by 5′ and 3′-UTRs. The HCV ORF encodes a 3000 amino acid polyprotein, which undergoes proteolytic processing by host and viral proteases to yield four structural (Core, E1, E2 and p7) and six non-structural (NS2, NS3, NS4A, NS4B, NS5A and NS5B) proteins.[1–3] HCV variants span six genotypes that display phylogenetic heterogeneity, differences in infectivity and interferon sensitivity.[4] However, despite a wealth of concerted research, a precise understanding of the molecular mechanisms underlying HCV pathology remains elusive.

Most genes and proteins function in a complex web of interactions. Thus, the study of protein–protein interactions (PPIs) is critical to understanding the cellular networks that regulate the physiology of a living organism. The increasing availability of PPI data for human and host–pathogen interactions has led to increasing efforts in understanding the network basis of human diseases and pathogenesis.[5,6] In particular, the increasing availability of large scale interaction data between viral and human host proteins is likely to lead to a better understanding of viral pathogenesis and help identify novel targets for experimental and therapeutic intervention.[7–9] Comprehensive analyses of yeast two-hybrid (Y2H) screens have been employed to investigate the interactions of HCV,[7] Epstein–Barr virus[10] herpesviral[11] proteins with host factors. Analysis of such interactions suggests that viral (and bacterial) pathogens preferably interact with host proteins either engaged in a large number of interactions or critical to the integrity of the host cellular networks.[7,10]

Here, we report the host biological processes likely to be perturbed by HCV Core and NS4B proteins by virtue of inferred PPI networks. Core, also known as capsid protein, is spliced from the polyprotein by signal peptidase and further processed into a highly conserved 21 kDa mature form by the signal peptide peptidase; this processing facilitates its transfer to the detergent-resistant membrane fraction where virus replication and assembly take place.[12] Core also localises to the nucleus, which is essential for efficient viral propagation and development of HCV pathogenicity.[3,13] Core is a multi-functional protein implicated in RNA binding and as a pathogenic factor, which induces steatosis and HCC and thus, a promising target for anti-HCV therapy.[14,15] NS4B, the least

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† Electronic supplementary information (ESI) available: PPI networks analysed in the study and their functional associations. See DOI: 10.1039/c0mb00103a

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The ESCRT System Is Required for Hepatitis C Virus Production

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Abstract

Background: Recently, lipid droplets have been found to be involved in an important cytoplasmic organelle for hepatitis C virus (HCV) production. However, the mechanisms of HCV assembly, budding, and release remain poorly understood. Retroviruses and some other enveloped viruses require an endosomal sorting complex required for transport (ESCRT) components and their associated proteins for their budding process.

Methodology/Principal Findings: To determine whether or not the ESCRT system is needed for HCV production, we examined the infectivity of HCV or the Core levels in culture supernatants as well as HCV RNA levels in HuH-7-derived RSc cells, in which HCV-JFH1 can infect and efficiently replicate, expressing short hairpin RNA or siRNA targeted to tumor susceptibility gene 101 (TSG101), apoptosis-linked gene 2 interacting protein X (Alix), Vps4B, charged multivesicular body protein 4b (CHMP4b), or Brox, all of which are components of the ESCRT system. We found that the infectivity of HCV in the supernatants was significantly suppressed in these knockdown cells. Consequently, the release of the HCV Core into the culture supernatants was significantly suppressed in these knockdown cells after HCV-JFH1 infection, while the intracellular infectivity and the RNA replication of HCV-JFH1 were not significantly affected. Furthermore, the HCV Core mostly colocalized with CHMP4b, a component of ESCRT-III. In this context, HCV Core could bind to CHMP4b. Nevertheless, we failed to find the conserved viral late domain motif, which is required for interaction with the ESCRT component, in the HCV-JFH1 Core, suggesting that HCV Core has a novel motif required for HCV production.

Conclusions/Significance: These results suggest that the ESCRT system is required for infectious HCV production.

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Introduction

Hepatitis C virus (HCV) is a causative agent of chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma. HCV is an enveloped virus with a positive single stranded 9.6 kb RNA genome, which encodes a large polyprotein precursor of approximately 3,000 amino acid residues. This polyprotein is cleaved by a combination of the host and viral proteases into at least 10 proteins in the following order: Core, envelope 1 (E1), E2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B [1]. HCV Core, a highly basic RNA-binding protein, forms a viral capsid and is targeted to lipid droplets [2–6]. The Core is essential for infectious virion production [7]. NS5A, a membrane-associated RNA-binding phosphoprotein, is also involved in the assembly and maturation of infectious HCV particles [8,9]. Intriguingly, NS5A is a key regulator of virion production through the phosphorylation by casein kinase II [9]. Recently, lipid droplets have been found to be involved in an important cytoplasmic organelle for HCV production [4]. Indeed, NS5A is known to colocalize with the Core on lipid droplets [5], and the interaction between NS5A and the Core is critical for the production of infectious HCV particles [3]. However, the host factor involved in HCV assembly, budding, and release remains poorly understood.

Budding is an essential step in the life cycle of enveloped viruses. Endosomal sorting complex required for transport (ESCRT) components and associated factors, such as tumor susceptibility gene 101 (TSG101, a component of ESCRT-I), charged multivesicular body protein 4b (CHMP4b, a component of ESCRT-III), and apoptosis-linked gene 2 interacting protein X (ALIX, a TSG101- and CHMP4b-binding protein), have been found to be involved in membrane remodeling events that accompany endosomal protein sorting, cytokinesis, and the budding of several enveloped viruses, such as human immunodeficiency virus type 1 (HIV-1) [10–12]. The ESCRT complexes I, II, and III are sequentially, or perhaps concentrically recruited to the endosomal membrane to sequester

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Hepatitis C Virus Hijacks P-Body and Stress Granule Components around Lipid Droplets[▽]

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The microRNA miR-122 and DDX6/Rck/p54, a microRNA effector, have been implicated in hepatitis C virus (HCV) replication. In this study, we demonstrated for the first time that HCV-JFH1 infection disrupted processing (P)-body formation of the microRNA effectors DDX6, Lsm1, Xrn1, PATL1, and Ago2, but not the decapping enzyme DCP2, and dynamically redistributed these microRNA effectors to the HCV production factory around lipid droplets in HuH-7-derived RSc cells. Notably, HCV-JFH1 infection also redistributed the stress granule components GTPase-activating protein (SH3 domain)-binding protein 1 (G3BP1), ataxin-2 (ATX2), and poly(A)-binding protein 1 (PABP1) to the HCV production factory. In this regard, we found that the P-body formation of DDX6 began to be disrupted at 36 h postinfection. Consistently, G3BP1 transiently formed stress granules at 36 h postinfection. We then observed the ringlike formation of DDX6 or G3BP1 and colocalization with HCV core after 48 h postinfection, suggesting that the disruption of P-body formation and the hijacking of P-body and stress granule components occur at a late step of HCV infection. Furthermore, HCV infection could suppress stress granule formation in response to heat shock or treatment with arsenite. Importantly, we demonstrate that the accumulation of HCV RNA was significantly suppressed in DDX6, Lsm1, ATX2, and PABP1 knockdown cells after the inoculation of HCV-JFH1, suggesting that the P-body and the stress granule components are required for the HCV life cycle. Altogether, HCV seems to hijack the P-body and the stress granule components for HCV replication.

Hepatitis C virus (HCV) is the causative agent of chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma. HCV is an enveloped virus with a positive single-stranded 9.6-kb RNA genome, which encodes a large polyprotein precursor of approximately 3,000 amino acid (aa) residues. This polyprotein is cleaved by a combination of the host and viral proteases into at least 10 proteins in the following order: core, envelope 1 (E1), E2, p7, nonstructural 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B (12, 13, 21). The HCV core protein, a nucleocapsid, is targeted to lipid droplets (LDs), and the dimerization of the core protein by a disulfide bond is essential for the production of infectious virus (24). Recently, LDs have been found to be involved in an important cytoplasmic organelle for HCV production (26). Budding is an essential step in the life cycle of enveloped viruses. The endosomal sorting complex required for transport (ESCRT) system has been involved in such enveloped virus budding machineries, including that of HCV (5).

DEAD-box RNA helicases with ATP-dependent RNA-unwinding activities have been implicated in various RNA metabolic processes, including transcription, translation, RNA splicing, RNA transport, and RNA degradation (32). Previously, DDX3 was identified as an HCV core-interacting protein by yeast two-hybrid screening (25, 29, 43). Indeed, DDX3 is required for HCV RNA replication (3, 31). DDX6 (Rck/p54) is also required for HCV replication (16, 33). DDX6 interacts with an initiation factor, eukaryotic initiation factor 4E (eIF-4E), to repress the translational activity of mRNP (38). Furthermore, DDX6 regulates the activity of the decapping enzymes DCP1 and DCP2 and interacts directly with Argonaute-1 (Ago1) and Ago2 in the microRNA (miRNA)-induced silencing complex (miRISC) and is involved in RNA silencing. DDX6 localizes predominantly in the discrete cytoplasmic foci termed the processing (P) body. Thus, the P body seems to be an aggregate of translationally repressed mRNPs associated with the translation repression and mRNA decay machinery.

In addition to the P body, eukaryotic cells contain another type of RNA granule termed the stress granule (SG) (1, 6, 22, 30). SGs are aggregates of untranslating mRNAs in conjunction with a subset of translation initiation factors (eIF4E, eIF3, eIF4A, eIFG, and poly(A)-binding protein [PABP]), the 40S ribosomal subunits, and several RNA-binding proteins, including PABP, T cell intracellular antigen 1 (TIA-1), TIA-1-related protein (TIAR), and GTPase-activating protein (SH3 domain)-binding protein 1 (G3BP1). SGs regulate mRNA translation and decay as well as proteins involved in various aspects of mRNA metabolisms. SGs are cytoplasmic phase-dense structures that occur in eukaryotic cells exposed to various environmental stress, including heat, arsenite, viral infection, oxidative conditions, UV irradiation, and hypoxia. Impor-

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ARTICLE

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Leucettines, a Class of Potent Inhibitors of cdc2-Like Kinases and Dual Specificity, Tyrosine Phosphorylation Regulated Kinases Derived from the Marine Sponge Leucettamine B: Modulation of Alternative Pre-RNA Splicing

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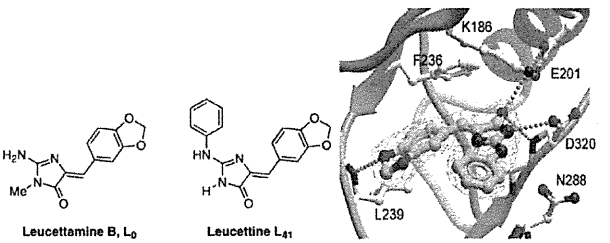
Supporting Information

ABSTRACT: We here report on the synthesis, optimization, and biological characterization of leucettines, a family of kinase inhibitors derived from the marine sponge leucettamine B. Stepwise synthesis of analogues starting from the natural structure, guided by activity testing on eight purified kinases, led to highly potent inhibitors of CLKs and DYRKs, two families of kinases involved in alternative pre-mRNA splicing and Alzheimer's disease/Down syndrome. Leucettine **L41** was cocrystallized with CLK3. It interacts with key residues located within the ATP-binding pocket of the kinase. Leucettine **L41** inhibits the phosphorylation of serine/arginine-rich proteins (SRp), a family of proteins regulating pre-RNA splicing. Indeed leucettine **L41** was demonstrated to modulate alternative pre-mRNA splicing, in a cell-based reporting system. Leucettines should be further explored as pharmacological tools to study and modulate pre-RNA splicing. Leucettines may also be investigated as potential therapeutic drugs in Alzheimer's disease (AD) and in diseases involving abnormal pre-mRNA splicing.



INTRODUCTION

Marine organisms constitute a rich source of high diversity chemical structures.[1] Marine natural products are being explored for multiple biomedical applications, mostly as potential anticancer agents but also in other therapeutic indications such as inflammation, neurodegenerative diseases, parasites, viral infections, and renal pathologies.[2] Among the many marine natural products that have been isolated, a number of molecules share a 2-aminoimidazolone ring structure (Figure 1). The best studied molecules of this series include leucettamine B,[3,4] polyandrocarpamines,[5] dispacamide,[6] aplysinopsine,[7] and hymenialdisine.[8] The marine sponge alkaloid (*Z*)-hymenialdisine was found to be a nanomolar inhibitor of various protein kinases including glycogen synthase-3β (GSK-3 β), casein kinase 1 (CK1), and different cyclin-dependent kinases (CDKs),[9] mitogen-activated protein kinase 1 (MEK-1),[10] and checkpoint kinase 1.[11] Protein kinases are the enzymes which catalyze protein phosphorylation, a key cellular regulatory mechanism which is frequently deregulated in human diseases. Consequently, protein kinases represent interesting

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Analysis of Interferon Signaling by Infectious Hepatitis C Virus Clones with Substitutions of Core Amino Acids 70 and 91▽§

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Substitution of amino acids 70 and 91 in the hepatitis C virus (HCV) core region is a significant predictor of poor responses to peginterferon-plus-ribavirin therapy, while their molecular mechanisms remain unclear. Here we investigated these differences in the response to alpha interferon (IFN) by using HCV cell culture with R70Q, R70H, and L91M substitutions. IFN treatment of cells transfected or infected with the wild type or the mutant HCV clones showed that the R70Q, R70H, and L91M core mutants were significantly more resistant than the wild type. Among HCV-transfected cells, intracellular HCV RNA levels were significantly higher for the core mutants than for the wild type, while HCV RNA in culture supernatant was significantly lower for these mutants than for the wild type. IFN-induced phosphorylation of STAT1 and STAT2 and expression of the interferon-inducible genes were significantly lower for the core mutants than for the wild type, suggesting cellular unresponsiveness to IFN. The expression level of an interferon signal attenuator, SOCS3, was significantly higher for the R70Q, R70H, and L91M mutants than for the wild type. Interleukin 6 (IL-6), which upregulates SOCS3, was significantly higher for the R70Q, R70H, and L91M mutants than for the wild type, suggesting interferon resistance, possibly through IL-6-induced, SOCS3-mediated suppression of interferon signaling. Expression levels of endoplasmic reticulum (ER) stress proteins were significantly higher in cells transfected with a core mutant than in those transfected with the wild type. In conclusion, HCV R70 and L91 core mutants were resistant to interferon *in vitro*, and the resistance may be induced by IL-6-induced upregulation of SOCS3. Those mechanisms may explain clinical interferon resistance of HCV core mutants.

Hepatitis C virus (HCV) is one of the most important pathogens causing liver-related morbidity and mortality. Approximately 3% of the worldwide population is infected with HCV, which represents 170 million people, and 3 million to 4 million individuals are newly infected each year (33, 47, 62). There is no therapeutic or prophylactic vaccine available for HCV. Antiviral treatment has been shown to improve liver histology and decrease the incidence of hepatocellular carcinoma in chronic hepatitis C (CHC) (17, 64). Current therapies for CHC consist of treatment with pegylated interferon (peg-IFN), which acts both as an antiviral and as an immunoregulatory cytokine, and ribavirin (RBV), an antiviral prodrug that interferes with RNA metabolism (16, 31). However, less than 50% of patients infected with HCV genotype 1 treated in this way achieve a sustained virological response (SVR) or a cure of the infection (14, 16). Given this situations, gaining a detailed understanding of the molecular mechanisms of interferon (IFN) resistance has been a high priority in academia and industry.

The response to peg-IFN-plus-RBV treatment is affected by several viral and host factors, including age, gender (22, 23), grade of liver fibrosis (21, 42), HCV genotype, and serum viral load (14, 59). Several viral genetic factors influence treatment outcomes, including mutations in NS5A-interferon sensitivity determining region (ISDR) (13, 38) and the core region (4, 6). Akuta et al. reported that HCV-core amino acid substitutions at positions 70 and 91 are significantly correlated with poor responses to peg-IFN-plus-RBV therapy (6) and with increased hepatocarcinogenesis (2, 3). Furthermore, it was reported recently that the core amino acid 70 and amino acid 91 substitutions are associated with a poor response to peg-IFN, RBV, and telaprevir combination therapy, respectively (1). However, the underlying molecular mechanisms of such distinct biological properties of the core 70/91 mutations are poorly understood.

In this study, we have analyzed virus infection and replication kinetics and response to interferon treatment using the HCV-JFH1 cell culture system (HCVcc) (60, 65). We constructed HCVcc expressing virus with substitutions of core amino acid 70 and amino acid 91 (R70Q, R70H, and L91M). The core mutant HCV clones were compared in terms of intracellular replication, infectious virus production, and sensitivity to alpha interferon (IFN-α). Here we have shown that the differences in sensitivity to IFN are attributable to upregulated overexpression of the cellular interferon signal attenuator SOCS3 and that this upregulation is caused by overexpression of interleukin-6 (IL-6).

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Expression of human factors CD81, claudin-1, scavenger receptor, and occludin in mouse hepatocytes does not confer susceptibility to HCV entry

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ABSTRACT

No suitable mouse model is available for studying chronic liver disease caused by hepatitis C virus (HCV). CD81, claudin-1, scavenger receptor class B type I, and occludin were recently reported to be the important factors in HCV entry into hepatocytes. We made transgenic mice (Alb-CCSO) expressing the four human proteins and examined whether HCV from a patient serum or HCV pseudoparticles (HCVpp) were capable of infecting them. HCV was not detected in the mouse serum after injecting the mice with HCV from a patient serum. We also found no indications of HCVpp entry into primary hepatocytes from Alb-CCSO mice. In addition, HCV-infectible Hep3B cells were fused with HCV-resistant primary mouse hepatocytes and the fused cells showed 35-fold lower infectivity compared to wild-type Hep3B cells, indicating that primary mouse hepatocytes have the inhibitory factor(s) in HCVpp entry. Our results suggest that the expression of the human factors does not confer susceptibility to HCV entry into the liver.

Hepatitis C virus (HCV) causes a chronic liver disease and affects an estimated 3% of the world population. No vaccine is available for preventing HCV, and the current treatment is often ineffective. The study of HCV has been hampered by the lack of suitable animal models. Chimpanzees are the only available *in vivo* experimental systems, but their use is limited by ethical concerns and high costs. HCV infection and replication in human hepatocytes has been confirmed in chimeric mice, the first reported mouse model (19). Although various factors, such as CD81, claudin-1 (CLDN1), scavenger receptor class B type I (SR-BI), and occludin (OCLN) have been reported to mediate HCV infection (8, 11, 21, 22), CD81 and OCLN must be human proteins to render mouse cells permissive to HCV infection (22). CD81 alone is insufficient to confer susceptibility to HCV in CD81 transgenic mice (16). CD81 and SR-BI interact with the E2 glycoprotein of HCV and have been suggested to play an initial role in attachment of the virus to cells (21, 23). After attachment, receptor-virus complexes are required to enter the cells as CD81-CLDN1-HCV complexes (13, 15) and OCLN-HCV complexes (2). Although HCV is known to enter cells via a pH- and clathrin-dependent endocytic pathway (3, 6, 14, 17, 26), the precise mechanisms are not fully elucidated. In the present study, we generated CD81, CLDN1, SR-BI, OCLN transgenic mice and examined whether HCV infect the liver using HCV from a patient serum and HCV pseudoparticles (HCVpp).

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Pretreatment prediction of response to peginterferon plus ribavirin therapy in genotype 1 chronic hepatitis C using data mining analysis

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Abstract
Background This study aimed to develop a model for the pre-treatment prediction of sustained virological response (SVR) to peg-interferon plus ribavirin therapy in chronic hepatitis C.
Methods Data from 800 genotype 1b chronic hepatitis C patients with high viral load (>100,000 IU/ml) treated by peg-interferon plus ribavirin at 6 hospitals in Japan were randomly assigned to a model building ($n = 506$) or an internal validation ($n = 294$). Data from 524 patients treated at 29 hospitals in Japan were used for an external validation. Factors predictive of SVR were explored using data mining analysis.
Results Age (<50 years), alpha-fetoprotein (AFP) (<8 ng/mL), platelet count ($\geq 120 \times 10^9$/l), gamma-glutamyl-transferase (GGT) (<40 IU/l), and male gender were used to build the decision tree model, which divided patients into 7 subgroups with variable rates of SVR ranging from 22 to 77%. The reproducibility of the model was confirmed by the internal and external validation ($r^2 = 0.92$ and 0.93, respectively). When reconstructed into 3 groups, the rate of SVR was 75% for the high probability group, 44% for the intermediate probability group and 23% for the low probability group. Poor adherence to drugs lowered the rate of SVR in the low probability group, but not in the high probability group.

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BASIC STUDIES

Anti-ulcer agent teprenone inhibits hepatitis C virus replication: potential treatment for hepatitis C

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Keywords
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Abstract

Background: Previously we reported that 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, statins, inhibited hepatitis C virus (HCV) RNA replication. Furthermore, recent reports revealed that the statins are associated with a reduced risk of hepatocellular carcinoma and lower portal pressure in patients with cirrhosis. The statins exhibited anti-HCV activity by inhibiting geranylgeranylation of host proteins essential for HCV RNA replication. Geranylgeranyl pyrophosphate (GGPP) is a substrate for geranylgeranyltransferase. Therefore, we examined the potential of geranyl compounds with chemical structures similar to those of GGPP to inhibit HCV RNA replication. *Methods:* We tested geranyl compounds [geranylgeraniol, geranylgeranoic acid, vitamin K_2 and teprenone (Selbex)] for their effects on HCV RNA replication using genome-length HCV RNA-replicating cells (the OR6 assay system) and a JFH-1 infection cell culture system. Teprenone is the major component of the anti-ulcer agent, Selbex. We also examined the anti-HCV activities of the geranyl compounds in combination with interferon (IFN)-α or statins. *Results:* Among the geranyl compounds tested, only teprenone exhibited anti-HCV activity at a clinically achievable concentration. However, other anti-ulcer agents tested had no inhibitory effect on HCV RNA replication. The combination of teprenone and IFN-α exhibited a strong inhibitory effect on HCV RNA replication. Although teprenone alone did not inhibit geranylgeranylation, surprisingly, statins' inhibitory action against geranylgeranylation was enhanced by cotreatment with teprenone. *Conclusions:* The anti-ulcer agent teprenone inhibited HCV RNA replication and enhanced statins' inhibitory action against geranylgeranylation. This newly discovered function of teprenone may improve the treatment of HCV-associated liver diseases as an adjuvant to statins.

Hepatitis C virus (HCV) infection frequently causes persistent hepatitis and leads to cirrhosis and hepatocellular carcinoma (HCC). Currently, the combination therapy of pegylated interferon (IFN) with ribavirin is available for patients with chronic hepatitis C (CH C) and yields a sustained virological response rate of about 50% (1). However, about half of CH C patients are still susceptible to the progression of the disease to fatal cirrhosis and HCC. Therefore, the development of more effective reagents for the treatment of HCV infection is urgent.

To overcome this problem, we developed a genome-length HCV RNA (strain O of genotype 1b) replication system (OR6) with luciferase as a reporter, which facilitated the prompt and precise monitoring of HCV RNA replication in hepatoma cells (HuH-7-derived OR6 cells) (2). Using this OR6 system, we recently reported that 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, statins, inhibited HCV RNA replication efficiently (3–5). Among five statins – fluvastatin (FLV), atorvastatin (ATV), simvastatin (SIV), pravastatin (PRV) and lovastatin (LOV) – FLV exhibited the strongest anti-HCV activity, while PRV had no effect on HCV RNA replication (3, 6). More recently, Bader *et al.* (7) demonstrated that FLV inhibited HCV RNA replication

*Contributed equally.

MTOpen

Efficient Generation of Hepatoblasts From Human ES Cells and iPS Cells by Transient Overexpression of Homeobox Gene *HEX*

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Human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) have the potential to differentiate into all cell lineages, including hepatocytes, *in vitro*. Induced hepatocytes have a wide range of potential application in biomedical research, drug discovery, and the treatment of liver disease. However, the existing protocols for hepatic differentiation of PSCs are not very efficient. In this study, we developed an efficient method to induce hepatoblasts, which are progenitors of hepatocytes, from human ESCs and iPSCs by overexpression of the *HEX* gene, which is a homeotic gene and also essential for hepatic differentiation, using a HEX-expressing adenovirus (Ad) vector under serum/feeder cell-free chemically defined conditions. Ad-HEX-transduced cells expressed α-fetoprotein (AFP) at day 9 and then expressed albumin (ALB) at day 12. Furthermore, the Ad-HEX-transduced cells derived from human iPSCs also produced several cytochrome P450 (CYP) isozymes, and these P450 isozymes were capable of converting the substrates to metabolites and responding to the chemical stimulation. Our differentiation protocol using Ad vector-mediated transient HEX transduction under chemically defined conditions efficiently generates hepatoblasts from human ESCs and iPSCs. Thus, our methods would be useful for not only drug screening but also therapeutic applications.

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INTRODUCTION

Human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are able to replicate indefinitely and differentiate into most cell types of the body,[1–4] and thereby have the potential to provide an unlimited source of cells for a variety of applications.[5] Hepatocytes are useful cells for biomedical research, regenerative medicine, and drug discovery. They are particularly applicable to drug screenings, such as for the determination of metabolic and toxicological properties of drug compounds in *in vitro* models, because the liver is the main detoxification organ in the body.[6] For these applications, it is necessary to prepare a large number of functional hepatocytes from human ESCs and iPSCs. Many of the existing methods for cell differentiation of human ESCs and iPSCs into hepatocytes employ undefined, serum-containing medium and feeder cells.[7–9] Preparation of human ESC- and iPSC-derived hepatocytes for therapeutic applications and drug toxicity testing in humans should be done in nonxenogenic culture systems to avoid potential contamination with pathogens. Furthermore, the efficiency of the differentiation of the human ESCs and iPSCs into hepatocytes is not particularly high using these methods.[9–14]

In vertebrate development, the liver is derived from the primitive gut tube, which is formed by a flat sheet of cells called the definitive endoderm.[5,15] Shortly afterwards, the definitive endoderm is separated into endoderm derivatives containing the liver bud, the cells of which are referred to as hepatoblasts. The hepatoblasts have the potential to proliferate and differentiate into both hepatocytes and cholangiocytes. In the process of hepatic differentiation, the maturation is characterized by the expression of liver- and stage-specific genes. For example, α-fetoprotein (AFP) is an early hepatic marker, which is expressed in hepatoblasts in the liver bud until birth, and its expression is dramatically reduced after birth.[16] In contrast, albumin (ALB), which is the most abundant protein synthesized by hepatocytes, is initially expressed at lower levels in early fetal hepatocytes, but its expression level is increased as the hepatocytes mature, reaching a maximum in adult hepatocytes.[17] Furthermore, isoforms of cytochrome P450 (CYP) proteins also exhibit differential expression levels according to the developmental stages

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ORIGINAL ARTICLE

Analysis of the complete open reading frame of hepatitis C virus in genotype 2a infection reveals critical sites influencing the response to peginterferon and ribavirin therapy

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Abstract
Purpose A proportion of patients infected with genotype 2a hepatitis C virus (HCV) cannot achieve a sustained virological response (SVR) to pegylated-interferon plus ribavirin therapy (PEG-IFN/RBV) but the reason remains unclear. The present study aimed to clarify the possible correlation between viral sequence variations and final outcome.
Methods The pretreatment complete open reading frame (ORF) sequences of genotype 2a HCV were determined by direct sequencing for two independent groups of patients (43 patients as test; group 1 and 35 as validation; group 2), and the correlation with the final outcome was explored.
Results Patients with SVR ($n = 58$) and with non-SVR ($n = 20$) differed significantly in pretreatment HCV RNA level ($p = 0.002$), fibrosis score ($p = 0.047$), and cumulative RBV dosage ($p = 0.003$). By comparison of all amino acid positions in the complete HCV ORFs, threonine at amino acid (aa) 110 in the core region was remarkably frequent in SVR ($p = 0.01$ for group 1, $p = 0.004$ for group 2, and $p = 5E{-}05$ for combined). A sliding window analysis revealed that the total number of amino acid variations within the NS5A aa 2258–2306 region were significantly high in SVR compared to non-SVR patients ($p = 0.01$ for group 1, $p = 0.006$ for group 2, and $p = 0.0006$ for combined). Multivariate analyses revealed that core aa 110 ($p = 0.02$), NS5A aa 2258–2306 ($p = 0.03$), and cumulative RBV dosage ($p = 0.02$) were identified as independent variables associated with the final outcome.
Conclusions The outcome of PEG-IFN/RBV therapy is significantly influenced by variation in the core and NS5A regions in genotype 2a HCV infection.

Abbreviations

EVR	Early virological response
IFN	Interferon
IRRDR	Interferon ribavirin resistance determinant region
ISDR	Interferon sensitivity determinant region
ORF	Open reading frame
PEG-IFN	Pegylated-interferon
PePHD	PKR-eIF2 phosphorylation homology domain
PKR-BD	Double-stranded RNA-activated protein Kinase binding domain
RBV	Ribavirin
RVR	Rapid virological response
SVR	Sustained virological response

Introduction

Worldwide, 180 million of people are estimated to be infected with hepatitis C virus (HCV), and HCV is a major cause of chronic hepatitis, liver cirrhosis, and

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Analysis of the Complete Open Reading Frame of Genotype 2b Hepatitis C Virus in Association with the Response to Peginterferon and Ribavirin Therapy

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Abstract

Background and Aims: Patients infected with genotype 2b hepatitis C virus (HCV) generally can achieve favorable responses to pegylated-interferon plus ribavirin therapy (PEG-IFN/RBV). However, a proportion of patients show poorer responses and the correlation between viral sequence variation and treatment outcome remains unclear.

Methods: The pretreatment complete open reading frame (ORF) sequences of genotype 2b HCV determined by direct sequencing were investigated for correlation with the final outcome in a total of 60 patients.

Results: In this study group, 87.5% (14/16) of non-sustained virological response (non-SVR) patients (n = 16) were relapsers. Compared to sustained virological response (SVR) patients (n = 44), non-SVR patients were older and could not achieve prompt viral clearance after the therapy induction. Comparing each viral protein between the two groups, viral sequences were more diverse in SVR patients and that diversity was found primarily in the E1, p7, and NS5A proteins. In searching for specific viral regions associated with the final outcome, several regions in E2, p7, NS2, NS5A, and NS5B were extracted. Among these regions, part of the interferon sensitivity determining region (ISDR) was included. In these regions, amino acid substitutions were associated with the final outcome in an incremental manner, depending upon the number of substitutions.

Conclusions: Viral sequences are more diverse in SVR patients than non-SVR patients receiving PEG-IFN/RBV therapy for genotype-2b HCV infection. Through systematic comparison of viral sequences, several specific regions, including part of the ISDR, were extracted as having significant correlation with the final outcome.

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Introduction

Worldwide, 180 million people are estimated to be infected with hepatitis C virus (HCV), a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) [1]. In HCV-infected patients with chronic hepatitis, treatment with interferon (IFN)-based therapy can result in viral clearance as well as biochemical and histological improvements [2]. In this IFN-based therapy, HCV genotype is the most significant factor affecting treatment responses [3,4].

In genotype 2b HCV infection, 80% of patients with high viral titers can achieve a sustained virological response (SVR) to the regimen of pegylated-interferon (PEG-IFN) plus ribavirin (RBV) for 24 weeks [5,6]. This response is high considering that much lower percentages of patients infected with other genotypes can achieve SVR, especially with genotype 1 [1]. However, in other words, 20% of patients infected with genotype 2b HCV still cannot clear the virus and remain at risk of developing HCC. On the other hand, although various studies have been undertaken to clarify the factors contributing to the response to IFN-based therapy in genotype 1 infection, it remains poorly understood which patients with genotype 2b HCV infection will show unfavorable responses. Recently, the significance of IL28B single nucleotide polymorphisms (SNPs) in determining the response to PEG-IFN/RBV therapy was demonstrated in genotype 1 HCV infection [7,8]. However, the significance of IL28B SNPs was rather weak in genotype 2 HCV infection [9].

In terms of the association between HCV sequence variation and treatment responses, previous studies have reported that

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Involvement of cyclophilin B in the replication of Japanese encephalitis virus

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ABSTRACT

Japanese encephalitis virus (JEV) is a mosquito-borne RNA virus that belongs to the *Flaviviridae* family. In this study, we have examined the effect of cyclosporin A (CsA) on the propagation of JEV. CsA exhibited potent anti-JEV activity in various mammalian cell lines through the inhibition of CypB. The propagation of JEV was impaired in the CypB-knockdown cells and this reduction was cancelled by the expression of wild-type but not of peptidylprolyl *cis-trans* isomerase (PPIase)-deficient CypB, indicating that PPIase activity of CypB is critical for JEV propagation. Infection of pseudotype viruses bearing JEV envelope proteins was not impaired by the knockdown of CypB, suggesting that CypB participates in the replication but not in the entry of JEV. CypB was colocalized and immunoprecipitated with JEV NS4A in infected cells. These results suggest that CypB plays a crucial role in the replication of JEV through an interaction with NS4A.

Introduction

The genus *Flavivirus* within the family *Flaviviridae* comprises over 70 viruses, many of which are predominantly arthropodborne viruses, such as Japanese encephalitis virus (JEV), West Nile virus (WNV), Murray Valley encephalitis virus, dengue virus (DENV), yellow fever virus (YFV), and tick-borne encephalitis virus. JEV is one of the most important flaviviruses in the medical and veterinary fields and exists in a zoonotic transmission cycle among mosquitoes, pigs, and birds mostly in Eastern and Southeast Asia. This virus spreads to dead-end hosts, including humans, through the bite of JEV-infected mosquitoes, and around 30,000–50,000 cases and up to 15,000 deaths are reported annually (Ghosh and Basu, 2009; Mackenzie et al., 2004; Solomon et al., 2003). JEV has a single-stranded positive-sense RNA genome of approximately 11 kb, which is capped at the 5′ end but lacks a 3′ polyadenine tail. The genome RNA is translated into a single large polyprotein at the endoplasmic reticulum (ER) membrane, then cleaved by the host- and virus-encoded proteases into three structural proteins, the capsid, precursor membrane (prM), and envelope (E) proteins, and seven nonstructural (NS) proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Sumiyoshi et al., 1987).

Flavivirus infection causes extensive rearrangement of cellular membranes to form two distinct membrane structures called the vesicle packet and convoluted membrane (Mackenzie et al., 1996; Miller and Krijnse-Locker, 2008). Whereas the vesicle packet is believed to contain the replication complex in which viral RNA synthesis takes place, the convoluted membrane is the putative site for viral polyprotein processing (Mackenzie et al., 1999). A recent tomography study clarified that the ER, convoluted membrane, and outer membrane of the vesicle packet were connected together to form a continuous membrane, with the vesicle packet being observed as an invagination of the ER with NS proteins and viral RNA, suggesting that viral replication occurred on the surface of the ER (Welsch et al., 2009). The structures of the convoluted membrane can be observed by infection with the WNV strain Kunjin virus or expression of the DENV NS4A protein alone (Miller et al., 2007; Roosendaal et al., 2006). Previous studies have indicated that NS4A localizes to both the vesicle packet and convoluted membrane and interacts with NS1, indicating that NS4A plays an important role as an integral scaffold of the replication complex (Lindenbach and Rice, 1999; Mackenzie et al., 1998).

In addition to NS proteins, flavivirus RNA replication is known to be regulated by several host factors, such as eEF1A, TIA/TIAR, HMGCR, and cyclophilin (Cyp) A (Davis et al., 2007; Emara and Brinton, 2007; Mackenzie et al., 2007; Qing et al., 2009). RNAi screening has identified various host factors involved in the replication of RNA viruses, including the hepatitis C virus (HCV), human immunodeficiency virus (HIV), and influenza A virus (Karlas et al., 2010; Konig et al., 2010, 2008; Tai et al., 2009). Host factors essential for viral replication might be an ideal target for antiviral development because the frequency of appearance of resistant viruses is lower by this method than when using antivirals targeted to the viral proteins.

In this study, we identified CypB as a host factor involved in the propagation of JEV. CypB is a member of the Cyp family, is ubiquitously expressed in most cells, and predominantly resides in the ER through the ER retention signal sequence in the C-terminus (Price et al., 1994,

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Sequences in the Interferon Sensitivity-Determining Region and Core Region of Hepatitis C Virus Impact Pretreatment Prediction of Response to PEG-Interferon Plus Ribavirin: Data Mining Analysis

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The aim of the present study was to clarify the significance of viral factors for pretreatment prediction of sustained virological response to pegylated-interferon (PEG-IFN) plus ribavirin (RBV) therapy for chronic hepatitis C using data mining analysis. Substitutions in the IFN sensitivity-determining region (ISDR) and at position 70 of the HCV core region (Core70) were determined in 505 patients with genotype 1b chronic hepatitis C treated with PEG-IFN plus RBV. Data mining analysis was used to build a predictive model of sustained virological response in patients selected randomly (n = 304). The reproducibility of the model was validated in the remaining 201 patients. Substitutions in ISDR (odds ratio = 9.92, $P < 0.0001$) and Core70 (odds ratio = 1.92, $P = 0.01$) predicted sustained virological response independent of other covariates. The decision-tree model revealed that the rate of sustained virological response was highest (83%) in patients with two or more substitutions in ISDR. The overall rate of sustained virological response was 44% in patients with a low number of substitutions in ISDR (0–1) but was 83% in selected subgroups of younger patients (<60 years), wild-type sequence at Core70, and higher level of low-density lipoprotein cholesterol (LDL-C) (≥120 mg/dl). Reproducibility of the model was validated ($r^2 = 0.94$, $P < 0.001$). In conclusion, substitutions in ISDR and Core70 of HCV are significant predictors of response to PEG-IFN plus RBV therapy. A decision-tree model that includes these viral factors as predictors could identify patients with a high probability of sustained virological response. ***J. Med. Virol. 83:445–452, 2011.***

KEY WORDS: data mining; decision-tree model; ISDR; core region; PEG-interferon

INTRODUCTION

The combination of pegylated-interferon (PEG-IFN) plus ribavirin (RBV) is currently the most effective therapy for chronic hepatitis C, but the rate of sustained virological response after 48 weeks of therapy is about 50% in patients with HCV genotype 1b and a high HCV

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Pretreatment prediction of anemia progression by pegylated interferon alpha-2b plus ribavirin combination therapy in chronic hepatitis C infection: decision-tree analysis

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Abstract
Background This study aimed to develop a model to predict the development of severe anemia during pegylated interferon alpha-2b plus ribavirin combination therapy.
Methods Data were collected from 1081 genotype 1b chronic hepatitis C patients who were treated at 6 hospitals in Japan. These patients were randomly assigned to a model-building group ($n = 691$) or an internal validation group ($n = 390$). Factors predictive of severe anemia (hemoglobin, Hb < 8.5 g/dl) were explored using data-mining analysis.
Results Hb values at baseline, creatinine clearance (Ccr), and an Hb concentration decline by 2 g/dl at week 2 were used to build a decision-tree model, in which the patients were divided into 5 subgroups based on variable rates of severe anemia ranging from 0.4 to 11.8%. The reproducibility of the model was confirmed by the internal validation group ($r^2 = 0.96$). The probability of severe anemia was high in patients whose Hb value was <14 g/dl before treatment (6.5%), especially (a) in those whose Ccr was <80 ml/min (11.8%) and (b) those whose Ccr was ≥80 ml/min but whose Hb concentration decline at week 2 was ≥2 g/dl (11.5%). The probability of severe anemia was low in the other patients (0.4–2.5%).
Conclusions The decision-tree model that included Hb values at baseline, Ccr, and an Hb concentration decline by

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Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

Evolution of hepatitis B genotype C viral quasi-species during hepatitis B e antigen seroconversion

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Background & Aims: Although the evolution of viral quasi-species may be related to the pathological status of disease, little is known about this phenomenon in hepatitis B, particularly with respect to hepatitis B e antigen (HBeAg) seroconversion.

Methods: Nucleotide sequences of the hepatitis B virus (HBV) *X/precore/core* region was analyzed at five time-points in four groups of chronic hepatitis B patients, interferon-induced seroconverters (IS, $N = 9$), interferon non-responders (IN, $N = 9$), spontaneous seroconverters (SS, $N = 9$), and non-seroconverters (SN, $N = 9$) followed during 60 months on an average. Only patients with genotype C were studied.

Results: Analysis of 1800 nucleotide sequences showed that there was no statistical difference between the nucleotide genetic distances of seroconverters (IS and SS; 6.9×10^{-3} substitutions (st)/site and 6.7×10^{-3} st/site, respectively) and those of non-seroconverters (IN and SN; 5.3×10^{-3} st/site and 3.8×10^{-3} st/site, respectively) before seroconversion. Compared to non-seroconverters (IN and SN; 5.1×10^{-3} st/site and 5.9×10^{-3} st/site, respectively), the sequence diversity of seroconverters (IS and SS; 10.9×10^{-3} st/site and 9.9×10^{-3} st/site, respectively) was significantly higher after seroconversion ($p < 0.05$), and was higher in seroconverters after seroconversion than before seroconversion ($p < 0.05$), while this changed very little in non-seroconverters during the observation period. Phylogenetic trees showed greater complexity in secoconverters than non-seroconverters. Parsimony-based estimation of the direction of sequence change between descendants and ancestors before HBeAg seroconversion, revealed higher frequencies of transversional A to T substitution in seroconverters (0.06 *vs.* 0.02, $p = 0.0036$) that coincided with the dynamics of quasi-species possessing A1762T mutation.

Conclusions: The distinctly greater viral diversity in HBeAg seroconverters after seroconversion could be related to escape mutants resulting from stronger selection pressure.

Keywords: Chronic hepatitis B; Quasi-species; Hepatitis B e antigen seroconversion.

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Abbreviations: SC, seroconversion; ALT, alanine aminotransferase; CHB, chronic hepatitis B; HBV, hepatitis B virus; IFN, interferon; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; IS, interferon-induced HBeAg seroconverters; IN, IFN non-responders; SS, spontaneous seroconverters; SN, non-seroconverters.

Introduction

Hepatitis B virus (HBV) is a major human pathogen which can cause severe hepatic disease, including chronic hepatitis, cirrhosis (LC), and hepatocellular carcinoma (HCC). Quasi-species comprises a complex and dynamic distribution of non-identical but related genomes [1]. The evolution of viral quasi-species has been reported as important in the pathogenesis of RNA viruses such as hepatitis C virus [2–6] and human immunodeficiency virus [7–10], but little is known about HBV. HBV is a hepatotropic, non-cytopathic DNA virus replicated by an error-prone polymerase through an RNA intermediate. Because of this feature, the replication of HBV lacks fidelity. This results in a complex distributions of genomes with naturally-acquired mutations or mutations selected by either antiviral therapy or the immune response of the host. HBV quasi-species have not been subjected to detailed investigation, especially in the context of hepatitis B e antigen (HBeAg) seroconversion (SC), an immunologically mediated event. Whether there is a causal relationship between HBV seroconversion and HBV quasi-species remains unclear. HBV-related disease is known to be mediated both virologically and immunologically. Several studies have depicted the dynamic evolution of HBV quasi-species during lamivudine resistance or multiple drug resistance. This highlights the importance of HBV molecular evolution in revealing the mechanism of drug resistance [11,12]. HBV-specific cytotoxic T-cells play a significant role in the control of replication of HBV, which has been well documented in the literature [13–16].

Precore/core protein is the target of immunologically mediated HBeAg seroconversion. When the *precore/core* gene in HBV DNA is transcribed and translated, HBeAg is produced and secreted into the circulation [17,18]. But the synthesis and secretion of HBeAg are aborted by the emergence of a point mutation from G to A at nucleotide (nt)1896 (G1896A). Convincing lines of evidence have indicated a close association between HBeAg/anti-HBe seroconversion and the emergence of *precore* and *core* promoter mutations [19,20].