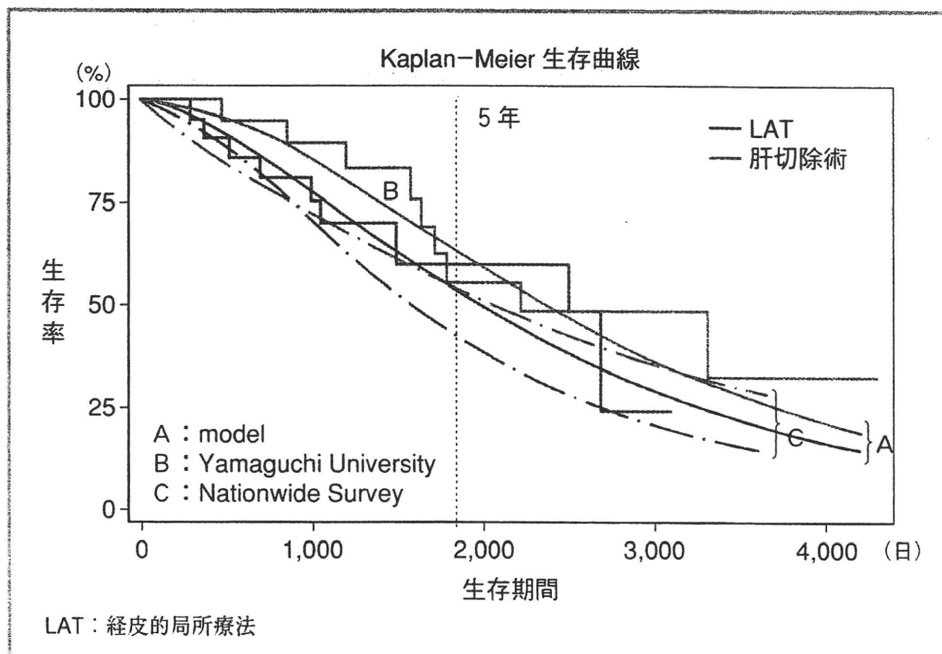
表5 各種治療における効用値

	median	平均（SD）	最小	最大	等価喪失日
肝切除					
RS	0.50	0.43（0.20）	0.20	0.70	8.6
TTO	0.25	0.33（0.13）	0.15	0.50	10.1
エタノール局所注入					
RS	0.70	0.72（0.13）	0.40	0.90	4.2
TTO	0.50	0.49（0.17）	0.15	0.75	7.6
ラジオ波熱凝固術					
RS	0.70	0.65（0.14）	0.30	0.80	5.2
TTO	0.50	0.75（0.42）	0.33	1.50	3.8
TACE					
RS	0.60	0.56（0.15）	0.20	0.70	6.5
TTO	0.67	0.78（0.36）	0.40	1.50	3.3
TACE＋LAT					
RS	0.50	0.47（0.17）	0.05	0.65	7.9
TTO	0.25	0.30（0.16）	0.10	0.50	10.6
化学療法					
RS	0.30	0.32（0.22）	0.01	0.70	10.1
TTO	0.17	0.22（0.16）	0.05	0.50	11.7

TACE：化学的腫瘍塞栓術，RS：評点尺度法，TTO：時間得失法

図2 生存曲線（モデル・実患者データ・全国原発性肝癌追跡調査）



LAT：経皮的局所療法

表6 治療に伴う費用（円）

年間治療の費用		
CH	232,000	
LC	316,000	
非代償性肝硬変症	1,439,000	

入院治療費用

治療	CH / LC	非代償性 LC
肝切除術	1,476,000	—
エタノール局所注入	570,000	1,073,000
ラジオ波熱凝固術	851,000	1,213,000
TACE＋LAT	1,017,000	1,028,000
TACE	639,000	762,000
化学療法	617,000	1,500,000
対症療法	624,000	624,000

CH：慢性肝炎，LC：代償性肝硬変症，TACE：化学的腫瘍塞栓術，
LAT：経皮的局所療法

表7 モデル，実データ，文献データにおける肝切除術，経皮的局所療法の3，5年生存率

初回治療法	モデル	実データ (Kaplan－Meier)	原発肝癌追跡調査
肝切除術			
3年	0.84	0.89（0.63～0.97）#	
5年	0.67	0.55（0.28～0.76）	0.77* ～0.59**
経皮的局所療法			
3年	0.68	0.70（0.45～0.85）	
5年	0.55	0.60（0.31～0.80）	0.65* ～0.49**

#：Greenwood による 95％信頼区間
*：単発・腫瘍径<2 cm，Child A　**：単発・腫瘍径 2～5 cm，Child A

plan－Meier 曲線に類似したものであった．3年および5年生存率で比較したのが表7である．Kaplan－Meier 曲線による 95％信頼区間は，症例数が少ないため非常に大きな範囲となっている．実データの5年生存率は肝切除に比べて内科的局所療法が高くなっているが，信頼区間からすると有意な差とは認められない．モデルの3年および5

表8 単発小肝細胞癌における経皮的局所療法と肝切除の費用対効果

初回治療	費用（円）	期待余命（年）	増分費用対効果比（円／年）
割引なし			
経皮的局所療法	8,230,000	6.44	
肝切除術	10,074,000	7.54	1,676,000
割引あり（3％／年）			
経皮的局所療法	7,301,000	5.63	
肝切除術	8,918,000	6.48	1,902,000

表9 単発小肝細胞癌における経皮的局所療法と肝切除の費用対効果（QOL 補正）

初回治療	費用（円）	QALYs	増分費用対効果比（円／QALY）
割引なし			
経皮的局所療法	8,230,000	4.43	
肝切除術	10,072,000	5.32	2,069,000
割引あり（3％／年）			
経皮的局所療法	7,301,000	3.88	
肝切除術	8,916,000	4.59	2,274,000

QALYs：生活の質（QOL）で補正した期待余命

年生存率は Kaplan－Meier 曲線によるものとほぼ同等と考えられ，モデルとしては妥当と考えられた．

6．費用対効果比

医療費をモデルに入れて求めた費用対効果分析の結果を表8に示す．肝切除術は割引（discount）なしの条件で内科的局所療法に比し約1.1年の期待余命の延長をもたらし，約180万円の生涯医療費の増加により，増分費用対効果は167万円／年となった．また，年3％の割引条件では，約190万円/年の増分費用対効果であった．さらに，QOL の指標である効用値（standard reference gamble）の平均値を組み入れた費用対効果の結果を表9に示す．割引なしの条件で207万円／QALY，年3％の割引条件で約227万円／QALY の増分費用対効果比であり，基本ケース（reference case）解析では，単発小肝細

胞癌の初回療法として外科的切除は内科的局所療法に比べて費用対効果的であるという結果であった．

おわりに

本研究はさらに多数例での検討を行うため，厚生労働省研究班『病期別にみた肝がん治療法の費用対効果および QOL の観点からみた有効性に関する研究』（主任研究者：沖田 極）において久留米大学，広島大学の症例も組み込んで解析を行っている．したがって，今後の解析結果によっては結果が異なる可能性も否定はできないが，少なくとも今回作成したモデルは妥当性のあるものと考えられた．従来の肝細胞癌治療の評価と異なり，このような費用対効果からの解析は肝細胞癌という疾患の特異性，複雑性から容易なものではないが，今後の肝細胞癌治療の選択基準の1つとなることを願っている．

日野啓輔・石田　博・井上裕二
沖田　極・坂井田功

文献

1) Partel D, et al: Cost-effectiveness of hepatocellular carcinoma surveillance in patients with hepatitis C virus-related cirrhosis. Clin Gastroenterol Hepatol 3: 75-84, 2005.

2) 石田 博, 他: 肝細胞がんにおける合併症と治療を考慮した医療費推定の試み. 医療情報学 24 (6): 667-674, 2005.

山形大学教授　河田純男
熊本大学教授　佐々木 裕

現場の疑問に答える

肝臓病診療 Q&A

中外医学社

Question 1

肝炎検診によるウイルス肝炎の実態はどうなっているのか？

Key point

- 老人健康法に基づく肝炎ウイルス検診（節目検診，節目外検診）の受診率は平均 26 %であった．
- 肝炎ウイルスキャリアの医療機関受診率は 80 %前後であったが，その約半数はかかりつけ医へ受診していた．
- HCV キャリアに開始された治療のうちインターフェロン治療の割合は年々増加傾向であったが，5 年間の平均は 17 %であった．

わが国の肝炎ウイルス感染者の掘り起こしを目的として，2002 年度から 2006 年度までの間に老人健康法に基づく保健事業である肝炎ウイルス検診（節目検診，節目外検診）が施行された．この 5 年間に数多くの肝炎ウイルスキャリアが発見されたが，その後のフォローアップ体制など種々の問題点も浮き彫りとなった．2008 年度からは新たなシステムによる肝炎ウイルス検診が始まろうとしているが，より効果的な検診を行うためにもこれまでの肝炎ウイルス検診の実態ならびに問題点の把握が必要である．

❶ 検診受診率と発見された肝炎ウイルスキャリア

肝炎ウイルス検診事業の実施主体は市町村であり，原則として基本健康診査時にあわせて実施される．節目検診では 40 歳から 70 歳までの 5 歳刻み，すなわち 40・45・50・55・60・65・70 歳に該当する者を対象者とし，5 年間で 40 歳から 70 歳まですべての基本健康診査対象者を網羅する．一方，節目検診の対象とならない者のうち，過去に肝機能異常を指摘されたことのある者，広範な外科的処置を受けたことのある者または妊娠・分娩時に多量に出血したことのある者であって定期的に肝機能検査を受けていない者を対象とするのが節目外検診である．

肝炎ウイルス検診のなかで節目検診受診者の割合は 2002 年度の 30 %を最高に徐々に低下し，5 年間の平均では 26 %であり，合計 6,280,111 人が検診を受けた（図 1）．5 年間の肝炎ウイルス検診（節目検診，節目外検診）受診者のうち C 型肝炎ウイルス（HCV）キャリアは 99,950 人，B 型肝炎ウイルス（HBV）キャリアは 100,983 人が新たに発見された．

JCLS 498-14204

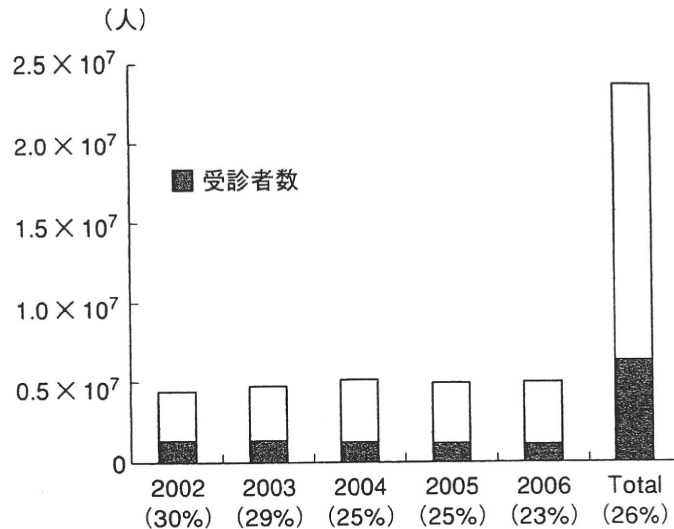


図 1 節目検診（2002 ～2006 年）受診対象者と受診者数

❷ 肝炎ウイルスキャリアの医療機関受診状況

厚生労働省の研究班の一員として，新たに発見された肝炎ウイルスキャリアの医療機関受診状況について全国調査を行った．5 年間を通じて都道府県からの回答率は 83 ～ 94 %と高かったが，残念なことに政令市からの回答率は 31 %と低かった．医療機関受診の有無を把握できたキャリアのうち実際に医療機関を受診した割合は初年度（2002 年度）を除けばおおむね 80 %強であり比較的高率であった（表 1，2）．実際にどの病院を受診したか，あるいは受診先が肝臓専門医の所属する病院か否かを把握することは困難であったため，いわゆるかかりつけ医に受診した割合を調査することで，どの程度のキャリアが肝臓専門医を受診していないかを推定した．医療機関としてかかりつけ医を受診した割合は 50 %弱であったが，2006 年度はいくぶん高い傾向がみられた（表 1，2）．さらに 5 年間に医療機関を受診した HCV キャリアのなかで何らかの治療を受けたのは 7,633 人であった．治療の内訳は表 3 に示すとおりで，特にインターフェロン治療の占める割合は平均で 17 %であった．しかし，2002 年度以降インターフェロン治療の割合は増加しており，2006 年度には 25 %に達した．

表 1　C 型肝炎ウイルスキャリアの医療機関受診に関する調査結果

	2002 年度	2003 年度	2004 年度	2005 年度	2006 年度
医療機関受診の有無確認	17,090	9,081	6,455	4,682	5,662
発見されたキャリアに対する割合（%）	71	45	50	41	43
医療機関受診者数	11,177	7,769	5,282	3,784	4,210
医療機関受診率（%）	65.4	85.6	81.8	80.8	79.3
かかりつけ医受診者数	—	3,491	2,548	1,765	2,478
かかりつけ医受診率（%）	—	44.9	48.2	46.6	55.2

表2 B型肝炎ウイルスキャリアの医療機関受診に関する調査結果

	2002年度	2003年度	2004年度	2005年度	2006年度
医療機関受診の有無確認	13,318	8,108	6,859	5,440	7,241
発見されたキャリアに対する割合（%）	71	43	45	40	42
医療機関受診者数	7,045	6,247	5,047	4,029	5,323
医療機関受診率（%）	52.9	77.0	73.6	74.1	73.5
かかりつけ医受診者数	—	2,989	2,490	1,928	3,229
かかりつけ医受診率（%）	—	47.8	40.3	47.9	60.7

表3 2002年度から2006年度までの二次医療機関受診者の治療内訳

	経口薬	注射薬（IFN以外）	インターフェロン（IFN）	その他または不明
2002年度	663(38%)	269(16%)	239(14%)	556(32%)
2003年度	785(43%)	258(14%)	244(13%)	609(33%)
2004年度	521(34%)	162(11%)	269(18%)	549(37%)
2005年度	401(36%)	112(10%)	217(20%)	381(34%)
2006年度	507(36%)	184(13%)	353(25%)	354(25%)
計	2,877(38%)	985(13%)	1,322(17%)	2,449(32%)

おわりに

この5年間の肝炎ウイルス検診の受診率は節目検診に限れば検診対象者の約4人に1人であり，キャリアと判明した人の約80%は医療機関を受診したものの，その半数近くはいわゆるかかりつけ医を受診しており肝臓専門医への受診率は低いと想定された．この影響があるのか否かは明らかでないがHCV感染に対する治療のなかでインターフェロン治療の施行率も低かった．このようにいくつかの課題を残して節目検診，節目外検診は終了したが，厚生労働省は老人保健法に基づく節目・節目外検診の結果を受けて新たに各自治体単位での肝炎ウイルス検診の充実やインターフェロン治療への財政的支援を打ち出している．

なお，本研究は厚生労働省科学研究費補助金肝炎等克服緊急対策研究事業，C型肝炎ウイルスの感染者に対する治療の標準化に関する臨床的研究（熊田班，分担研究者 沖田 極）ならびに肝炎ウイルス検診の現状把握と評価および今後のあり方に関する研究（吉澤班，分担研究者 日野啓輔）の一部として行われたことを付記する．

〈日野啓輔 沖田 極〉

Chapter 7

RNA Replication of Hepatitis C Virus

Hideki Aizaki[†] *and Tetsuro Suzuki*[*,†]

A precursor polyprotein of hepatitis C virus (HCV) is processed by host signalases and viral proteases into at least ten matured proteins. Nonstructural proteins, the NS2-3 protease, the NS3 serine protease, the NS3 helicase, and the NS5B RNA-dependent RNA polymerase function in the polyprotein processing and viral replication. Although studies on HCV replication have been hampered by the absence of a productive cell culture system, establishment of cell cultures that are highly permissive for replication of subgenomic and full-length HCV RNAs has opened new avenues to study the initiation of the viral replication. The use of the culture system has recently generated considerable information on HCV RNA replication, and as a result the formation of an intracellular membrane-associated replication complex (RC) composed of viral and cellular proteins as well as replicating RNA is proposed. Intensive research is being carried out to understand the cellular pathways for HCV RC formation and its biochemical function.

Introduction

Hepatitis C virus (HCV) is the most important causative agent of posttransfusion and sporadic non-A, non-B hepatitis. It is a positive-stranded RNA virus belonging to *Flaviviridae*, genus *Hepacivirus*, approximately 9.6 kb in length. Persistent infection with HCV is

*Corresponding author.

[†]Department of Virology II, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan.

associated with development of chronic hepatitis, hepatic steatosis, cirrhosis, and hepatocellular carcinoma. There are currently 170 million or more HCV carriers worldwide. In past years, anti-hepatitis C therapy has modestly improved; however, a currently available combination therapy, consisting of interferon (IFN)-α and the nucleoside analogue ribavirin, shows a sustained response in only less than half of the treated patients. The development of innovative treatment alternatives for patients infected with HCV is urgently required, and a better understanding of the mechanism of HCV replication should allow the identification of novel targets for antiviral intervention specific to HCV.

Cell Culture Systems for HCV Replication

Studies on HCV replication have long been hampered by the lack of efficient cell culture systems. Although many attempts have been made to achieve full replication of HCV in cell cultures, all systems including cell lines derived from human hepatoma, B cells and T cells, primary hepatocytes and peripheral blood mononuclear cells suffer from low virus yield and are not robust enough to allow genetic analyses of the HCV life cycle. The development of HCV subgenomic replicons (self-replicating RNAs) has allowed examination of viral RNA replication in cell culture.[1] Expression systems of heterologous virus genes based on RNA replicons have been established in a variety of positive-strand RNA viruses such as polio virus,[2–5] alphaviruses semliki forest virus,[6] sindbis virus,[7–10] kunjin virus,[11] human rhinovirus 14,[12] and bovine viral diarrhea virus.[13] In general, advantages of replicon systems are based on (1) a high level of gene expression and RNA replication, (2) easy construction of recombinants, and (3) the permitting of a wide host range.

The HCV replicons are typically composed of the 5′-nontranslated region (NTR), which directs translation of the gene encoding the neomycin phosohotransferase (Neor); the internal ribosome entry site (IRES) of the encephalomyocarditis virus (EMCV), which directs translation of HCV NS3 through NS5B region; and the 3′-NTR. Following transfection of RNA transcribed from the above bicistronic constructs into a human hepatoma cell line Huh-7, antibiotic G418-resistent cells could be obtained in which the subgenomic RNA replicated autonomously. RNA replication was at first detected at low

frequency, followed by the identification of replicons harboring cell culture-adaptive mutations yielding higher replication efficiency.[14–16] Some of the most adaptive mutations are located at highly conserved serine residues within NS5A upstream of the region putatively involved in IFN sensitivity. A combination of adaptive mutation in NS3 and NS5A resulted in the highest level of replication of particular HCV genotype 1b isolate.[16] Later work, however, has indicated that adaptive mutations can arise in most of the viral nonstructural proteins.[17,18] The mechanisms by which adaptive mutations increase RNA replication efficiency are not well understood.

Huh-7 cells have been permissive for adapted HCV replicons, although variability in the permissiveness for replicons has been observed for these cells. Recently, some studies showed that HCV subgenomic RNAs could replicate in human hepatic HepG2 and IMY-N9 cells, human cervical carcinoma HeLa cells, human embryonic kidney 293 cells,[19,20] a human T cell line MT-2C[21] and mouse hepatoma cells.[22] These findings suggest that host factors required for HCV RNA replication are not hepatocyte specific and are not restricted to cells of human origin.

Finally, taking advantage of cell culture-adaptive mutations, full-length dicistrinic HCV genomes that efficiently and stably replicate in Huh-7 cells have been developed. By immuno-electron microscopy, the core protein expressed in the cells harboring the genomic HCV RNA was localized mainly to the boundary of cytoplasmic lipid storage vesicles and was also found at the endoplastic recticulum (ER).[23] In the cells, viral envelope proteins E1 and E2 formed heterodimers and existed at the ER and cis-Golgi compartments. Cell culture systems based on the selectable subgenomic and genome-length dicistronic HCV RNAs, which produce abundant viral RNA and nonstructural proteins, open avenues of biochemical and genetic studies for HCV replication.

Polyprotein Translation and Processing

The HCV genome carries a 5′-NTR, an open reading frame (ORF) that encodes a polyprotein with a length of ~3010 amino acids, and a 3′-NTR. The precursor polyprotein is co-translationally or post-processed by both viral and host proteases into at least ten viral products.

These viral products include i) structural proteins consisting of the following virus particles: core, E1, and E2, which are encoded at the N terminus; and ii) nonstructural proteins involved in viral RNA replication, which encompass the remainder of the viral polyprotein and consist of NS2, NS3, NS4A, NS4B, NS5A, and NS5B.

The ~340 nucleotide long 5′ NTR is functionally characterized as IRES to direct cap-independent translation of the genome. The HCV 5′ NTR is highly conserved in the genome and the secondary structural models reveal four distinct RNA domains in the region.[24] The IRES has been shown to cover most of the 5′-NTR, with the 5′-end being located in between (nt) 28 and 69. By analogy with other RNA viruses with IRES-mediated expression, the HCV 5′-NTR has been expected to contain not only determinants for translation, but also cis-acting elements for RNA replication. Recent studies demonstrated that i) the sequence upstream of the IRES is essential for the viral RNA replication, ii) sequences within the IRES are required for high-level HCV replication, and iii) the stem-loop domain II of the IRES is crucial for the replication.[25]

The 3′-NTR also contains significant predicted RNA structure with three distinct domains: a variable region (VR) of ~40 nucleotides, a variable length poly(U/UC) tract, and a highly conserved 98-nt 3′ terminal segment (3′X) that putatively forms three stem-loop structures.[26–28] The viral RNA replication was not detected when any of the three putative stem-loop structures within the 3′X region or whole of poly(U/UC) were deleted.[29] The VR segment also contributes to efficient RNA replication.[30]

Nonstructural proteins of HCV are processed by two viral proteases: the junction of NS2 and NS3 is cleaved by the NS2-3 protease, which spans NS2 and the N-terminal domain of NS3; the remaining four junctions are cleaved by the serine protease located at the N-terminal 180 residues of NS3 protein. NS2 protein is highly hydrophobic and the autoproteolytic reaction presumably occurs in a membrane compartment. Deletion of NS2 from the HCV nonstructural polyprotein did not abolish the replication of HCV RNA in cell culture, indicating that it is not required for viral RNA replication.[1,14] NS3 is a multifunctional molecule. Besides its N-terminal protease

activity, the helicase and nucleotide triphosphatase (NTPase) activities reside in the C-terminal 500 residues of the NS3 protein.[31–35] Helicase/NTPase activities are essential for the replication of the HCV genome. The helicase activity is presumed to be involved in unwinding a putative double-stranded replication intermediate or to remove regions of secondary structure so that the RNA-dependent RNA polymerase of NS5B (see below) can copy both strands of the viral RNA. It is likely that the NTPase activity is coupled with the helicase function, supplying the energy required for disrupting RNA duplexes.

NS4A functions as a cofactor of the NS3 serine protease and is required for efficient polyprotein processing. There are significant differences in the stability and activity of the NS3 protease in the presence and absence of NS4A. NS3 protein is relatively unstable when expressed in cells in the absence of NS4A.[36] Structural studies by NMR and X-ray methods indicate that the NS3-4A complex has a more highly ordered N-terminal domain, and NS4A binding leads the NS3 protease to a rearrangement of the active site triad to a canonical conformation.[37] It has been predicted that the N-terminus of NS4A forms a transmembrane helix, which presumably anchors the NS3-4A complex to the cellular membrane.[38] NS4B is a hydrophobic, membrane-associated protein, which colocalizes predominantly with ER markers.[39–42] Although relatively little is known to date about functions of NS4B in the HCV life cycle, inhibitory activities in the translation[43,44] and modulation of NS5B enzymatic function[45] have been reported. It has also been shown that NS4B protein can induce a membranous web consisting of small vesicles embedded in a membranous matrix,[46] and that the newly synthesized HCV RNA exists in these membrane webs and speckles.[47,48] NS4B may play an important role in the formation of the HCV RNA replication complex.[49]

NS5A is a phosphoprotein, which is mainly phophorylated on serine residues[50] mediated by one or more cellular serine-threonine kinases.[51–54] The role of NS5A phosphorylation in HCV replication is so far not clear; however, it has been thought that the protein is important for the viral lifecycle since phosphorylation of NS5A is a

conserved feature among different HCV isolates and among other members of the *Flaviviridae*. Evidence indicating an involvement of NS5A in the viral RNA replication is now accumulating. A hot spot of the cell culture-adaptive mutations that increase replication efficiency of HCV RNA is located in the central region of NS5A.[14–16] The membrane association of NS5A through its N-terminal transmembrane domain[55] and the interaction between NS5A and 5B[56] are essential for the RNA replication. Several cellular proteins interacting with NS5A have been identified, and human vesicle-associated membrane protein-associated protein A (h-VAP-A) is likely to play a key role in the RNA replication through the interacting with NS5A, as demonstrated by experiments using RNA interference and dominant-negative protein fragments.[49] In addition to its function related to the viral replication, NS5A appears to be implicated in resistance of HCV-infected cells to the antiviral effect of IFN. At least for some HCV isolates, NS5A interacts structurally and functionally with an IFN-induced protein kinase PKR, leading to the inhibition of the kinase function and hence blocking the translation reduction in the IFN-treated cells.[57,58]

NS5B is an RNA-dependent RNA polymerase (RdRp), a key enzyme involved in viral replication.[27,59–64] The RdRp activity of NS5B as well as its three-dimensional structure have been demonstrated using recombinant gene products prepared from a variety of expression systems. The optimal temperature, pH requirements and concentration of bivalent cations for the activity were found to be similar to those for the poliovirus 3D polymerase.[61] The X-ray studies have reported that while the enzyme has the typical right-handed "finger-palm-thumb" domains of the polymerase, extensive interactions of the fingers and thumb lead to a more fully enclosed active site tunnel, unlike other RdRp.[65–67] NS5B contains a hydrophobic domain at its C terminal 21 residues, which is a transmembrane segment. Recent studies have demonstrated that the C-terminal domain of NS5B appears to serve dual functions in the viral RNA replication, both through its role as a membrane anchor and through its involvement in RNA synthesis in a sequence-specific manner.[68,69]

RNA Replication Model

RNA replication of most RNA viruses involves certain intracellular membrane structures, including the ER,[70–72] Golgi,[73] endosomes and lysosomes.[74] Although NS5B protein has RdRp activity *in vitro*, its recombinant product alone is presumably short of the strict template specificity and fidelity, which are essential for the viral RNA synthesis. It is highly likely that other viral and/or host factors are important for conferring proper RNA replication and that the replication complexes (RCs) that are composed of NS5B and additional components required for modulating polymerase activity are involved in catalyzing HCV RNA synthesis during the replication process.

Several coprecipitation and immunostaining studies revealed that the newly synthesized HCV RNA was localized to distinct speckle-like structures, where all of the viral nonstructural proteins coexisted.[48] These distinct structures may be equivalent to the membranous webs, as reported by Gosert *et al.*[45] and described above. Expression of all structural and nonstructural proteins in the context of the entire HCV polyprotein induced similar membrane changes.[46] It is of interest that morphologically similar structures, termed sponge-like inclusions,[75] have been found by electron microscopy in liver cells of HCV-infected chimpanzees. Thus, the membranous web may comprise the HCV RC in infected cells.

Recently, several groups have succeeded in showing the *in vitro* replication activities of the HCV RCs in crude membrane fractions of cells harboring the subgenomic replicons.[76–80] These cell-free systems provide a valuable complement to the *in vitro* RdRp assays for biochemical dissection of HCV RNA replication and a useful source for isolation of viral RCs. The replication activity in the crude membrane fraction, which contains HCV ribonucleoprotein complexes associated with cellular membranes, is measured by incorporation of radiolabeled nucleotides into newly synthesized RNA *in vitro*, and the products can be resolved from replicative intermediates by native or denaturing gel electrophoresis. The RNA synthesis can be initiated in the absence of added negative-strand template RNA, suggesting that preinitiated template RNA copurifies with the RC.[77–79] Although the

newly synthesized single-strand RNA can be used as a template for a further round of double-strand RNA synthesis, no exogenous RNA can be served as a template for HCV RC preparation.[79] The added RNA templates might not access the active site of the HCV RCs due to sequestration by membranes. The HCV RCs contain both minus- and plus-strand RNAs,[77,78] with the plus-strand RNA being approximately 10-fold more abundant than the minus strand.[76] This is consistent with the replication process for other positive-strand RNA viruses, which produce a negative-sense replication intermediate in smaller amounts than that of the genomic RNA. It has also been reported that the cell-free replication activity increases at temperatures ranging from 25°C to 40°C, and divalent cations (Mn^{2+} and Mg^{2+}) can be used in the reaction.[78,79]

Shi *et al.*[48] have demonstrated that newly synthesized HCV RNA and the nonstructural proteins colocalized on distinct speckle-like structures in the cytoplasm of the replicon-containing cells. Membrane flotation analysis and replication assay have shown that the viral RNA and proteins were present in detergent-resistant membrane (DRM) structures, most likely a lipid-raft structure, and that RNA replication activity was detected even after treatment with detergent.[76,81] Lipid rafts are cholesterol- and sphingolipid-rich microdomains and are characterized by their detergent insolubility.[82–84] The structures are known to play a critical role in a number of biological processes such as regulators and organizing centers of signal transduction and membrane traffic pathways, including virus entry and assembly of, for example, influenza virus,[85–87] human immunodeficiency virus type-1 (HIV-1),[88–90] Ebola, Marburg virus,[91] enterovirus,[92] avian sarcoma and leukosis virus,[93] Coxsackie B virus, adenovirus,[94] measles virus,[1] and respiratory syncytial virus.[95] However, HCV may be the first example of the association of lipid rafts with viral RNA replication.

On the other hand, it has been widely thought that most of the HCV life cycle, including the protein processing and genome replication, takes place at the ER, where cholesterol-sphingolipid rafts are not assembled.[46,96–98] Several studies using the replicon system have indicated that the nonstructural proteins were associated with the ER.[97,99] Nevertheless, it is still possible that HCV nonstructural proteins that

are synthesized at the ER relocalize to lipid raft membranes when they are actively engaged in RNA replication. It has been shown by the membrane separation analysis that HCV nonstructural proteins existed both in the ER and the Golgi, but the activity of viral RNA replication was detected mainly in the Golgi fraction.[76,100] Further studies to elucidate where and how the HCV genome replicates in the infected cells are needed.

Following is a model for the HCV RC formation proposed by Aizaki *et al.*[76] HCV nonstructural proteins are processed from precursor polyprotein by the viral proteases and localized at the ER. A part of the nonstructural proteins and host factors interacted are transported to the Golgi, where they associate directly or indirectly, via protein-protein interactions, with lipid rafts (Fig. 1). NS4B may bind to lipid rafts first and then recruit other viral proteins consecutively to form RCs, since it appears that NS4B, but not other HCV proteins, associate with lipid rafts when expressed alone. A vesicle-associated protein hVAP-33, which possibly binds to NS5A and NS5B,[98] may also contribute to the formation of HCV RCs. Rafts harboring putative RCs may then be stabilized and combined to create DRM structures, presumably involved in the nature that NS4B can be oligomerized.[101] During the process of RC polymerization, HCV RNA is enclosed within the membrane complex. This process, which permits high local concentration of NS5B and the viral RNA, may be advantageous in order to confer template specificity and ensure RNA synthesis with necessary fidelity during the viral replication.

Host Factors Possibly Involved in HCV RNA Replication

Studies with the RNA replicons have demonstrated that the viral RNA level is highest in the growth phase of the cells and drops significantly when cells reach a confluent state, suggesting that HCV replication and/or translation are tightly linked to host cell metabolism.[99] Huh-7 cells in which adapted replicons are cured by treatment with IFN are able to yield cell populations that are more permissive for the replicon tested. Thus, it is likely that some interplay between

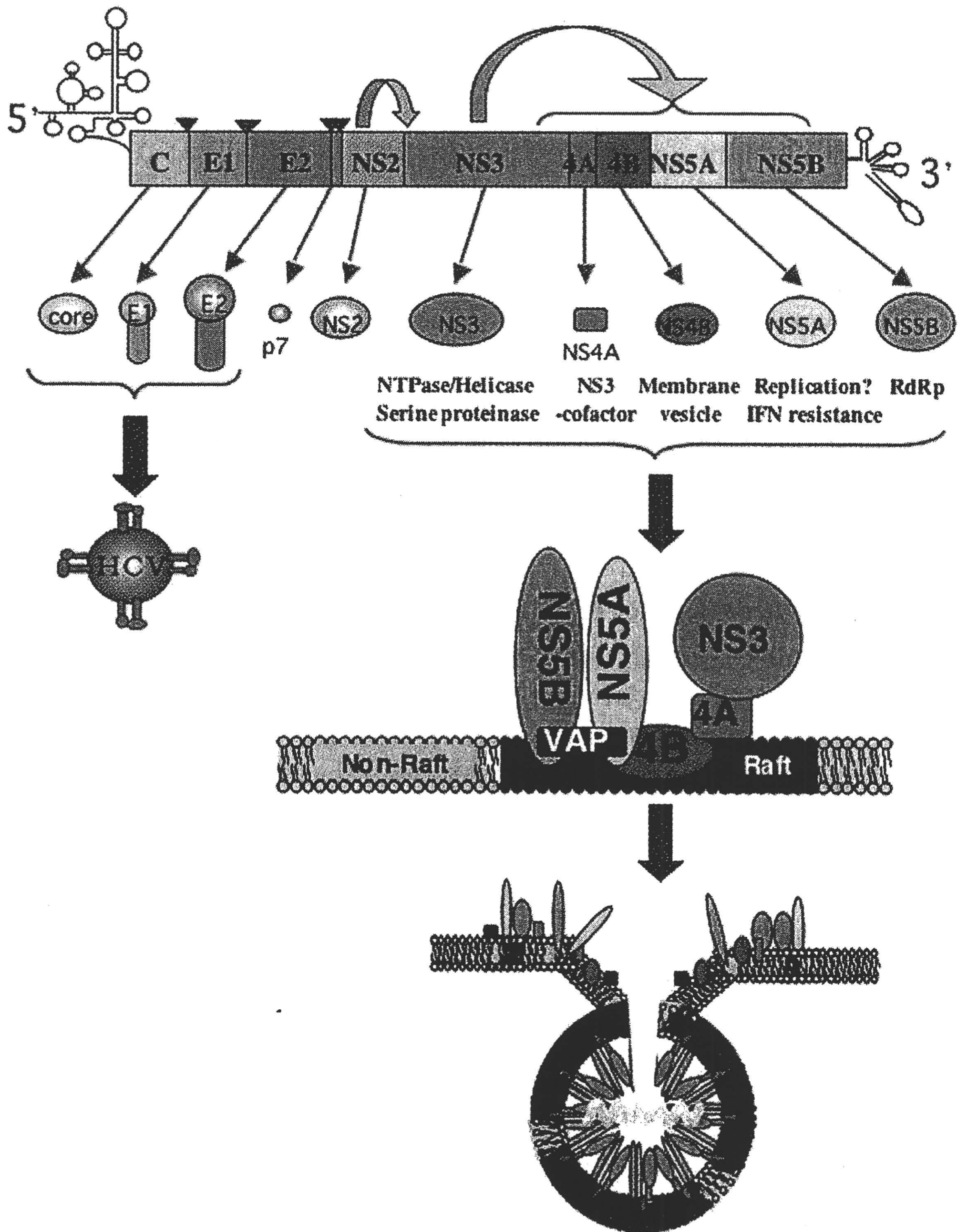


Fig. 1. Illustration of the HCV genome and replication complex. A schematic presentation of the HCV genome is given at the top. The individual cleavage products of the polyprotein are given in the second level down. The functions of the viral proteins are given in the third level down. A model of the formation of HCV replication complex on the lipid raft is shown at the bottom.

the cellular environment and particular adaptive mutations of the viral RNA is important for efficient RNA replication of HCV.

There is increasing experimental evidence that shows specific interactions between various cellular factors and HCV nonstructural proteins and/or RNA, possibly playing roles in HCV replication and/or translation. Several cellular proteins interacting with NS5A, such as hVAP-33,[98] growth factor receptor-bound protein 2 adaptor protein,[102] transcription factor SRCAP,[103] and karyopherin b3,[104] have been identified. Among them, it is of interest that hVAP-33, a SNARE-like protein, can interact with NS5B as well; NS5A binds to the C-terminus of hVAP-33, whereas NS5B binds to the N-terminus.[98] Down-regulation of hVAP-33, either through siRNA or expression of a truncated, dominant negative fragment of this protein, has lead to inhibition of HCV RNA replication, suggesting that protein-protein interactions among NS5A, NS5B and hVAP-33 are critical for formation of HCV RC and viral RNA replication (Fig. 1).[49] Another NS5B-interacting protein identified is a human RNA helicase, p68.[105] Expression of NS5B alone or of all the nonstructural proteins induced the redistribution of endogenous p68 from the nucleus to the cytoplasm. It has also been shown that knockdown of p68 reduces the negative-strand synthesis of the HCV genome, suggesting that the NS5B-p68 interaction and the subsequent relocalization of cellular proteins may serve to mediate HCV replication processes.

Cellular components binding to HCV RNA are also possibly involved in viral replication. Some candidates are La autoantigen, polyprimidine tract-binding protein (PTB), NS1-associated protein 1 (NSAP1), human heterogeneous nuclear ribonucleoprotein (hnRNP) L,[106] 40S ribosomal subunit protein,[107] and the gamma subunits of human eukaryotic initiation factors 2B (EIF2B-gamma) and 2 (EIF2-gamma).[108] La antigen, which is known to bind to several viral RNAs,[109–111] has been demonstrated to bind to the 5′-NTR of HCV RNA and stimulate HCV IRES-mediated translation.[112] PTB, a pre-mRNA splicing factor, is also known to bind to many viral RNAs at several different sites, such as the IRES of poliovirus[113] and hepatitis A virus.[114] It has been shown that PTB has an ability to

interact with the HCV 5′-UTR,[115] 3′-UTR[116–118] and core-protein-coding region of HCV RNA.[26] These reports have suggested that the interaction between the HCV RNA genome and PTB may provide both positive and negative regulation of the viral translation. A recent work has demonstrated that NSAP1, which is highly homologous to hnRNP R, enhances HCV IRES activity through an interaction with the core-protein-coding sequence of the viral genome.[119] Interestingly, the knockdown of endogenous La, PTB, or hVAP-33 with siRNA has been shown to efficiently inhibit HCV RNA replication in cultured cells.[120] It has been proposed that some translation regulatory factors may participate in viral RNA synthesis by either interacting directly with the viral RNA or associating with RdRp in other RNA viruses.[121] Thus, it may be possible that cellular proteins capable of binding to HCV RNA play roles in the viral transcription as well as the translation, although further meticulous experiments to separate viral transcription from viral translation are needed.

Future Perspectives

In addition to biochemical and structural analyses of HCV components carried out using heterologous expression systems, molecular biological studies with cell culture systems based on bicistronic, subgenomic and genomic HCV RNAs have provided us much information about the HCV lifecycle. Nevertheless, a number of questions concerning HCV replication still remain. For instance, cellular pathways involved in the formation of HCV RC are the focus of active investigation, but works to define host cellular factors using a variety of techniques are just beginning. While we are learning a lot from studying HCV replicon systems, current cell cultures harboring the selectable full-length HCV genome unfortunately still do not allow the production of viral particles and re-initiation of an infectious cycle. Development of a robust cell culture system that supports full replication of HCV would bring a further understanding of the viral life cycle.