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Recent advances in hepatitis C virus research

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Introduction

Much effort has been expended in attempts to identify the causative agent of non-A, non-B hepatitis, which had been suggested to be a virus. Finally, the genome of the major blood-borne form of non-A, non-B viral hepatitis was cloned by immunoscreening a cDNA library derived from the plasma of a chimpanzee infected with contaminated human factor VIII concentrates in 1988.[1] This virus, now designated hepatitis C virus (HCV), was the first to be discovered by making use of the sophisticated molecular biological techniques that have progressed so dramatically in recent years. However, the HCV particle was still unidentified by electron microscopy. The cloning of the HCV genome allowed the development of an assay for antibody to the virus, using a yeast-derived recombinant antigen (C100-3).[2] The anti-HCV assay revealed that HCV was a major cause of non-A, non-B hepatitis worldwide.[2] Furthermore, anti-HCV was also detected at a high rate in patients with hepatocellular carcinoma, especially in Japan.[3]

The HCV genome appeared to be a positive stranded RNA, about 10 kilobases in length,[4] and distantly related to the genera of the flaviviruses and the pestiviruses in the flavivirus family.[5] Several sequences of the entire HCV genome have been reported, suggesting the existence of multiple HCV genotypes.[6–10] The HCV genome contains a large open reading frame encoding a polyprotein precursor of 3010 or 3011 amino acids and noncoding sequences in the 5′ and 3′ end of the genome. The putative organization of the HCV genome includes: the 5′ untranslated region (UTR), the core protein (C), the glycoprotein envelope 1 (E1), the glycoprotein envelope 2/nonstructural proteins 1 (E2/NS1), the nonstructural proteins 2–5 (NS2–NS5), and the 3′ UTR, in order from the 5′ end of the genome.[6–10] Recent studies, using molecular biological techniques, have revealed the characteristics of each component.

Here we present a summary of recent advances in HCV research, which continues to progress rapidly.

Hepatitis C virus genome

Since the first nucleotide sequence of the HCV genome was reported as a European patent by the Chiron group,[4] data on the variations of nucleotide sequences in the HCV genome have been increasing. The complete nucleotide sequence of at least 14 HCV isolates is available at present.[6–17] Analysis of the nucleotide sequence data revealed that HCV could be classified into several genotypes. Several groups have proposed similar, but not necessarily identical, classifications of the HCV genotype based on the nucleotide sequence data.[18–27] For one, Enomoto et al.[18] reported that HCV could be classified into two genotypes, named K1 and K2, and each genotype was further classified into two minor types. However, the nomenclature of the HCV genotype is not uniform, and this has caused the present confusion. Moreover, several new types of HCV genome have been reported in quick succession.[21–26] A universal nomenclature for the HCV genome is urgently needed. The types of genome claimed by different groups are summarized in Table 1.

The genotyping of HCV has been performed either by slot blot hybridization,[18] restriction fragment length polymorphisms (RFLPs),[28] or polymerase chain reaction (PCR) with type specific primers.[20,29] Among patients with type C liver disease in Japan, 5% were classified as having type I, 60% as type II, 23% as type

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Table 1. Genotyping of hepatitis C virus

Nakao et al.[28]	PT	K1	K2a	K2b	K3	K3		
Okamoto et al.[20]	I	II	III	IV				
Mori et al.[21]	I	II	III	IV	V	VI		
Cha et al.[23]	GI	GII	GIII	GIII	GIV	GIV	GV	
Chayama et al.[24]	PT	K1	K2a	K2b		Tr		
Simmonds et al.[25]	1a	1b	2a	2b	3a	3b		4
Stuyver et al.[26]	1a	1b	2a	2b	3	3	4	
Tsukiyama-Kohara et al.[27]	I	I	II	II				

III, 10% as type IV, and 2% mixed type.[20] The genotyping of HCV was often correlated to clinical features and was used to pursue the route of HCV infection. Takada et al.[30] reported that the initial response to interferon treatment was significantly better in patients with type K2 (corresponding to types III and IV classified by Okamoto et al.[20]) than in patients with type K1-PT (corresponding to types I and II). Yoshioka et al.[31] had similar results, in that the complete response rate in patients with type III was significantly higher than that in patients with type II. However, it was also shown by Yoshioka et al.[31] that the amount of hepatitis C virus RNA in sera was significantly higher in type II than in type III. We and several other groups have reported that patients with a sustained complete response to interferon therapy had lower pre-treatment virus levels than partial responders and non-responders.[32–34] Factors that may influence the effect of interferon treatment should be further clarified to determine better ways to eliminate HCV.

Tsukiyama-Kohara et al.[35] classified HCV into two groups by serotype, based on the differences of nucleotide sequence in the putative NS3 and NS4 regions of the HCV genome. They showed that the antigens encoded by these regions reacted to group-specific antibodies.[35] In the same way, Machida et al.[36] reported that synthetic peptides deduced from the putative core protein of HCV defined two distinct serological subtypes. Typing the virus by determining the nucleotide sequence will soon be replaced by these serological typing methods.

The HCV genome is known to be heterogeneous within the same genotype and within the same individual.[14,37] The mutation rate of this genome was calculated to be $1.44–1.92 \times 10^{-3}$ base substitutions per site per year,[13,38] this value being similar to those of the majority of RNA viruses. The cause of the mutation rate being higher than that for eukaryotic DNA is that RNA-dependent RNA polymerase lacks a proofreading function for replication of the viral genome. A high rate of change has been observed in the E2/NS1 region of the HCV genome forming a hypervariable region (HVR).[38] In contrast, a high degree of conservation has been observed in the 5′ UTR and the 3′ half of the NS4 region.[38]

5′ Untranslated region

The 5′ UTR of HCV appears to be 341 nucleotides long. The nucleotide sequence of this region was found to be highly conserved among many isolates.[39] Therefore, the 5′ UTR was considered to be the most suitable region for performing reverse transcription-polymerase chain reaction (RT-PCR) assays to detect HCV RNA.[40] Within this region, there were highly conserved domains with completely invariant nucleotides among all HCV isolates. These conserved regions showed homology with the 5′ UTR of the pestiviruses. Moreover, HCV had a few short open reading frames; their relative position was similar to those of the pestiviruses.[41] The secondary structure of the 5′ UTR developed by comparative sequence and thermodynamic modeling is also similar to those of pestiviruses, bovine viral diarrhea virus, and hog cholera virus.[42] These features provided the evidence that HCV is a relative of the pestiviruses, and it was suggested that the 5′ UTR may play an important role in the control of polyprotein translation and viral replication. Recent studies by Tsukiyama-Kohara et al.[43] and Wang et al.[44] have shown that the internal ribosome entry site (IRES) resided in the 5′ UTR of HCV. The IRES is known to be in the 5′ UTR of the picornavirus and plays an important role in the cap-independent mechanism of initiation of mRNA translation.[45] The initiation mechanism of most eukaryotic cellular and viral mRNAs is the scanning ribosome mechanism, i.e. ribosomes initially recognize the cap structure at the 5′ end of mRNA, scan the first AUG sequence of the mRNA, and translate mRNA into protein efficiently. However, picornavirus mRNA is known to be uncapped and to have a long 5′ UTR containing many silent AUG sequences. It has recently been confirmed that picornavirus had the IRES at the 5′ UTR, where ribosomes bound to initiate RNA translation.[45] From the structural similarities between the 5′ UTR of HCV and that of picornavirus, Tsukiyama-Kohara et al.[43] predicted the existence of an IRES function in the 5′ UTR of HCV, and demonstrated it with cell-free translation systems. They reported that the IRES function required 232 nucleotides of the 3′ end of the 5′ UTR, and that of group II HCV was

more efficient than that of group I HCV. These results suggest that different strains may have different capacity to replicate; this feature could be related to differences in interferon effects among HCV genotypes. Wang et al.,[44] in a transfection study of cultured human cells, showed further evidence that the IRES existed within the 5′ UTR proximal to the initiator AUG.

However, Yoo et al.[46] have presented somewhat different data concerning the existence of IRES in the 5′ UTR. They did not find evidence of the existence of IRES within the 5′ UTR, and they suggested that the initiation was mediated in a 5′ end-dependent manner that favored the conventional cap-dependent ribosome scanning mechanism. Their conclusion is that the full-length HCV RNA is translationally inactive, while subgenomic RNA with the 5′ deletion is active, and the 5′ terminus represses translation, while the 3′ terminus enhances translation.[46] Further studies are required to clarify this problem.

Structural proteins

The putative structural proteins of HCV have been analyzed using an in vitro expression system.[47] The structural proteins are a protein of 22 kDa (p22), a glycoprotein of 35 kDa (gp35), and a glycoprotein of 70 kDa (gp70) from the amino-terminal end of the polyprotein precursor in the order. The p22 is considered to be the nucleocapsid (core) protein and the gp35 and gp70 are considered to be the envelope proteins. The cleavage of p22, gp35, and gp70 is mediated by the signal transpeptidase of the host cell, as is the processing of the structural proteins of the flaviviruses.[47]

The core protein is expressed in mammalian cells as a protein of 22 kDa (p22).[48] The antibody against this region was found to be more sensitive and more specific than the antibody against c100-3, the first-generation HCV antibody.[49] The commercial second-generation assay detects antibody to c22-3, the core antigen, and antibody to c200, the combined antigen of c100-3 with another structural protein, c33c. By using the second-generation assay, 90%–96% of chronic non-A, non-B hepatitis cases were shown to be HCV-related,[49] suggesting that this number could reach 100% when still more sensitive assays are developed.

It has been reported that HVR exists in the envelope protein of gp70.[50,51] There are two HVRs in Japanese isolates; these are amino acid residues of 391–400 (HVR1) and 474–480 (HVR2), and there is one HVR in isolates in the United States, amino acid residues of 386–411. In these regions, amino acid sequences showed 40%–100% diversity, while the amino acid sequences of other envelope protein regions showed diversity below 30%. These variants have been shown to coexist simultaneously in a single individual, and the existence of a variant specific epitope located in this region was confirmed by an antibody-epitope binding study.[52] There was specific antibody to HVR of the predominant variant, and no detectable antibody to the HVR of the second variant that become predominant later. This finding suggests that the variability of HVR may result from immune selection. A recent report shows that alterations of amino acids in HVR1 of gp70 occurred sequentially in the chronic phase of hepatitis at intervals of several months, and were associated with alterations of the predicted local secondary structure of the epitope regions.[53] This finding suggested that mutations in HVR1 were involved in the mechanism of chronic HCV infection by allowing HVR1 to escape from the immunosurveillance system. Actually, HVR1 has been shown to contain a sequence-specific immunological epitope that induces antibody production, and titers of anti-HVR1 antibodies usually reached maximal levels several months after the isolation of HCV that had the specific sequence of HVR1.[53] Anti-HVR1 may thus be involved in the genetic drift of HVR1 by immunoselection. Taniguchi et al.[54] also reported that HVR was structurally flexible and antigenically variable, providing the virus a way to escape from host immunity. These findings will have a considerable impact on vaccine development.

Non-Structural proteins

HCV polypeptides appear to be produced by the translation of a long open reading frame and by the subsequent processing by host and viral proteinases, as occurs in flaviviruses and pestiviruses. The vaccinia virus transient-expression assay showed that the non-structural proteins included NS2 (23 kDa), NS3 (70 kDa), NS4A (8 kDa), NS4B (27 kDa), NS5A (58 kDa), and NS5B (68 kDa).[55]

The NS3 region encodes viral proteinase. A serine proteinase domain is located in the N-terminal one-third of the NS3 protein. Mutation analysis in the proteinase catalytic triad (histidine-1083, serine-1165) has revealed that the cleavages of NS3/4A/4B/5A/5B were mediated by this serine proteinase.[56–58] The features that determine this proteinase substrate specificity include an acidic residue (aspartic or glutamic acid) at the P6 position, a cysteine or threonine residue at the P1 position, and a serine or alanine residue at the P1′ position. However, the cleavage at

the NS2/3 is mediated either by cellular enzymes or by an NS2 inherent proteinase activity.[56–58]

The NS3 protein contains motifs characteristic of NTPases and helicases in the C-terminal portion. The Gly-Asp-Asp motifs characteristic of RNA-dependent RNA polymerases are found in the NS5B protein. However, direct evidence of the functions of these proteins is lacking at present.

3′ Untranslated region

There are only a few reports on the analysis of the 3′ UTR of HCV RNA. This region of HCV RNA is known to be the least conserved within the HCV genome. However, Han et al.[59] have reported that the nucleotide sequence of the 3′ UTR was highly conserved within the same group, although it was poorly conserved between different groups. Moreover, a secondary structure search by computer showed four stem and loop structures in all groups. In a positive strand RNA virus such as HCV, the 3′ UTR is considered to be an entry site for RNA polymerase. Therefore, Han et al.[59] predicted that these secondary structures might be a recognition signal for RNA polymerase, and that the efficiency of viral replication might differ among different groups of HCV. This characteristic could be related to the difference of viral pathogenecity seen among different HCV groups.

Hepatitis C virus virion

To date, the HCV virion has not yet been visualized with certainty. Takahashi et al.[60] reported that they identified 33-nm core particles, which aggregated with anti-core antibody, by electron microscope.

On sucrose density gradient equilibrium centrifugation, intact HCV virions were detected in a fraction of 1.08 g/ml density and nucleocapsids were detected in a fraction of 1.25 g/ml density by measuring HCV RNA.[61] Recently, Hijikata et al.[62] reported that HCV (measured in terms of HCV RNA) obtained from serum with high in vivo infectivity was detected in fractions of about 1.06 g/ml, and HCV from serum with low in vivo infectivity was detected in fractions of about 1.17 g/ml. HCV with low in vivo infectivity obtained from the sera of patients with chronic type C hepatitis was precipitated with anti-human immunogloblin, suggesting that HCV was bound to anti-HCV antibody in the sera of patients with chronic type C hepatitis.

In vitro hepatitis C virus replication system

The study of HCV replication is hampered by the lack of an in vitro replication system. Recently, Shimizu et al.[63] presented data for the in vitro replication of HCV in a human T cell line, MOLT-4. Intracellular minus-strand HCV RNA, considered to be a replication intermediate, was detected intermittently, from 3 days after inoculation with a serum containing HCV until 3 weeks, by RT-PCR. The presence of minus-strand HCV RNA was also confirmed by in situ hybridization with a strand-specific RNA probe. The expression of core and NS4 antigens was confirmed by immunofluorescence tests.[63] An in vitro HCV replication system may be valuable for the further characterization of this virus and for the development of a vaccine or anti-viral drugs. The establishment of cell lines more susceptible to HCV is desirable.

Immunology of hepatitis C virus

The mechanism underlying the pathogenesis of HCV is unknown. In hepatitis B virus infection, cytotoxic T lymphocytes (CTLs) are considered to be responsible for the pathogenesis of type B hepatitis. Namely, CTLs recognize the hepatitis B virus antigen expressed on the infected hepatocytes and then lyses them. Shirai et al.[64] provided evidence that $CD8^+$ CTL recognized an epitope from the HCV sequence in association with a class I major histocompatibility complex molecule in mice. Koziel et al.[65] reported that HLA class I-restricted, $CD8^+$ lymphocytes cloned from liver-infiltrating lymphocytes were identified in two of two patients with chronic type C hepatitis evaluated, and these $CD8^+$ lymphocytes recognized epitopes in variable regions of the envelope and nonstructural proteins of the HCV genome. These studies provide evidence of the existence of HCV-specific CTL, and may thus help to elucidate the pathogenetic mechanism of type C hepatitis.

Although an enormous number of studies of HCV have been performed in the last several years, many unsolved problems remain; these include the mechanism of HCV-related oncogenesis and the development of a vaccine. Thorough analysis of the 9030 nucleotides of the HCV genome and its function may eventually open the way to the elimination of HCV in the future.

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