

REVIEW

Viral, host and interferon-related factors modulating the effect of interferon therapy for hepatitis C virus infection

Ke-Qin Hu,¹ John M. Vierling² and Allan G. Redeker³ ¹Department of Medicine and Transplantation Institute, Loma Linda University Medical Center and Jerry L. Pettis Memorial Veterans' Affairs Medical Center, Loma Linda, CA, ²Center for Liver Diseases and Transplantation, Cedars-Sinai Medical Center, UCLA, Los Angeles CA, and ³Division of Gastrointestinal and Liver Diseases, University of Southern California School of Medicine, Los Angeles, CA, USA

SUMMARY. The estimated prevalence of hepatitis C virus infection in the US is approximately 1.8%. Although interferon monotherapy and combination therapy of interferon with ribavirin represent mainstay for treating HCV infection, the rate of sustained virologic response remains suboptimal. The growing evidence suggested that the clinical sequence and treatment response of chronic hepatitis C are determined by a dynamic, complex tripartite relationship among HCV infection, the host immune response, and the effect of different interferon regimens. The treatment response is associated with various viral factors including the pretreatment viral level, dynamic change of viral level during treatment, viral genotype quasispecies and nucleotide mutation in

nonstructural protein 5A of hepatitis C virus. Host factors that may affect treatment response include age, gender, race, HLA alleles and the host immune responses. Interferon regimens, including type, dose, frequency and duration of treatment and combination of interferon with other anti-HCV agents also alter the therapeutic response. Understanding these complicated interaction may provide better insights into the mechanism(s) of interferon response, leading to more effective clinical application of interferon therapy.

Keywords: Hepatitis C virus, treatment of chronic hepatitis C, interferon therapy, viral level, viral quasispecies, and immune response.

INTRODUCTION

Hepatitis C virus (HCV) is the major causative agent of parenterally transmitted hepatitis in the USA. The estimated prevalence of anti-HCV in the general population of the United States is 1.8% [1–2]. Besides causing acute infection, HCV infection becomes chronic in approximately 85% of patients and may progress to cirrhosis [3–5] with an associated risk of developing hepatocellular carcinoma (HCC) [3–9]. The successful cloning of HCV cDNA in 1988 [10] has led to the characterization of the viral genome and proteins, develop-

ment of the specific diagnostic tests and assessment of virologic consequence of interferon (IFN) therapeutic regimens.

IFN had been the mainstay for the treatment of HCV infection until recently, when the combination regimen of IFN with ribavirin was demonstrated to be significantly more effective than the traditional IFN monotherapy [11–13]. However, the rate of HCV clearance, which is considered the best indicator of response to treatment, remains suboptimal and variable [11–15]. Nevertheless, long-term follow-up of patients who received IFN monotherapy for chronic HCV infection has shown favourable clinical and histological outcomes [16].

Extensive studies have investigated the underlying reasons for the low IFN response rate in patients with chronic HCV infection. Due to the unavailability of an *in vitro* HCV replication system, most of these studies have focused on the sequence characteristics of HCV RNA. Recent progress in the understanding of HCV viral dynamics during IFN administration [17,18] has provided new insights into the anti-HCV mechanism of IFN, and design and assessment of new anti-HCV treatment. It is well accepted that the clinical course of hepatitis C is determined by the dynamic status of both HCV infection and the host immune response. In addition, it is well documented that different regimens of IFN therapy have

Abbreviations: HCV, hepatitis C virus; IFN, interferon; HCC, hepatocellular carcinoma; UTR, untranslated region; aa, amino acid(s); HVR, hypervariable region; PKR, double-stranded RNA-dependent protein kinase; 2'-5' OAS, 2', 5'-oligoadenylate synthetase; HLA, human leucocyte antigens; MHC, major histocompatibility complex; ISDR, IFN-sensitivity determining region; CTLs, cytotoxic T lymphocytes; PBMC, peripheral blood mononuclear cells; (eIF-2 α), eukaryotic translation initiation factor-2 alpha.

Correspondence: Dr Ke-Qin Hu, Transplantation Institute, Loma Linda University Medical Center, 11234 Anderson Street, Room 1405, Loma Linda, CA 92354, USA.

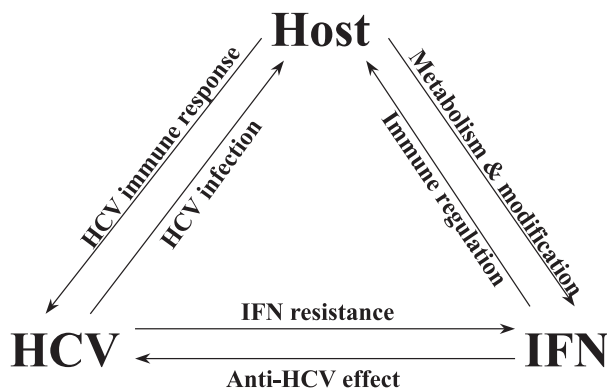


Fig. 1 Tripartite interactions of HCV infection, host immune response and IFN therapy.

resulted in variable response rates, suggesting that the type of IFN regimen should also be considered as a codetermining factor of IFN response. This review summarizes recent advances in our understanding of the complex tripartite relationships among HCV infection, the host immune response, and the effect of different IFN regimens (Fig. 1). A critical review of such interactions may provide better insights into the mechanism(s) of IFN response, leading to more effective clinical application of IFN therapy.

HCV GENOME ORGANIZATION, VIRAL PROTEINS AND GENOTYPES

HCV is a single-stranded RNA virus (Fig. 2) of positive polarity, with a genome of approximately 9500 nucleotides (nt) [19,20]. The genome contains a large open reading frame (ORF) flanked by highly conserved untranslated regions (UTR) at both the 5' and 3' termini. The HCV ORF encodes a precursor polyprotein of approximately 3000

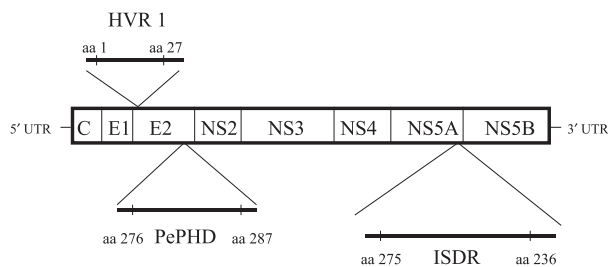


Fig. 2 Genetic organization of HCV genome and regions related to IFN response. A large ORF encodes a polyprotein precursor from which various viral proteins are produced. UTR, untranslated region; aa, amino acid counted from each individual HCV protein; HVR 1, hypervariable region 1; ISDR, interferon sensitivity-determining region (i.e. aa 2009–2248 of HCV polyprotein); PePHD, PKR-eIF2 α phosphorylation homology domain.

amino acids (aa), which is further processed to produce a series of viral proteins. Structural proteins include core, E1 and E2 that are located at the N-terminal region of the polyprotein precursor, while nonstructural (NS) proteins include NS2, NS3, NS4A, NS4B, NS5A and NS5B located at the C-terminus.

Most HCV viral proteins have been expressed *in vitro* and their functions have been extensively reviewed [20]. Two of these proteins, E2 and NS5A, deserve special attention because of their potential to influence IFN responses. The E2 protein interacts with the E1 protein to form the putative HCV envelope protein complex. A high degree of variation in the aa sequences occurs in the N-terminus of the E2 protein (i.e. aa 1–27 of E2). This region is referred to as the hypervariable region 1 (HVR 1), as shown in Fig. 2 [19,20]. HVR 1 epitopes are probably presented on the surface of the virion as a part of the viral envelope, and contain a neutralizing epitope, which is susceptible to immune pressure and the selection of escape mutants. The function of NS5A protein has not been defined, although it is assumed to be important for RNA replication. Studies have indicated that mutations in this region may be related to IFN responsiveness (Fig. 2) [21,22], and NS5A may promote IFN resistance by repressing IFN-induced double-stranded RNA-dependent protein kinase (PKR) [23].

Comparison of HCV cDNA nucleic acid sequences has led to a better understanding of the variation of HCV RNA genome. Variations as high as 34% occur among the most divergent strains [24]. Based on the observed variations, HCV can be further classified into different genotypes and subgenotypes. Genotypes are defined as major branches in the HCV phylogenetic tree, whereas subgenotypes represent more closely related sequences within a major genotype. Six different HCV genotypes have been identified and designated as types 1–6. Subgenotypes are usually designated by letters as a, b or c [24].

ANTI-VIRAL MECHANISMS OF IFN

There are two types of IFNs. Type I includes IFN- α , IFN- β and IFN-1 omega, while type II includes IFN- γ [25,26]. Type I IFNs are released within a few hours after viral infection and are involved in the inhibition of viral replication as a component of the host's nonspecific immune response. In contrast, type II IFN is primarily produced by antigen-activated lymphocytes and is involved in antigen-specific immune responses. The IFN- α family possesses the greatest antiviral activity against HCV infection.

In general, the antiviral effects of IFNs can be classified as direct and indirect. The direct antiviral effects are initiated by interaction of IFN and its receptor, which leads to production of antiviral polypeptides. In contrast, the indirect antiviral effects result from the host immune response against infected cells. Although the detailed mechanisms of the antiviral effects of type I IFNs are incompletely understood, recent

studies have demonstrated that the process involves a series of signal transductions, induced by IFN- α binding to its receptor on the cell surface [25–29]. This interaction activates phosphorylation of Janus kinases (JAKs), a family of tyrosine kinases, in the cytoplasm, which leads to phosphorylation of a family of proteins referred to as signal transducers and transactivators (STATs). This is referred to as JAK–STAT pathway, a signalling pathway also stimulated by numerous other cytokines. Consequently, a multisubunit transcription factor called IFN-stimulated gene factor 3 (ISGF3) is formed in the cytoplasm and translocated to the nucleus. ISGF3 contains two components, ISGF3 α and ISGF3 γ . The former consists of two STAT proteins and the latter is a DNA-binding protein. This complex binds to IFN-stimulated response element (ISRE), an upstream regulatory sequence in IFN-sensitive genes, and subsequently activates transcription of these IFN-sensitive genes. Using human osteosarcoma cell lines expressing HCV proteins, it has recently been demonstrated that HCV proteins inhibit IFN- α -induced signalling through JAK–STAT pathway [30]. It remains to be defined whether the inhibition of JAK–STAT pathway observed in the *in vitro* system contributes to the lower rate of IFN response and persistence of HCV infection in patients with chronic hepatitis C.

Some of IFN-sensitive genes produce IFN-inducible proteins with well-documented antiviral activities. For instance, the enzyme 2',5'-oligoadenylate synthetase (2'-5' OAS) is produced soon after IFN- α stimulation and initiates antiviral effects by synthesizing 2',5'-oligoadenylates that activate an RNase L capable of cleaving double-stranded viral RNA [25,31]. Studies have indicated that 2'-5' OAS inhibits the replication of a narrow range of viruses, including picornaviruses [31]. Peripheral blood mononuclear cells (PBMC) isolated from patients with HCV viraemia have shown reduced baseline and post-IFN-stimulated 2'-5' OAS activity, which was associated with lower *in vitro* response to IFN treatment [32]. PKR is another IFN- α -induced double-stranded RNA-dependent protein kinase, which leads to phosphorylation and inactivation of the eukaryotic translation initiation factor-2 alpha (eIF-2 α). The latter is required for efficient viral protein synthesis [29,33]. Other IFN-induced proteins with antiviral properties include Mx protein, which has been shown to inhibit the replication of influenza and other viruses [30]. Mx protein is specifically induced by human type I IFNs in a dose-dependent manner [25].

In vitro studies have shown that type I IFNs may also suppress viral growth by augmenting cell-mediated immune responses [25]. It is well known that human leucocyte antigens (HLA) play an important role in the molecular signal transduction of lymphocytes during antigen presentation and cytotoxic reaction against target cells [25,34]. By inducing expression of HLA class I molecules, IFN likely enhances the presentation of viral antigens during infection [27]. However, treatment with IFN- α caused increased

intrahepatic expression of HLA class I molecules only in patients with chronic hepatitis B, but not those with chronic hepatitis C [35]. Intrahepatic expression of HLA class II molecules was unaltered by IFN- α therapy in either group of disease. The importance of IFN-mediated indirect antiviral effects is also supported by the evidence that IFN-induced clearance of HBV, and sometimes HCV, is preceded by increased hepatocyte injury as manifested by an increase of serum aminotransferase [36,37].

VIRAL FACTORS AND IFN RESPONSE

HCV viral level

Patients with chronic hepatitis C exhibit persistent viraemia. The serum HCV viral level in patients with chronic hepatitis C is the consequence of a dynamic balance between virus production and clearance. These factors can be estimated by serial measurements of serum HCV RNA concentration following IFN- α -induced perturbation of the balance. Based on such *in vivo* modeling, the estimated half-life of serum HCV ranged from 2.7 hours to 2.7 ± 1.3 days. The pretreatment virus production and clearance rate was estimated to be in the range from 10^{10} to 10^{12} virions/day in patients with chronic hepatitis C [17,18,38].

A number of studies have shown that pretreatment HCV viral level is a determinant of response to both IFN monotherapy and combination therapy of IFN and ribavirin [11–13,39–42]. Using competitive RT–PCR techniques, it was found that lower pretreatment viral level was associated with sustained IFN responses [40]. A 60–70% viral response rate was reported in patients with pretreatment viral level less than 2×10^5 copies/ml. However, the viral response rate dropped to 11% in patients with higher pretreatment viral levels [41]. These results were validated using a branched DNA (bDNA) assay [42]. The predictive value of intrahepatocytic HCV level to IFN response remains unclear. One report showed that viral level in single hepatocyte was significantly correlated with the IFN response [43]. However, intrahepatocytic viral level appeared not to be correlated with the histological activity and severity in patients with chronic HCV infection [44].

Recent studies have emphasized the importance of monitoring the kinetics of HCV viral level during IFN therapy [17,18,38]. Clinical observation has shown that early HCV RNA clearance is associated with a higher rate of sustained IFN responses [45,46]. Thus, both pretreatment viral level and the kinetics of reduction of viral level are predictive factors for IFN response.

HCV genotypes

The response to both IFN monotherapy and combination therapy of IFN and ribavirin varies among different HCV genotypes [39,47–51]. The frequency of long-term normal-

ization of serum aminotransferase after IFN treatment was lower in patients infected with HCV-1 than those infected with either HCV-2 or -3. In addition, infection with HCV-1b was associated with the lowest IFN response rate [49–51]. In patients with acute HCV infection, the chronicity rate was as high as 92% in patients with HCV-1b infection, although the overall chronicity rate was 59.5% [52]. HCV-1b is also more prevalent in patients with cirrhosis, suggesting that it causes a more progressive disease [53].

Theoretically, the differences in IFN response could be secondary to either differences in the viral virulence and/or replication rate among HCV genotypes, or the difference in the host immune response to different HCV genotypes. HCV-1b infection has been more frequently associated with higher circulating viral levels than other genotypes [51]. It has also been suggested that HCV-1b has a greater replicating efficiency than other genotypes. Using a mathematical model, it was demonstrated that the IFN-induced HCV clearance rate was significantly higher for HCV-2 and -3 than for HCV-1 [54]. Thus, genotype-specific differences in virus clearance rate may partially explain the difference of IFN response in patients infected with different genotypes. HCV core-specific T cell response was significantly enhanced by IFN treatment in patients with HCV-2c, but not HCV-1b infection [55]. Using a murine model, both the humoral and cell-mediated immune responses to HCV NS4 protein were influenced by both HCV genotype and major histocompatibility complex (MHC) molecules [43]. These data indicated that both HCV genotype and host immune response are involved in determining IFN response.

HCV viral quasispecies

HCV infection is associated with a predominant viral species and a variable mixture of highly related, but genetically distinct variants [57–60]. This phenomenon is due to a distribution of dynamic mutants referred to as viral quasispecies. Indeed, quasispecies are present in a variety of RNA viruses [58]. It is believed that quasispecies in HCV HVR 1 may alter B cell or T cell epitopes and favour persistence of HCV infection. Two major techniques are currently used to identify HCV quasispecies, i.e. single strand conformation polymorphism (SSCP) and direct sequencing of HCV PCR products [21,22,59,60]. SSCP can detect minor variants representing at least 4–5% of the HCV population [59,60]. Whereas SSCP provides an overall view of quasispecies, sequencing of HCV PCR products is required for a detailed analysis of nucleotide variation.

Studies of HCV quasispecies have usually focused on HVR 1 (Fig. 2) since this region represents a target site for potential neutralizing antibodies and is characterized by dynamic hypervariability. Manzin *et al.* investigated dynamic changes of HCV quasispecies in three patients with acute HCV infection [61]. They found that early HCV infection was oligoclonal and then diverged into multiple

quasispecies in the later phase of infection. These data indicated that as HCV infection becomes persistent, viral genetic evolution enters an adaptive phase with selection of variants. On the other hand, comparison of the quasispecies among these three patients showed differences in variants, indicating that the adaptation to a persistent HCV infection varies among patients. This strongly implicates the importance of the host immune response in determining the spectrum of HCV quasispecies, the clinical course of disease, and possibly response to IFN. However, a similar study of patients with chronic hepatitis C revealed that HVR 1 quasispecies, although variable among individual patients, were stable over time, associated with lower ALT levels and absence of cirrhosis, indicating HVR 1 variation may be associated with a favourable prognosis of disease [62].

A longitudinal study showed that HCV quasispecies fluctuated following IFN treatment, suggesting that the impact of IFN varied among the quasispecies [59]. Other reports [60,63–65], but not all [66,67], indicated that greater numbers of quasispecies were associated with lower IFN response rates and possibly with IFN resistance. It should be noted that both multivariate analyses and dynamic observation have revealed that the complexity of HCV quasispecies parallels viral level, but is a less important predictor of IFN response than the viral level [39,61].

Besides quasispecies of HVR 1, studies have also assessed the relationship between the variation of the nucleotide sequence of the HCV 5' UTR and IFN response [68,69]. One study showed that the extent of variation of the 5' UTR sequence was correlated with the viral level and predicted the efficacy of IFN therapy [68], but the contradictory results were also reported [69].

Mutations in NS5A region of HCV genome

The potential role of NS5A mutation in determining IFN response was initially reported by Enomoto *et al.* [21,22]. By analyzing full-length cDNA sequences of HCV-1b in three patients who received IFN therapy, they found that in patients who failed to respond to IFN therapy, the persistent HCV viraemia was associated with new HCV quasispecies, suggesting that the therapy resulted in selection of IFN-resistant quasispecies. Although both the N-terminal region of E2 and the C-terminal region of NS5 contained variations in these three patients, the most predominant and consistent changes were located in the C-terminal region of NS5A between aa 2209–2248, the so-called 'IFN-sensitivity determining region (ISDR)' as shown in Fig. 2 [21,22]. These studies led to a hypothesis that the HCV-1b wild-type without mutation in NS5A region is IFN-resistant, while mutation in the NS5A region confers IFN-sensitivity. Consequently, patients infected with wild-type HCV-1b have a lower response rate to IFN therapy than those infected with mutant strains. Although such association has not been found in

HCV-1a, 2c, and 3a [70–75], it was recently demonstrated in Japanese patients with HCV-2a infection [76].

Extensive studies have assessed the importance of ISDR in determining IFN response in the past few years. The results have been quite variable [22,70–72,74–83]. Although studies from Japan supported Enomoto and his colleagues' finding [72,77–79], most, but not all, studies from Western countries failed to confirm the finding [74,75,80–83]. The reasons for the discrepant results may be multifactory. First of all, many dissimilarities existed among the studies. For instance, the dose, route of administration and duration of IFN therapy varied widely. Although most studies included patients with a clinical or pathological diagnosis of chronic hepatitis C, some lacked definitive enrollment criteria or included cirrhotic patients. Due to the relatively low prevalence of NS5A mutations in Europe and the USA, most of these studies enrolled only a small number of such patients, which may have compromised the power of statistical analysis. Secondly, a recent report from Europe showed a positive correlation of NS5A-1b mutation to the sustained virological response in patients who received combination therapy of IFN and ribavirin, indicating that the previous discrepant results may be due to a relatively lower response rate of IFN monotherapy [84]. Thirdly, based on the epidemiological difference and variation of nucleotide sequence in the E1 region, Nakano *et al.* further divided HCV-1b into three different subgroups [85]. While the W type is commonly seen in both Japan and Western countries, the J and NJ groups are preferentially distributed in Japan and the Western countries, respectively. The J group represents a high correlation between ISDR type and IFN response. However, the clinical value of the HCV-1b regroup remains to be defined.

The mechanism by which NS5A mutations alter IFN response remains unclear. As discussed above, PKR causes phosphorylation of the α -subunit of eIF-2 α , and a global cessation of protein synthesis and a concomitant block of viral replication [27,86]. Studies have shown that NS5A protein represses PKR activity through a direct interaction with the catalytic domain of PKR kinase [23,87,88]. Furthermore, deletion mutations indicated that such interaction and consequent repression requires the ISDR of the NS5A protein. Studies have also shown that an additional 26 aa carboxyl to the ISDR are required for formation of NS5A–PKR complex [89]. The mutation of NS5A in the PKR-binding region, including ISDR, resulted in disrupted dimerization of PKR and inhibition of PKR-mediated eIF-2 α phosphorylation [89].

Recently, NS5A-expressing cell lines have been established by introducing various NS5A fragments isolated from IFN-responsive and IFN-nonresponsive patients [90–92]. The cell lines can be used to develop a *trans* rescue assay to determine the functional IFN resistance of NS5A protein. Reports from several research groups have shown that NS5A-1b expression resulted in a variable degree of inhibi-

tion of antiviral activity of IFN, which was positively correlated with the level of NS5A expression [90–92]. However, when compared cell lines containing NS5A protein derived from different patients, it was found that NS5A from a IFN responder produced higher inhibition of antiviral activity than that from a IFN non-responder [92]. This finding suggested that detected inhibition of antiviral activity of IFN by NS5A expression may not necessarily correlate with patterns of clinical response to IFN treatment in patients with chronic HCV infection. Additionally, although full-length NS5A-1b protein was capable of inhibiting antiviral activity of IFN, NS5A-1a protein, which lacks an ISDR was still capable of partially inhibiting antiviral activity of IFN [90]. This finding suggested that other ISDR-independent factor(s) may also be involved in IFN resistance. It should be noted that interpretation of these results must be cautious, since all these studies have utilized non-hepatocytic cell lines, and attempts to express NS5A in human hepatoma cell lines have not been successful [92]. The observed toxic effect and alteration of growth rate in the cell lines expressing NS5A protein [90,92] raised the question whether the system of *in vitro* overexpression of NS5A protein represents a natural condition during HCV infection in human beings.

Several lines of evidence suggested that the mechanisms other than ISDR–PKR interaction may also be involved in the IFN resistance during HCV infection.

1. A study on dynamics of NS5A mutation failed to demonstrate the correlation of specific ISDR sequences in the pretreatment isolates with sustained HCV clearance, although the IFN response rate was correlated with the genetic diversity of NS5A [93]. In addition, variable aa substitutions in NS5A region existed before and after IFN treatment. Furthermore, the proportion of synonymous mutations (i.e. nucleotide substitutions that do not change aa) was always significantly higher than that of nonsynonymous mutations (i.e. nucleotide substitutions that do change aa). These data suggested that NS5A mutations may not intrinsically determine sensitivity to IFN therapy. Instead, IFN resistance could be associated with a combination of diverse mutations, which may be patient-specific and located at different positions throughout the HCV genome, rather than limited to the NS5A region.
2. Although NS5A from both HCV-1a and -1b strains were able to bind to PKR [89], the wild-type HCV-1a appeared not necessarily associated with IFN resistance at least in North American patients [80]. However, NS5A-1a protein was capable of rescuing virus challenge from IFN-mediated inhibition, although it was less broad and potent than HCV-1b [90].
3. Recent clinical studies have revealed inconsistent results in the correlation of NS5A mutation in the PKR-binding domain with IFN response [94,95].
4. It was recently demonstrated that HCV E2 protein also contains a stretch of sequence homologous to IFN-

inducible PKR and eIF-2 α , which is called PKR-eIF2 α phosphorylation homology domain (PePHD, Fig. 2). This region may confer IFN resistance by inhibiting PKR activity in patients with HCV-1 infection [96]. However, the same consensus sequence of PePHD has recently reported irrespective of patterns of response to IFN treatment [97], indicating that other mechanisms may also be involved in developing IFN resistance during HCV infection.

5. A recent study showed that expression of HCV proteins resulted in inhibition of IFN- α -induced signalling through JAK-STAT pathway, suggesting a potential involvement of this mechanism in IFN non-responsiveness and HCV persistence [30].
6. It was well documented that HCV level may serve as a co-contributory factor in ISDR-mediated IFN resistance [21, 76–78, 98].

A transcriptional activity has been demonstrated with N-terminal truncated NS5A protein [98–101]. These data raised the possibility that NS5A transcriptional activation may be involved in determining IFN sensitivity. However, it seems unlikely that NS5A transactivation activity observed *in vitro* under these special conditions (i.e. with N-terminal deletion) is also present during natural HCV infection. In patients infected with HCV-1b, ISDR mutation from H-2218 to R-2218 was also correlated with the presence of anti-NS5A antibodies. Both the ISDR mutation and anti-NS5A antibodies were reported to be associated with IFN response [73,102].

It should be emphasized that despite evidence indicating the possible influence of NS5A mutations on IFN response, this conclusion remains tentative and controversial. Although quasispecies of the NS5A region are detectable during HCV infection, the prevalence of NS5A mutations remains low, even in Japanese patients. Indeed, the rate of nucleotide substitution in NS5A was estimated to be very low, resulting in the occurrence of only one aa substitution in the ISDR region every 24 years in each patient [74]. The highest prevalence of mutations in the NS5A region that were associated with increased IFN response rates was reported in studies from Japan, but paradoxically, this was not observed in paediatric patients in Japan [103]. In addition, a comparative study demonstrated that IFN therapy leads to increased mutation rate in both HVR 1 and ISDR of NS5A. However, the mutation rate was significantly higher in HVR 1 (9/9 patients) than in ISDR of NS5A (2/9 patients) [104]. These data suggested that IFN therapy may exert quite different pressures on both regions.

Overall, it is clear that viral factors play a key role in determining IFN response in chronic HCV infection. As discussed above, HCV viral level and its dynamic changes are probably the most documented and extensively measured determinants. Analysis of HCV genotype provides additional predictive value and may serve as a codeterminant. Both

quasispecies and NS5A mutation of HCV also have demonstrated predictive value, but their application is currently limited by technical complexity of assays and geographical variation of HCV genotype and NS5A mutation.

HOST FACTORS AND IFN RESPONSE

Age, gender, race and body weight

Studies have shown that age is an important predictive factor for prognosis in chronic HCV infection. Elderly patients with HCV infection usually present with more progressive disease [9,105–107]. A possible correlation between age and IFN response rate was more evident in women than in men [108]. The rate of sustained IFN response was slightly higher in men (27.1%) than in women (24.1%), but the difference was not statistically significant. Female gender and age of less than 40 years more recently reported as favourable predictors to the combination therapy. It has been well documented that patients in Japan have higher rates of IFN response than patients in North America and Europe, but it is unclear if the differences are related to ethnicity or other factors. Evaluation of the role of race and ethnicity in IFN response showed that the rate of sustained virological IFN response was significantly higher in Caucasians, Hispanics and Asians than that in African-Americans [109]. This implies that race may play a major role in determining IFN response. Increased body weight, as measured with body mass index, was correlated with steatosis in chronic hepatitis C, which may contribute to fibrosis [110]. Obesity has also been reported as a negative predictive factor of IFN response [111].

HLA alleles

It is well known that HLA molecules play key roles in antigen presentation, immune modulation and clearance of virus-infected cells during viral infection. Studies of an *in vitro* culture assay showed that the endogenous core protein was presented to CD4⁺ T cells through HLA class II along with B7/BB1 costimulatory signals [112]. HLA DQB1*0301, an HLA class II molecule, has been associated with the T helper cell response to HCV viral proteins [113]. HLA class I-restricted HCV-specific CD8⁺ cytotoxic T lymphocytes (CTLs) are also detectable in the peripheral blood and the liver in patients with chronic hepatitis C [114].

The relationship between HLA haplotype, natural history of disease and the IFN response has also been assessed. However, the results remain controversial and inconclusive. For instance, one study failed to correlate HLA class II haplotype (i.e. HLA DRB1*0403, DQA1*03 and DQB1*0302 alleles) with the stage of the disease in North European with chronic HCV infection [115]. Other studies indicated the association of HLA alleles with persistent HCV infection (i.e. HLA DRB1*0301 and DRB1*1301/2 alleles) [116], and progression of liver

injury (i.e. HLA B54 alleles) [117]. Other HLA alleles have been associated with self-limited HCV infection (i.e. HLA DQB1*0301 and DRB1*1101/4 alleles) [118,119], lower hepatitis activity (i.e. DRB1*1302–DQB1*0604 alleles) [120], and low incidence of chronic hepatitis C and HCV-cirrhosis (i.e. HLA DR5 alleles) [120,121]. However, it appears that other factors, including age, sex and HCV genotype are significantly more important than HLA alleles in correlation with the severity of the disease [122].

Studies of the correlation between HLA phenotype and IFN response have been limited. In the USA, a sustained response to IFN therapy was seen more frequently in patients with HLA DR7 [123]. However, in Europe, IFN response was associated with HLA DRB1*01, *04 and *11, and HLA DRQ1*0501 alleles [124,125]. Although HLA haplotypes may differ geographically and are subject to variation related to the techniques used for HLA typing, current data appear to support an association of HLA alleles with the severity of disease and the rate of IFN response, but further study is needed to define the details of this association and its mechanism.

Host immune responses

The accumulated data indicated that HCV appears not directly cytopathic. Instead, the host's immune response to viral antigens is essential for HCV-related liver injury and viral clearance during HCV infection [126,127]. Insufficiency in either cellular-, humoral- or cytokine-mediated immune response may contribute to persistence of the infection and development of chronic hepatitis C. A transient normalization of transaminase and surge in viraemia has been reported in patients with chronic HCV infection and immunosuppression [126,128]. This provided additional evidence that liver cell injury and HCV replication rate are immunologically mediated. As discussed above, during chronic HCV infection, the virus mutation may also facilitate HCV to escape host's immune elimination.

The development of HLA class II-restricted T-helper cell (D C4⁺) responses to HCV antigens may be a critical determinant of disease resolution and virus clearance in patients with acute hepatitis C [129,130]. A strong, persistent, and multispecific response of T helper 1 response to both structural and nonstructural HCV is associated with spontaneous resolution of acute HCV infection [129,130]. However, insufficient or transient HCV-specific CD4⁺ T cell response may result in persistence or recurrence of HCV infection [131]. HLA class I-restricted HCV specific CD8⁺ T cell response, which mediates CTLs, has been detected in peripheral blood lymphocytes and in liver-infiltrated lymphocytes in patients with chronic hepatitis C [132,133]. However, the frequency of circulating CTLs that specifically react against individual HCV epitopes is suboptimal in these patients [132]. Chronicity of HCV infection was also associated with the emergence of HCV variants with epitope sequences of HVR 1 that antagonize CTLs activity [134,135]. The CTLs activities were

also inversely correlated with HCV viral level [136]. These data further emphasize the importance of HCV mutations and HCV-specific T cell response, and suggest that a deficient virus-specific CD4⁺ T-helper function in association with an inefficient CD8⁺ CTLs and virus mutation results in persistence of the infection and chronic hepatitis C.

The correlation of HCV-specific T cell activity with the rate of IFN response has been assessed by several research groups. Although a predominant CTLs response to the HCV core protein has been reported to correlate with IFN response [137–139], results remain controversial. For instance, a higher frequency of HCV core-specific circulating T-helper cell precursors defined by a limiting dilution assay and CTLs response of intrahepatic CD8⁺ T cells was reported to be significantly higher in patients with sustained IFN response [140,141]. Another study indicated that both HCV core and envelope-specific CTLs were correlated with the IFN response [140]. However, the same results were not observed using a lymphocyte proliferation assay [139]. In addition, correlation of IFN response with proliferative T-helper cell responses to HCV NS3 and NS4, but not core protein, was also reported [141]. Indeed, the same study found that increased T cell responses against HCV core and NS5 antigens were paradoxically associated with viral persistence [141]. It is worthy of note that HCV viral level may be a co-contributory factor in T cell response determined IFN response [140].

The role of host's humoral immune response in providing protection against HCV infection is questionable since the presence of anti-HCV antibodies failed to protect rechallenge of convalescent chimpanzee with homologous or heterologous HCV strains [142]. The relatively low titre and delayed appearance of HCV-specific antibodies in most patients with HCV infection also suggested that the humoral immune response may play relatively minor roles [143]. However, recent studies demonstrated that pooled human sera containing polyclonal antibodies to HCV may protect patients or chimpanzee from HCV infection [144,145]. The role of neutralizing antibodies during HCV infection was recently assessed using a neutralization of binding assay to evaluate the inhibition of binding of HCV E2 protein to human cells [146]. Prolonged high antibody titres were correlated with spontaneous resolution of chronic hepatitis C, suggesting an important role for the humoral immune response in HCV clearance. The data on correlation of HCV antibody profile with IFN response remains limited. It has been reported that in HCV-1b infection, a broad anti-HVR 1 reaction is associated with higher viral level and lower IFN response rate [147].

Cytokines are soluble hormone-like proteins associated with inflammation, immune responses, tissue injury, repair, and organ dysfunction [148]. Activated CD4⁺ T-helper cells (Th) can be divided into two subgroups based on their profiles of cytokine secretion. CD4⁺ Th1 cells produce IL-2 and IFN- γ and participate in cell-mediated immune responses, while CD4⁺ Th2 cells produce IL-4 and IL-10 and mediate humoral

immune responses [34,126,148]. The role of cytokines in immune-mediated HCV clearance is suggested from the finding that virus clearance more likely occurs in patients displaying a T-helper 1 cytokine profile than those displaying T-helper 2 cytokines profile during acute hepatitis C [149]. CD4⁺ T cell clones prepared from liver tissue of patients with chronic hepatitis C produced T-helper 1 cytokines following stimulation with recombinant HCV proteins [150,151]. This finding suggests that intrahepatic secretion of T-helper 1 cytokines likely stimulates CD8⁺ T cell response. Although lower expression of IFN- γ and IL-4 has been observed in patients who failed to respond to IFN therapy [152], increased expression of IL-4, IL-10 and TGF- β 1 and/or exhaustion of IFN- γ has also been reported in these patients [153]. Patients with genetical predisposition to high IL-10 production, as determined by heterogeneity in the promoter region of the IL-10 gene, have a poor initial IFN response [154]. However, the finding was complicated by other clinical parameters, making the interpretation difficult.

The predictive value of hepatic IFN receptor gene expression for IFN response has recently been assessed [155–157]. These studies indicated that higher level of hepatic IFN receptor mRNA was associated with a more favourable IFN response. This conclusion is supported by an immunohistochemical study, which showed enhanced hepatocytic expression of IFN- α/β receptors in IFN responders [158]. Correspondingly, increased expression of IFN receptor mRNA and IFN response was also associated with a lower HCV level [157].

Apoptosis, a physiological pathway of programmed cell death, is a highly conserved evolutionary process for deleting senescent, damaged, redundant or deleterious cells. It has been well documented that apoptosis plays a key role in liver injury during HCV infection [159]. HCV core protein may enhance both Fas-mediated and tumour necrosis factor (TNF)-induced apoptosis [160,161]. Thus, HCV core protein may promote cell death during HCV infection through various signalling pathways. However, the core protein has also been shown to inhibit TNF- α -induced apoptotic cell death [162].

Duration and status of HCV infection

In patients with acute HCV infection, IFN treatment results in a higher response rate than in chronic HCV infection [14]. This may, in part, be due to the spontaneous viral clearance during acute HCV infection. Alternatively, it may also reflect a possible relationship between the duration of HCV infection and the IFN response rate. This possibility is supported by the finding that patients with HCV-related cirrhosis (a sequela of long-term chronic HCV infection) have lower IFN response rates [163].

The detailed mechanism of HCV replication remains unclear, although it is presumed that minus-stranded HCV RNA functions as a replicating intermediate [19,20]. Minus-stranded HCV RNA is detectable in some patients with

chronic HCV infection [164,165]. Using HCV PCR assay, it has been reported that by the end of IFN treatment absence of minus-stranded HCV in the liver predicts a sustained response to the treatment [166]. HCV quasispecies in HVR 1 have been demonstrated in serum, liver and PBMC in individual patients. The pattern and degree of HCV genetic variability in serum may differ from that in liver and PBMC [167,168], indicating the complexity of the status of HCV infection in these patients. The presence of HCV in PBMC has suggested that PBMC may serve as a reservoir for HCV relapse after treatment with IFN [169,170]. The importance of clearing intracellular HCV from PBMC for favourable treatment outcomes is further emphasized by a report indicating increased sensitivity of detecting HCV RNA in whole blood, rather than serum, following IFN therapy [171].

Histological activity, aminotransferase levels and iron overload

Patients with HCV-related cirrhosis constitute an important therapeutic challenge. Clinically, these patients progress steadily, eventually developing complications of decompensation, hepatic failure or HCC. However, the justification of IFN therapy in this group of patients remains controversial due to low sustained response rates and higher rates of treatment breakthrough and adverse events [163,172]. Furthermore, the benefit of IFN on survival or reduction of the incidence of HCC has not been convincingly demonstrated. Studies have shown that lesser degrees of piecemeal necrosis, portal inflammation and fibrosis are associated with higher IFN response rates [173,174]. Pretreatment histological staging (the extent of fibrosis), rather than grading (inflammatory activity), was also correlated with long-term response rates [175]. Decreased response rate to the combination treatment was associated with portal fibrosis. It should be noted, however, that HCV viral level and genotype are more important predictors of long-term response than the histological score [175]. Virologic and biochemical responses to IFN therapy are usually accompanied by histological improvement [174,176]. In a recent report of IFN treatment in cirrhotic patients, the IFN response rate, histological improvement, and tolerability were similar in both cirrhotic and noncirrhotic patients [177]. Importantly, cirrhotic patients may clear HCV without normalizing aminotransferases.

Management of patients with chronic HCV infection and persistently normal alanine aminotransferase (ALT) represents another challenge. These patients are usually asymptomatic but almost all have histological evidence of chronic hepatitis on liver biopsy [178]. More importantly, the effectiveness of IFN therapy in this group of patients has been inferior to that in patients with chronic hepatitis C and elevated ALT [178,179]. In fact, a pilot randomized controlled study showed no treatment benefit with respect to viral clearance and ALT normalization in this group [180]. Moreover, a higher relapse rate was also reported in this group among

patients who had initial response [178,181]. A recent study evaluating demographic characteristics of these patients found that significant histological liver damage may occur regardless of clinical symptoms, ALT levels, HCV viral load, or genotype [182]. In contrast, a better IFN responsive rate was also observed in the same group of patients [183]. A large randomized clinical trial should be conducted in this group of patients to assess the safety and efficacy of IFN treatment.

It has been well documented that serum iron levels were elevated in about 40–50% patients with chronic HCV infection [184–186]. It was also reported that a significantly inverse correlation exists between hepatic iron stores and the IFN response rate. This finding suggested that elevated serum and/or hepatic iron levels are associated with an unfavourable outcome with IFN therapy. Interestingly, cirrhotic patients who have lower IFN response rates also have significantly greater frequency of stainable iron in their liver [184]. Recently, it was demonstrated that haemochromatosis gene (HFE) mutations, which are responsible for genetic haemochromatosis, are associated with hepatic iron accumulation in chronic hepatitis C [187]. Iron depletion alone has led to an improvement in aminotransferase levels in patients with chronic hepatitis C, but HCV viral load did not decrease [188,189]. An increased virologic response rate to IFN therapy was also observed in patients who received phlebotomy prior to IFN treatment, although the difference was not statistically significant.

In summary, the efficacy of IFN therapy for HCV infection may be modified by a variety of host factors, including age, gender, race, and HLA alleles, both cellular and humoral immune responses and cytokine signal transduction. In addition, the response rates are also altered by duration and status of HCV infection, severity of the histological activity, and presence of iron overload.

INTERFERON-RELATED FACTORS AND IFN RESPONSE

IFN forms used for anti-HCV therapy and their pharmacokinetics

There are four major forms of IFN- α being used for treating hepatitis C: alpha-2a, alpha-2b, alpha-n1, and alphacon-1 [15]. Both alpha-2a and alpha-2b are recombinant products derived from human leucocyte genes. Alpha-n1 is a mixture of nine interferon subtypes produced by a human B lymphoblastoid cell line. Alphacon-1 is a synthetic, recombinant IFN- α developed by assigning the most commonly observed aa in each position of several IFN- α nonallelic subtypes to generate a consensus sequence [190]. Data comparing the efficacy of different forms of IFNs have been limited since only few trials were designed for this purpose. However, some forms of IFN have been reported to be superior to the others [190–192].

After subcutaneous or intramuscular administration, the maximal serum level of IFN is reached between 3 and 12 h. The half-life of IFN is as short as 2–3 h, and it is primarily cleared by renal metabolism. Intravenous administration of IFN reaches the peak level at the end of infusion, but it is cleared from the circulation in 4 h [193].

Detectable antibodies to IFN develop in approximately 10% patients during IFN therapy. Both neutralizing and non-neutralizing antibodies have been identified. Among patients with detectable neutralizing antibodies, 10% demonstrated clinical resistance to IFN. The development of neutralizing anti-IFN antibodies was related to breakthroughs on therapy and relapse after treatment [193–195].

Impact of IFN on HCV dynamics

The impact of IFN administration on HCV dynamic represents one of the most important advances in our knowledge of IFN therapy for chronic HCV infection. HCV dynamic studies have confirmed a short half-life of HCV and the rapid rate of the virus production, which is greater than that in HIV-infected individual [18]. The analysis also indicated that the major initial effect of IFN is to block virion production or release [18]. Further, the effect of IFN- α on HCV clearance is dose-dependent [18,196]. Daily subcutaneous administration of various doses of IFN in patients infected with HCV-1 produced a biphasic decline in viral load. The first phase was characterized by a rapid slope that persisted through day 2. The absence of correlation between this phase and baseline viral load or ALT level suggested that it was determined by the clearance of free virions and therapy efficacy. The second phase was characterized by a slower slope occurring between 2 and 14 days of the treatment. This phase correlated inversely with baseline viral load and positively with the baseline ALT level. Thus, this phase appeared to be related to the rate of elimination of HCV-infected cells (i.e. cell death rate) and therapy efficacy, which showed a great variation among patients [18]. The second phase of HCV decline appears also dose-dependent [197], indicating prolonging duration of induction therapy may improve anti-HCV efficacy. A comparable biphasic HCV dynamics has also been demonstrated with IFN alphacon-1 in 15 μ g once a day regimen [198]. These results indicated that adequate serum concentrations of IFN are essential for the viral clearance and initial aggressive HCV treatment may increase the success of therapy. This concept has prompted recent modifications of current IFN regimens (discussed in detail below). Although the ratio of the viral load on day 0 and day 2 may serve as a good estimate of the antiviral efficacy [18], it is unclear if it has a predictive value for long-term responses. Nevertheless, the data derived from close monitoring of viral loads during initial IFN treatment appear to support the hypothesis [45,46].

IFN regimens and response rate

The finding that the conventional IFN regimens have a low response rate had stimulated extensive investigation of modification of current therapeutic regimens. Most studies have been directed towards either modification of current regimens or development of new regimens. It is now well accepted that longer-term therapy (i.e. 12 months) with standard doses of IFN is superior to previous regimen of 6-month duration [199–202]. Higher-dose regimens also produced higher response rates [202,203]. Alternatively, some specific forms of IFNs, when used as retreatment regimens, were associated with higher rates of response than the other forms of IFNs [191,192].

Various new regimens of IFN therapy are being developed and tested, based on emerging knowledge of HCV dynamics. For instance, several studies have assessed the effect of regimens containing daily induction in naive, relapsed, and nonresponsive patients [204–208]. The results have shown increased rates of IFN response in these patients. However, the length of induction and optimal doses of IFN for daily induction were quite different in these studies. The higher virologic responses observed with daily induction might be lost when converted to routine regimen of three times per week, indicating the limitation of the daily IFN induction [206]. Higher rates of intolerance were also associated with high dose, daily IFN induction [207,208]. Another report assessed the effect of adjusting doses of IFN based on the body weight and HCV genotype [209]. The preliminary results showed this regimen to be more effective than conventional fixed dose regimens. Randomized clinical trials with long-term follow-up will be required to evaluate the true value of these new regimens.

Limited information exists regarding prediction of a sustained response in patients retreated with IFN. Besides low pretreatment viral load, a history of relapse after prior IFN therapy has been well documented as a positive predictor of sustained response after retreatment [191,192,199,204]. The effect of retreatment is also regimen-dependent [13,191,192]; both longer duration and higher dose regimens, and addition of ribavirin to IFN treatment have resulted in improved sustained response rates. These findings emphasize the importance of the interaction of the factors of host, virus and IFN as illustrated in Fig. 1. Relapses may represent a condition in which the tripartite interaction produces a status of borderline virus clearance. If IFN were stopped too soon (such as the 6-month conventional regimen) in these patients, relapse would be expected. On the other hand, with either prolonged duration, increased dose of treatment, or addition of second anti-HCV agent in these patients, the balance of the interaction would favour more effective virus clearance and therefore a sustained response. In contrast to relapsers, nonresponders may represent the opposite extreme where the consequence of interaction of the host, virus and IFN favours the persistence of HCV infection. As a result,

retreatment of these patients even with modified regimens would not improve dramatically the rates of IFN response.

COMBINATION TREATMENT AND OTHER NEW ANTI-HCV REGIMENS

Perhaps, the most important advance in HCV treatment is the development of combination regimen of IFN with ribavirin [11–13]. Ribavirin is a synthetic guanosine analogue with a broad activity spectrum against DNA and RNA viruses. The mechanism of ribavirin action in combination with IFN remains unclear. Monotherapy with ribavirin produced an initial effect neither on HCV level nor on HCV quasispecies development [210]. In primary culture of both human and rat hepatocytes, ribavirin has been shown to inhibit cellular DNA synthesis [211]. Ribavirin has also been demonstrated to promote CD4⁺ Th1 cytokine-mediated immune response [212], indicating that ribavirin may enhance IFN anti-HCV activity through an immune modulatory mechanism. Ribavirin may also have direct anti-HCV effect through its misincorporation into the viral RNA molecules [213]. Several large clinical trials have proved that combination therapy significantly increased the rate of sustained biochemical and virologic responses in both untreated (na) and previously treated, but relapsed patients [11–13]. Currently, the combination therapy has become a first-line regimen of HCV treatment.

Amantadine is a chiral tricyclic amine that has been demonstrated to inhibit the replication of the influenza A. A pilot study of amantidine monotherapy appeared to improve ALT level, but not HCV viraemia in a short-term treatment for chronic hepatitis C [214]. Preliminary results from two clinical trials indicated that combination of IFN with amantidine seemed not superior to that of IFN with ribavirin in treating chronic hepatitis C [215,216]. Another open-label pilot study appeared not to support the application of the triple combination regimen of IFN with ribavirin and amantadine to patients with previous nonresponse to IFN and ribavirin treatment [217].

Based on the short half-life and dose-dependent virus suppression of IFN, it appears that stable serum concentrations of IFN may produce better viral clearance. Pegylated IFN (PEG-IFN) is a modified form of IFN. Pegylation results in reduced renal clearance of IFN molecule. Pharmacokinetics studies of PEG-IFN confirmed the adequacy of once weekly dosing in treating chronic HCV infection, including cirrhotic patients [218,219]. A phase II clinical trial has shown that PEG-IFN, administered at 180 µg weekly, is more effective and convenient than conventional IFN monotherapy [220]. Another phase II open-label study showed that combination of PEG-IFN and ribavirin appears to have acceptable tolerability and efficacy for treating chronic hepatitis C [221]. Apparently, these results are promising but preliminary and need further confirmation by longer-term follow-up.

The importance of host immune responses to IFN response suggests a potential role of immune modulators in increasing IFN response rate. For example, thymosin $\alpha 1$ is an immunomodulatory peptide produced by the thymus gland and other cells. Although a pilot trial of thymosin monotherapy was disappointing [222], a trial of combination therapy with thymosin and IFN showed an improved end-of-treatment response rate [223]. Obviously, a large patient group and longer-term follow-up is needed to assess the sustained response rate.

IFN therapy has been proved to be cost-effective in treating chronic hepatitis C [224,225], although the effectiveness of IFN therapy remains suboptimal and needs to be improved. Appropriate selection of patients for IFN treatment will likely play a key role in improving cost-effectiveness and response rate. Although some predictive factors can be used to select candidates, their clinical application is still limited. For instance, pretreatment liver biopsy may increase the cost without improving outcomes [226]. Using quantitative HCV PCR test to guide therapy may miss some potential sustained responders [226]. Thus, a more practical and cost-effective guideline for treatment indication is required.

CONCLUSION

Although IFN monotherapy and combination of IFN with ribavirin represent mainstay for treating HCV infection, the ability of the current regimens to achieve sustained response remains suboptimal. Studies have shown that a wide range of factors are involved in determining IFN response. These predictors can be classified as viral, host and IFN-related factors. Recent advances support the concept that the IFN response is the consequence of a dynamic tripartite interaction of the virus, host, and the IFN regimens (Fig. 1). Important viral factors include the pretreatment viral level, the dynamic change in viral level following introduction of IFN therapy, viral genotype, quasispecies, and NS5A mutation. Since most available data were derived from analyses of HCV nucleotide sequences rather than viral replication, the clinical importance and detailed mechanisms of these viral predictors need to be validated in the future by functional evaluation using *in vitro* HCV replicating system. Host factors that appear important in IFN response include age, gender, race, HLA alleles, and both humoral and cellular immune responses, including cytokines. IFN regimens, including type, dose, frequency and duration of the treatment, and combination of IFN with other anti-HCV agents are also involved in determining therapeutic response. Further understanding of the complex tripartite interactions should facilitate development of more effective therapeutic regimens. For instance, our insight into the virus dynamics during IFN therapy has resulted in the development of daily induction regimen. On the other hand, combination of IFN with other antiviral or immunoregulatory agent, which may modify anti-HCV efficacy of IFN or enhance host immune

responses to HCV, has shown improved effect for treating HCV infection. This may provide us with an alternative option to achieve an optimization of anti-HCV treatment.

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