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HCV-Related Transformation and New Therapeutic Strategies: An Update

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Abstract: The hepatitis C virus (HCV) is a single-stranded enveloped RNA virus, belonging to the Hepacivirus genus within the Flaviviridae family. HCV infection has become a major worldwide health problem because it causes a chronic hepatitis leading to hepatocarcinoma (HCC) and to non-Hodgkin's B-cell lymphoma (NHL). The absence of a reliable experimental model, which mimics the physiological effect of HCV infection in human subjects, hampered the analysis of the mechanisms by which HCV leads to cancer. Nevertheless, both *in vitro* expression systems and *in vivo* transgenic mice studies suggest that HCV persistent infection in the host is able to induce neoplastic transformation. The oncogenic properties of HCV are often related to the ability of HCV-encoded proteins to interfere with cell signaling through the interaction with different molecules involved in the control of cell proliferation, apoptosis and interferon (IFN)-signaling pathways. The present systematic review will mainly focus on the HCV proteins dependent pathogenetic effects on the most important regulatory proteins of cell homeostasis. Since poor efficacy of the current therapy, studying the mechanisms underlying HCV-induced cell transformation and immune evasion will help researchers to identify new therapeutic targets, which may be useful in the near future to develop more effective and better-tolerated therapies, capable of impairing or reversing the progression of HCV-related tumors.

Keywords: HCV, HCC, HCV-related NHL, IFN, treatment, gene therapy.

INTRODUCTION

Hepatitis C virus (HCV) is a major cause of post-transfusion and community-acquired hepatitis. The majority of HCV-infected individuals develop chronic hepatitis that may progress to liver cirrhosis and hepatocellular carcinoma (HCC). This neoplasia is a multistage disease whose occurrence is linked to environmental, dietary and life-style factors. HCC often has a fulminant course, poor response to conservative treatment, low resectability rate when symptomatic, high recurrence rate after resection and liver transplantation and dismal prognosis. Although cirrhosis of any cause increases the risk of developing HCC, cirrhosis associated with chronic hepatitis B (HBV) or C (HCV) virus infection or hemochromatosis carries the greatest risk. Moreover, it is relevant to notice the co-pathogenetic effects of HBV and HCV co-infection that seem to act synergistically in the HCC induction.

Another feature of HCV infection is its relationship with autoimmune manifestations, most notably essential mixed cryoglobulinemia (EMC), which is characterized by cutaneous vasculitis, nephritis, peripheral neuropathy and clonal B-cell proliferations. HCV infection has also been involved in a subset of non-Hodgkin's lymphomas (NHLs), even in the absence of EMC. The oncogenic process of HCV infection is typically slow and insidious. The hepatitis C virus is characterized by the capacity to transform a wide spectrum of cells (hepatocytes, lymphocytes and keratinocytes), through a process that probably requires multiple steps of genetic alterations with complex interactions between the virus and the host cell. Many of the proposed functions of the HCV gene products appear to be relevant to potential mechanisms of malignant transformation. The present review will focus on the viral oncogenic mechanisms based on the interaction of HCV proteins with host cellular signaling transduction pathways regulating cell growth and cell death, and on the possible developing therapeutic strategies to counteract HCV viral infection.

NATURAL HISTORY

HCV is an important health problem considering its extremely high prevalence, around 350 millions of chronically infected individuals worldwide; it is characterized by a high rate of chronic infection and a significant risk of severe chronic active hepatitis, cirrhosis and HCC among chronically infected subjects. In fact, HCV infection induces chronic infection in up to 60-80% of infected adults. The pathogenic effects of chronic HCV infections are very different from each other: for example, some patients show only minimal liver lesions while others develop, after 5-10 years follow-up, severe fibrosis and cirrhosis. 30-50% of HCV infected patients with cirrhosis develop HCC, after a 10 years follow-up [1]. Interestingly, HCV does not only infect hepatocytes but B and T cells as well. Long-term infection with HCV is associated with immune-mediated monoclonal gammopathies (MGs), such

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as type II mixed cryoglobulinemia (EMC), production of autoantibodies, the appearance of rheumatoid factors or development of B cell non-Hodgkin's lymphomas (B-NHL). MGs constitute a group of benign and malignant lymphoproliferative diseases (LPDs) characterized by the proliferation of a single clone of plasmacells producing a monoclonal protein. The prevalence of MGs in the normal healthy population is approximately 1% [2]. Given the high rate of re-infection of grafts after orthotopic liver transplantation in the patients with end-stage HCV induced liver disease, HCV replication in extrahepatic lymphocytes has been at the root of controversial opinions. In HCV infected patients, circulating immune complexes of HCV and anti-HCV antibodies with cryoprecipitating properties cause EMC, which is associated with polyclonal or monoclonal B cell expansion. Approximately one-third of HCV-infected patients have EMC that, although it is considered a non-neoplastic disorder, might evolve into lymphoma in 10% of patients [3-5].

VIROLOGY

HCV has been classified in the Hepacivirus genus within the Flaviviridae family. HCV isolates have been classified using the sequence divergence in genotypes and quasispecies. The term quasispecies refer to the genetic heterogeneity of the population of HCV genomes coexisting in an infected individual.

HCV is an enveloped virus and its genome is composed of a single-stranded RNA (9.6 kb) of positive polarity. Its genome contains a N-terminal non-coding region (50NCR), a long open reading frame (ORF), encoding a polyprotein precursor of about 3000 aminoacids, and a C-terminal non-coding region (30NCR) [6]. The polyprotein precursor is co and post-translationally processed by a combination of host and viral proteases into the mature structural and non-structural proteins, which play a different roles in virus life cycle [7, 8], (see Fig. **1** for details).

The structural proteins include the core protein (21 kDa) and the two envelope glycoproteins, E1 (27 kDa) and E2 (61 kDa). These are released from the polyprotein precursor by the endoplasmic reticulum (ER) signal peptidases. Host signal peptidases are also responsible for the biogenesis of p7, which is a membrane-associated protein, but its precise role in viral cycle is still unclear. The non-structural proteins include the NS2/3 autoproteases and the NS3 (68 kDa) serine protease, the NS4A polypeptide (6 kDa), the NS4B (26 kDa) and NS5A (56-58 kDa) proteins, and the NS5B (65 kDa) RNA-dependent RNA polymerase.

HCV has been identified since 1989 by the use of recombinant DNA technology [6], however, investigation of the viral life cycle has been retarded by the difficulty of creating an efficient cell culture system for HCV. As described in Fig. (**2**), the presumed life cycle of HCV includes: (1) binding to an as yet unidentified cell surface receptor and internalization into the host cell, (2) cytoplasmic release and uncoating of the viral RNA genome, (3) IRES-mediated translation and polyprotein processing by cellular and viral proteases, (4) RNA replication, (5) packaging and assembly, and (6) virion maturation and release from the host cell. Much work remains to be done with respect to the virion structure, the early and late steps of the HCV life cycle, the mechanism of RNA replication, and the pathogenesis of HCV-induced diseases.

ONCOGENIC FUNCTIONS OF HCV-ENCODED GENE PROTEINS

HCV proteins might contribute to HCV persistent infection and cancer development. This section will discuss how the single viral products may interfere with host cell homeostasis and immune functions to induce malignant transformation. A representation of the various types of interference between HCV proteins and cell proteins is reported in Fig. (**3**).

HCV Core Protein

The core protein is the first structural protein of the N-terminus of HCV polyprotein. It constitutes the virion nucleocapsid and most likely interacts with the viral RNA. HCV core protein is mainly cytoplasmic and perinuclear [9]. Whether or not a fraction of HCV core may also act in the nucleus during *in vivo* infection is still a matter for debate. C-terminally truncated core translocates to the nucleus and may exert distinct biological effects [10]. However, it has not been shown if such sequences are actually present during natural HCV infection.

In the previous years, an increasing number of reports indicated HCV core as a pleiotropic modulator of cell growth and viability. In fact, the core protein has been extensively studied and, in addition to its role in the packaging of viral RNA, it appears to play multiple roles in various cellular signaling pathways, and potentially in oncogenesis [11].

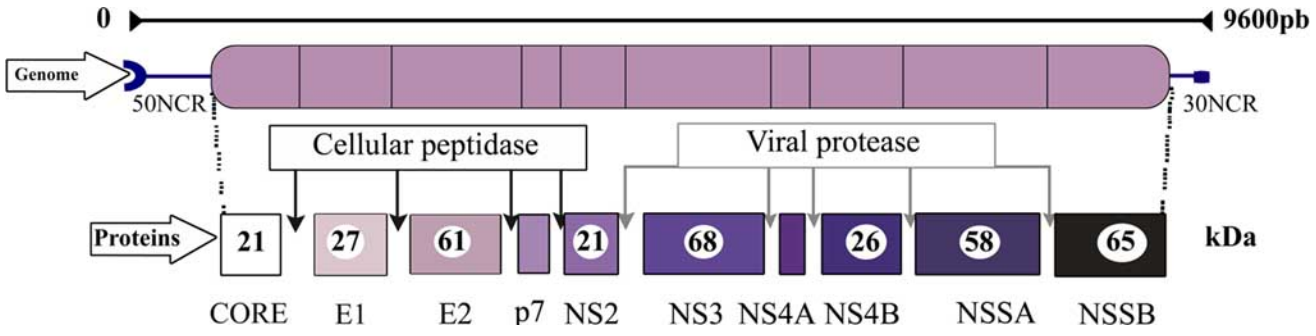

Fig. (1). Organization of HCV genome and structure of viral polyprotein.

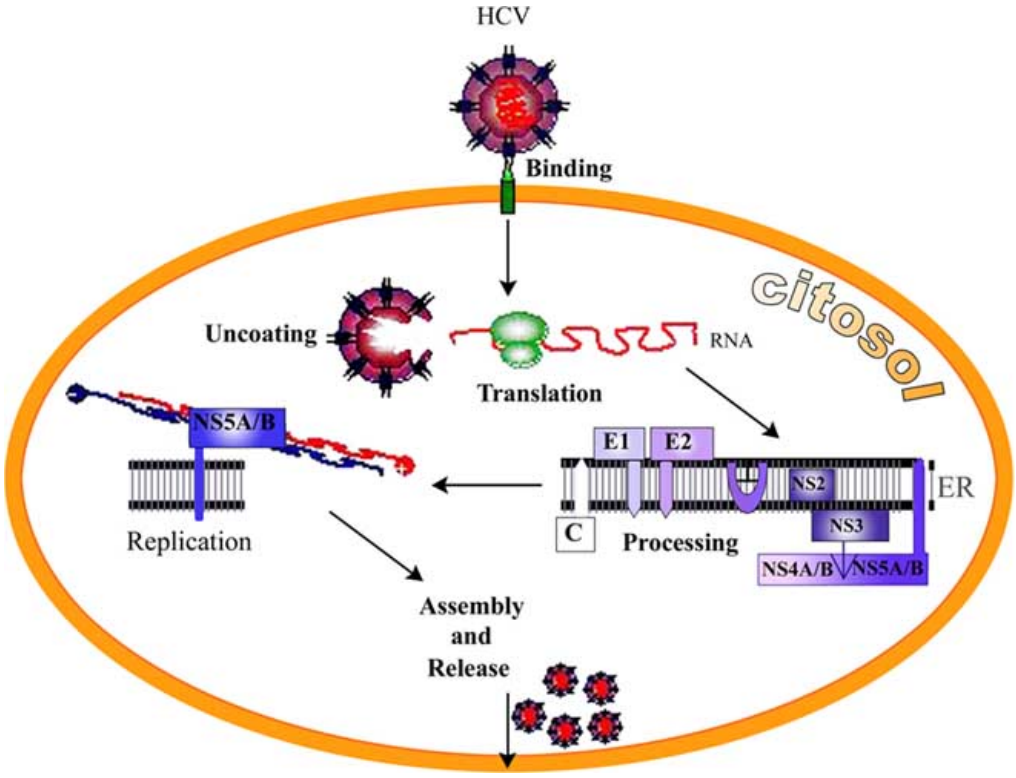

Fig. (2). Differential steps of HCV life cycle.

The HCV core transactivates a number of cellular promoters and activates transcription factors (as NF-kB, AP-1, SRE elements) [12]. The interference exerted by HCV core on Raf-MAPK (mitogen activated protein kinase) pathways has been also established by several groups in different cell types with different experimental models, which could partially justify some discrepancies in the obtained results [12-15]. According to this, HCV core can induce the transformation of immortalized Rat1 fibroblasts and BALB/3T3 cells [16] and, possibly, primary rat fibroblasts [17], in cooperation with v-Ha-ras. It has been also shown that HCV core can bind to Retinoid-X-Receptor-alpha (RXR-alpha) and modulate the expression of RXR-alpha-controlled genes [18]. Moreover, HCV core can modify cell cycle acting on different regulatory proteins such as pRb and all cyclins [19] (Alisi *et al*. 2005[1]). Inhibition of multiple pathways, which can lead to apoptosis (p53 superfamily, TNFR family, Fas/FasL and PKR), may play an important role during the multi-step process of hepatocarcinogenesis and lymphomagenesis. Interestingly, HCV core protein expression influences all the above cited apoptotic molecules either *in vitro* or *in vivo*. In studies using cell cultures, the HCV core protein reduces sensitivity to Fas and TNF (tumor necrosis factor), activating NF-kB, thus inhibiting apoptosis either constitutively or in response to cytokines [20]. In contrast, other *in vitro* studies have shown an opposite effect of core expression on TNF- and Fas-mediated apoptosis [21, 22]. HCV core protein modulates the expression and the activity of a variety of molecules involved in p53-related apoptosis (p53, p63 and p73). In fact, it has been reported that its direct modulation of different p53 and p73 functions plays a role in HCV-related HCC pathogenesis [23, 24].

In addition, lines of evidence indicate a relationship between malignant lymphoma and p63 gene [25, 26]; interestingly, HCV core protein modulates the expression and function of p63 and p73 different isoforms in HCV core constitutively expressing polyclonal B lymphocytes and in patients affected by HCV-related EMC and NHL (Alisi *et al*. 2005[2]).

Since HCV has the ability to interfere with the anti-viral and apoptotic effects of the interferons (IFNs), it is not surprising that HCV core protein is able to alter some functions of PKR, one of the most important components of IFN pathways, making such phenomena of particular relevance to the development of IFN resistance and cancer [27]. PKR is induced by IFN and activated by double-stranded RNA. Its activation by viral infection causes eIF2 phosphorylation and concomitant inhibition of protein synthesis [28]. Perturbation of PKR functionality may contribute to viral persistence and may affect many other cellular processes including transcription, signal transduction, apoptosis and cell growth. In fact, although HCV core protein has been proposed to be involved in PKR-induced apoptosis [29], recently, it has been demonstrated that in HCC cells, viral core protein causes an unconventional activation of PKR leading to a G2/M accumulation in the absence of apoptosis [30]. Such *in vitro* results have been substantiated by *in vivo* studies in

[1] Abstract (n. 418) presented in the 40th Annual Meeting of the EASL, 2005; published in J Hepatol Vol. 42, supplement 2.

[2] Abstract presented in the AISF Annual Meeting, 2005; published in Dig Liver Dis Vol. 37, pp. A34-A35.

transgenic mice, which demonstrated the induction of HCC by the expression of the full-length genome or of the core sequence only [19, 31]. The development of HCC in these animal models is preceded by steatosis in the absence of liver inflammation. However, other reports on HCV core-expressing transgenic mice, made using the same mice strain and the same promoter sequence, did not demonstrate such changes, and the reasons for these discrepancies remain unknown (Table **1**). Furthermore, the expression of the HCV core protein in transgenic mice is able to induce the development of malignant lymphoma with a high frequency (80%) in elderly animals [40], suggesting its own important role in inducing lymphocytes transformation. Oxidative stress has been indicated as another of the possible mechanisms of HCV induced hepatocarcinogenesis. *In vivo* data obtained in HCV core expressing transgenic mice provide evidence that elevated peroxide products potentially contribute to the development of HCC in older animals, where scavenger systems are less effective [41].

E1/E2 Proteins

Envelope proteins E1 and E2 are transmembrane proteins consisting of a large N-terminal ectodomain and a C-terminal hydrophobic anchor. E1 and E2 are post-translationally modified by extensive N-linked glycosylation [42]. They are believed to associate as a non-covalent heterodimer and are exposed on the virion surface [43]. E1 and core proteins can interact with each other, suggesting that the viral capsid is enveloped through this interaction. E2 mediates viral binding to the cells, as shown by a decrease of infectivity by incubation of the virus with anti-E2 antibodies [44], but the HCV receptor has not yet been identified. A number of cell surface molecules have been proven to be HCV receptors. For instance, human CD81, as well as low density lipoprotein receptor (LDLR) are putative HCV receptors binding specifically to E2 protein but do not mediate viral entry [45, 46]. In addition, human scavenger receptor class B type I and liver/lymph node-specific intercellular adhesion molecule-3-grabbing integrin have been recently characterized as novel receptors for HCV [47, 48]. Whether and how these cellular molecules transmit stimulation of HCV E2 protein to intracellular signaling pathways need to be further clarified. Since interaction of E2 protein with its receptors is a pivotal process taken by HCV for entry, it is reasonable to speculate that transmembrane signal transduction initiated by E2 may at least in part account for HCV pathogenesis; in fact, it has been documented that the E2 protein was capable to significantly promote cell proliferation both in Huh-7 and human T lymphocytes [49, 50].

Beside its structural role, E2 has been shown to modulate the IFN-alpha response. In fact, it contains a region that shares a high degree of sequence homology with the autophosphorylation sites of PKR and the phosphorylation site of eIF2 [51-53].

The precise role of HCV E2 protein in the development of cancer is currently unknown, but the interaction with PKR might be important in HCC development; on the other hand, the hypothesis that some HCV-associated lymphomas originate from B cells might explain the association between HCV infection and some B-cell lymphoproliferative disorders [54].

NS2, NS3 and NS4 Proteins

NS2 and NS3 are the two viral proteases responsible for the cleavage of all the NS proteins. Furthermore, NS3 has a helicase and a NTPase activity, suggesting that it plays a role in RNA replication [55]. HCV NS3 protein probably also has many kinds of potential biological effects, for example mediating cellular immune response, and modulating p53, protein kinase A (PKA) function, signal transducers and activators of transcription (STAT) and telomerase activities [56-61]. Although NS3 has been implicated in interaction with various cell constituents, resulting in phenotypic changes including malignant transformation, the precise pathogenic mechanism of HCV NS3 protein remains unclear. It has been reported that HCV NS3 protein is able to transform mouse fibroblast cell lines and induce tumors in nude mice [62, 63]. In addition, Kwun *et al*. [64] have demonstrated that the NS3 protein can specifically repress p21waf1/cip1 promoter activity. Recent experiments suggest that the transformation and tumorigenesis induced by HCV NS3 serine protease could be dependent on its ability to activate MAPK pathways. The precise mechanism by which NS3 activates these pathways is still unclear.

NS4A is a cofactor of NS3, with which it forms the NS3/4A heterodimer [66]. NS3/4A heterodimer permits HCV evasion from immune response by Toll-like receptor 3-mediated inhibition of IFN regulatory factor 3 (IRF-3) [67].

NS4B is an integral ER membrane protein. Its function is not yet known, but it may play a role in the anchorage of the replication complex to membrane, as observed for the replication of other RNA viruses [68]. As occurs for other HCV proteins, also HCV NS4B plays an important role in the malignant transformation; in fact, it transforms NIH3T3 cells in cooperation with the Ha-ras oncogene [69].

NS5A/NS5B Protein

NS5A is a phosphoprotein, which exists in differentially phosphorylated forms of 56 and 58 kDa with modifications of serine residues [70]. Probably, it plays a role in the replication cycle; in fact, interesting studies on HCV RNA replicon system have shown many adaptive mutations in NS5A, able to enhance viral replication [71, 72]. Although the physiological role of NS5A protein is still largely unknown, the direct role of this protein in liver cancer is supported by the observation that it is able to modulate gene transcription and modify the susceptibility of cultured cells to apoptotic signals [73-75].

NS5A interacts with a number of cellular proteins in mammalian cells, some of which have been identified and partially characterized. It was shown to associate with cellular serine/threonine kinase and adaptor proteins [76], including growth factor receptor bound protein 2 (Grb2), thereby interfering with cell signaling [77]. It also interferes directly with DNA binding of p53, repressing its transcriptional activity on promoters such as p21waf1/cip1 [78, 79]. NS5A also interacts with a newly identified cellular transcription factor, SRCAP (Snf-2-related CBP activator protein) [80] as well as other transcription factors (NF-kB and PCNA) that are related to the promotion of cell

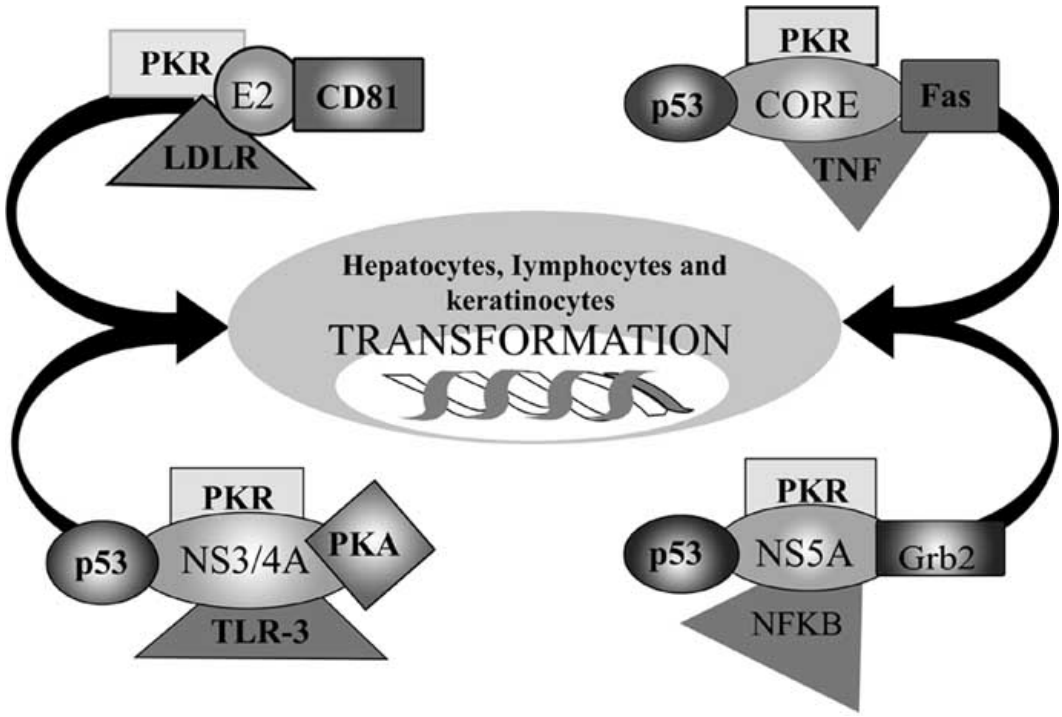

Fig. (3). Schematic representation of potential interplays between HCV proteins and cellular pathways.

proliferation [81]. In addition, NS5A mediates growth delay by blocking the G2/M transition [82, 83]. Several reports indicate that HCV NS5A could repress PKR functions, by which HCV could escape the anti-viral and antiproliferative effects of IFN [84, 85]. The interaction of HCV NS5A protein with this kinase, through its ISDR region prevents dimerization of PKR, which is critical for protein kinase functions [85, 86]. When HCV NS5A induces PKR dysfunction, sustained expression of eIF-2α in cells destabilizes cell growth and differentiation, and induces malignant transformation and development of tumors in nude mice.

Finally, it has been predicted that the NS5B protein encodes an RNA-dependent RNA polymerase (RdRp), which is the central catalytic enzyme of the HCV replicase [87]. It has a hydrophobic domain at its C-terminus, allowing its insertion into membrane [88]. NS5B has been reported to interact with NS proteins and some host proteins [89]. Such interaction(s) may modulate the activity of NS5B RdRp in many different ways. In fact, NS5B interacts with NS3 and NS4B as positive and negative regulator during HCV replication [90]. In this context, the amphipathic helix of the HCV NS5A is necessary for its membrane localization and for HCV RNA replication [91]. Although this protein also interacts with cellular proteins such as nucleolin [92] and eukaryotic initiation factor 4AII [93], a direct role for NS5B in cell transformation is lacking.

Summary

This part of the manuscript provides an overview of some of the common functions of HCV gene products, which contribute to viral persistence and HCV cancer pathogenesis. Literature has clearly shown a direct role of some of the HCV viral proteins (core, NS5A etc.) in the pathogenesis of HCV-related transformation. HCV proteins stimulate

Table 1. Some Models of Transgenic Mice

Strain	HCV protein	Promoter	Pathology
C57BL/6 [32]	Core	HBV elements	None
C57BL/6 [33]	Core	HBV elements	Insulin resistance
C57BL/6 [34,19,35]	Core	HBV elements	Steatosis, adenomas, HCC
CD1 [36]	*E1, E2*	*HBV elements*	*None*
CD1 [37]	*E1, E2*	*HBV elements*	*Exocrinopathy*
BALB/c [38]	Core, E1, E2, NS2	CMV-actin promoter	None
BALB/c [39]	Core, E1, E2, NS2	CMV-actin promoter	Hepatitis

Table 2. Pro-Oncogenic Functions of HCV Gene Products

HCV protein	Functions and Cellular Interferences
Core	Viral capside, Signal transduction, Apoptosis, Cell cycle, IFN actions, Immune suppression, Transcription.
E1, E2	Viral entry, Signal transduction, Cell proliferation, IFN actions.
NS3/4A	Serine protease and helicase, Signal transduction, Cell cycle, Immune evasion.
NS4B	Viral replication, Signal transduction.
NS5A/5B	Viral replication, Signal transduction, Apoptosis, Cell cycle, IFN actions, Transcription.

immune response and inflammation; they too interact with several cellular signal transduction pathways, regulating cell proliferation and apoptosis (in Table **2** all the HCV protein functions and cellular interferences are summarized).

CURRENT TREATMENT FOR HCV-RELATED CANCERS

Current Therapy for HCC

The therapeutic strategy for HCC patients has to take into account that in the vast majority of individuals, the tumor develops in a diseased liver with a variable degree of functional derangement. Thereby, it would be desirable for effective treatment to have a limited impact on liver function. In fact, if the degree of liver dysfunction is advanced, it will determine an unpredictable disease evolution with a dismal prognosis; at this point, response to the therapy will become absolutely irrelevant. There are no definitive molecular tools to characterize the biology of HCC. Actually, most treatment decisions are taken looking especially at size and tumor number, overall, because there is no clear and definitive molecular biology characterization of HCC. For this reason, it is necessary to follow accurately each advanced cirrhotic patient; in fact, patients diagnosed at an early stage of HCC may undergo effective treatments such as surgical resection, percutaneous ablation or transplantation. In any case, it is important to notice that the majority of HCC patients are treated with palliative treatments because the diagnosis is typically made at a late stage [94]. Inclusion of such patients with advanced liver disease in clinical trials of new anti-HCC agents will confound survival results, because of deaths from progressive liver disease that may be unrelated to the HCC or its therapy.

Here will be briefly described surgical and non-surgical aspects of currently strategies used as HCC treatments.

Liver Transplantation

At present, liver transplantation is considered the only curative treatment option for HCC, since the major challenge in liver transplantation for HCC is the decrease of the rates of tumor recurrence. It is also the treatment of choice for patients with early HCC in de-compensated cirrhosis. Patients with solitary tumors less than 5 cm or with up to 3 nodules of less than 3 cm achieve outcomes identical to patients who are transplanted, because of end stage cirrhosis without malignancy (70% at 5 years), with the recurrence rate less than with surgery [95]. Thus, transplantation might be used as the first therapeutic choice, but transplant has several drawbacks. Firstly, the lack of donors prompts a waiting time between enlistment and transplantation. During this time, the tumor may progress. Live donation may partially ameliorate the situation of waiting, but HCC patients waiting for a liver will still exist. To achieve the current criteria for transplantation and to prevent recurrences, sometimes it is important to restrict tumors before transplant can be performed [96]. Several pilot randomized studies have shown that subjects with advanced but locoregional HCC (i.e. disease in the absence of portal vein thrombosis) who are treated with adjuvant chemo-therapy achieve prolonged survival.

Predictors of tumor progression are not available, thus development of molecular profiling strategies using microarray analysis should provide a major help [97, 98]. Identification of prognostic markers [99-101] should also allow a more accurate choice in patient selection for transplantation as well as resection.

Graft rejection and viral re-infection remain major unsolved issues after transplant. HCV re-infection of the graft is frequent, if not always present, and almost 50% of patients will display a cirrhotic liver within 5 years [102]. Development of better anti-viral therapies and tools to prevent collagen deposition is needed.

Surgical Resection

Complete removal of HCC nodules can be achieved by surgical resection or by medical ablation. Surgical resection is the most common first option in patients with solitary tumors who have well-preserved liver function and normal portal pressure. Patients with these characteristics normally tolerate the resection of a hepatic segment well, and their liver will not be damaged by the immunological response and ischemia reperfusion injury related to surgery. On the other hand, it is important to underline that patients with cirrhosis generally are not considered good candidates for surgical resection because of high morbidity and mortality rates associated with cirrhosis and its complications. Moreover, it is well known that in those cirrhotic patients, who do undergo resection, recurrence rates are among the highest of any solid tumor, and approach 75% to 100% at 5 years [103].

Hence, the major problem related to the HCC total population treated with surgical resection is the high rate of recurrence (15-30% per year); although some studies demonstrate a favorable effect of interferon alpha in the prevention of HCC recurrence, future therapeutic approaches should be directed to ameliorate this shortcoming. If patients

with HCV-related HCC are treated with interferon after the complete ablation, the rates of second or third recurrence are different between the treated and untreated patients. The interferon-treated patients had a survival rate of 68% at 5 years and 53% at 7 years, whereas untreated patients had a survival rate of 48% at 5 years and 23% at 7 years [104]. The combination treatment with interferon plus ribavirin (RBV) seems to determine a better favorable prognosis if combined with curative ablation therapy or surgery.

The presence of vascular invasion or additional nodules is an important predictor of recurrence related to dissemination. Some type of molecular analysis is needed to give a more precise prognosis than conventional pathology. Higher recurrence rate has been related to p53 protein dysfunction, reduced expression of p27 or nm23-H1 and/or increased expression of AFP, Ki-67 or VEGF, among others, but further investigation is needed to identify genes that correlate with predictive risk of recurrence. Actually, antiangiogenetic agents, acyclic retinoids, interferon, immunotherapy, and internal radiation may have some preventive effect, however, their efficacy requires extensive studies (see references below). The high recurrence rates for stages II and III HCC post-resection have led to attempt by investigators to decrease these rates with agents that have known activity against HCC recurrence. Approaches to prevent recurrence have included chemoembolization before and neoadjuvant chemotherapy after surgery to deal with the microscopic disease that may be present in the non-resected portions of the liver; unfortunately, neither of these therapeutic approaches has proven to be beneficial. Transarterial embolization/chemoembolization (TACE) and ^{131}I-lipiodol seem to be reasonable approaches [105, 106]. Although many other agents have been studied, they have often been used at sub-therapeutic doses. Less toxic or non-toxic agents would be particularly attractive in this setting.

Ablative Localized Treatments

Percutaneous ablation is the third treatment option that may permit to observe a long-term relapse free period. It includes some techniques to ablate tumors by physical means such as Percutaneous Ethanol Injection (PEI), Radiofrequency Interstitial Thermal Ablation (RITA), cryotherapy and new kind of radio-wave therapies.

All are performed under image guidance, and their maximal activity is achieved in nodules < 3 cm, when complete response rates account for 80% of cases [107, 108]. Local ablative therapies are generally useful for patients with 1 or 2 tumor lesions with a maximum diameter of 3 cm. These local ablative therapies seem to be similar in applicability, and results are highly dependent on clinician skills and choice of patients.

The main problem is again the high rate of disease recurrence. Unfortunately, it often occurs near to the treated area, reflecting failure to provide adequate local control. This lack of local control is not an issue with surgical resection, which eliminates the tumor and the surrounding tissue, containing the local tumor spread. Thus, percutaneous ablation is an effective option, but is usually indicated if surgery is unfeasible.

Chemotherapy

The results of systemic treatments for hepatocellular carcinoma are currently disappointing. Almost 70-90% of newly diagnosed HCCs are non-resectable and non-transplantable as judged by the extent of the tumor. The management of such tumors is currently non-surgical and only chemotherapeutic or hormonal treatment is indicated. In any case, in patients with advanced disease, none of the tested treatments demonstrates an unequivocal benefit in terms of survival. Moreover, the trials including the largest numbers of patients have been consistently negative. The ideal chemotherapeutic agent for HCC, as noted above, would be effective against the tumor and non-toxic to the cirrhotic liver. Unfortunately, few agents exist that have such criteria. A notable number of randomized and nonrandomized clinical trials to evaluate the usefulness of single agents or combinations of agents of cytotoxic cancer chemotherapy have been published [109]. In particular, the two most widely used chemotherapeutic drugs for HCC are doxorubicin and cisplatin. Overall, cisplatin seems to be better tolerated in patients with cirrhosis and has less myelosuppressive activity. Some more recent combinations such as cisplatin, interferon alpha, adriamycin, and 5-fluorouracil are extremely toxic and yield response rates of only 20%, showing no survival advantage compared with supportive care alone [110]. Actually the better way to follow seems to be the hepatic arterial chemoembolization (TACE) that permits to deliver directly to the HCC higher concentrations of cancer chemotherapeutic agents. Despite enormous efforts in this area from multiple groups, the effect on survival is difficult to prove. A glimmer of light has been done by the recent publication of two trials [111, 112], showing a survival advantage for TACE using either doxorubicin or cisplatin, compared with supportive care only. Because both have shown a survival advantage for unresectable HCC, this approach could be considered a new standard with which other agents or combinations of treatments should be compared. Nonetheless, survival at 2 years still does not exceed 40%. In these patients, the lack of effect on survival might be caused by loss of control of tumor growth, although it appears to be more commonly caused by liver failure.

Randomized, controlled trials are clearly needed to establish confidence in the use of TACE for the treatment of unresectable HCC, because at present, there is no general agreement on an ideal agent or regimen for chemoembolization.

Hormonal Therapy

A majority of patients diagnosed with HCC have advanced disease at presentation and, based on the number, size, location of lesions and the severity of underlying cirrhosis, are not candidates for transplantation, surgical resection, or liver-directed therapies. The gender differences noted in HCC incidence rates have encouraged many investigators to examine tumor profiles for hormonal or growth factor receptors. Some clinical trials have been performed to study the influence of various hormonal therapies on HCC progression, including agents to inhibit

estrogen actions, such as tamoxifen, and anti-androgens, such as leuprolide acetate and flutamide. Unfortunately, a meta-analysis of studies investigating the use of tamoxifen did not provide support for its therapeutic use in advanced HCC. In fact, this analysis of 10 randomized trials with a total of 1709 patients highlighted the fact that tamoxifen has no effect on median survival or tumor response rate [113].

Despite many trials, the overall results have been disappointing and survival has remained poor [114-118]. Nevertheless, variations of such approaches continue to be somewhat attractive, because the agents are in general non-toxic, inexpensive, and easy to administer.

Prospective, randomized controlled trials using current therapies alone or in combination, are needed to better define the optimal management of HCC. Actually, however, we especially need new and effective therapeutic agents against HCC, which have to be non-cytotoxic and well tolerated by the typical patient with underlying cirrhosis.

Current Therapy for HCV-Related Lymphomas

As already mentioned, there are many important extrahepatic manifestations of HCV infection. Most of them are associated with autoimmune or lymphoproliferative disorders (LPDs) and may be related to the possibility that HCV is able to replicate in lymphocytes [119-120].

There are several studies that indicate HCV as responsible for the development of benign (EMC) and malignant LPDs associated with chronic HCV infection [121]. The association between EMC and chronic HCV infection is extremely strong with more than 95% of patients affected by EMC having serological evidence of a current or prior HCV infection. Between 10 and 54% of individuals with HCV infection can be affected by EMC, and cryoprecipitates from HCV infected patients usually contain large amounts of HCV antigens and antibodies [122].

Type II mixed cryoglobulinemia is often observed in conjunction with bone marrow findings consistent with indolent B-cell lymphoma [123] and evolves to frank B-cell malignancy in about 10% of cases [124]. In this respect, it has been reported that EMC may lead to lymphoma, especially low-grade B-NHL, after a long latent period [125]. Hence, HCV may be responsible for triggering a clonal B-cell proliferation that in some cases can progress to a malignant lymphoma [125, 126].

The following sections will summarize the current therapy used for benign (EMC) and malignant HCV-related lymphoproliferative diseases.

Anti-viral Therapy in HCV-Related Mixed Cryoglobulinemia

Anti-viral therapy with IFN has been reported to be efficacious for the treatment of HCV-associated cryoglobulinemia, although most studies lack a control group; therefore, controlled studies are necessary to establish whether the improvement in clinical features is related to anti-viral effect or to other effects of IFN [127-129].

The therapeutic efficacy of IFN in HCV associated cryoglobulinemia seems to be closely related to its anti-viral activity, thus supporting the idea that this disease is due to HCV viral infection. Therefore, it has been suggested that IFN treatment should be administered as soon as possible when HCV related cryoglobulinemia is diagnosed [130]. According to this, it has been suggested that the induction of the regression of mixed cryoglobulinemia-associated B-cell monoclonal proliferation with IFN therapy could reduce the prevalence of hematological malignancies [131] and induce remission of low-grade types of NHL (lymphoplasmocytic or lymphoplasmoid immunocytomas).

The mechanisms of action of IFN in mixed cryoglobulinemia are unclear at present. First, it is known that IFN has an antiproliferative effect, as indicated by the use of this drug in several myeloproliferative and lymphoproliferative disorders. However, the anti-viral property of IFN, and not only its antiproliferative effect, is arguably responsible for the beneficial effects in mixed cryoglobulinemia. In particular, the regression of the clonal B-cell disorder is obtained only in patients who achieve clearance of the virus [131]; the symptoms associated with cryoglobulin deposition disappear only in those who respond to the treatment with IFN-alpha and are negative for HCV-RNA [120]. Notably, virological relapse after treatment withdrawal is characterized by a recurrence of cryoglobulin-associated symptoms [127, 128, 132, 133].

A recent study has focused on the t(14,18) translocation, evaluating the effects of IFN-alpha plus RBV on viremia, B-lymphocyte HCV RNA and t(14,18) in peripheral blood mononuclear cells in HCV-infected patients without either mixed cryoglobulinemia syndrome or other lymphoproliferative disorders [134]. At the end of treatment, t(14;18) was no longer detected in 50% of patients with complete or partial virological response, whereas it was persistently detected in non-responders.

In summary, although it should be emphasized that not all patients with HCV infection and cryoglobulinemia respond to anti-viral treatment, it seems logical to recommend interferon-alpha (preferably pegylated interferon-alpha plus RBV) to achieve a sustained virological response in patients with chronic hepatitis C and cryoglobulinemia.

Anti-Viral Therapy in HCV-Related B-NHL

As previously reported, there is an epidemiological association of HCV infection with LPDs, particularly with B-cell NHL. Rarely, HCV-associated lymphoproliferative disorders have been observed in the absence of cryoglobulinemia, and in these patients, the response to IFN-alpha is largely unknown. Some observations have shown that anti-viral treatment appears to be effective in eliminating the clonal proliferation of B cells in patients with chronic HCV infection. Patients with HCV are more likely than healthy individuals to have the t(14,18) translocation with overexpression of the antiapoptotic bcl-2 proto-oncogene [135, 4] and bcl-2 rearrangements [136], suggesting its involvement in HCV-related B-NHL development.

Complete remission of LPD was often achieved. Encouraging results emerge from a recent report in which most patients with HCV and splenic lymphoma with villous lymphocytes (SLVL), characterized by a clonal expansion of B cells with villous projections and splenomegaly, entered complete remission after anti-viral treatment with IFN-alpha [137]. These results suggest that systematic screening for

HCV infection should be performed in patients who have been given a diagnosis of splenic lymphoma, as in some HCV-positive cases, anti-viral therapy may be an alternative to splenectomy, chemotherapy or both. Other studies have also reported regression of different types of lymphomas after IFN-alpha treatment, such as splenic and nodal marginal zone lymphomas [138] or HCV-associated immunocytoma [139]. Furthermore, treatment with IFN-alpha can achieve a concurrent disappearance of HCV-RNA and of lymphoma bone marrow infiltration [139]. Moreover, the regression of HCV-related gastric mucosa-associated lymphoid tissue (MALT) lymphoma after treatment with alpha interferon (IFN) plus RBV has been reported. Other authors have confirmed the beneficial effect of IFN treatment in patients infected by HCV and MALT (mucosa-associated lymphoid tissue lymphoma) of the salivary glands, the oral cavity, or the spleen [140, 141].

The inclusion of a control group integrated by patients with lymphoproliferative disease but without HCV infection [137] demonstrated that, in contrast with infected patients, HCV-negative subjects did not respond to IFN therapy. This observation indicates that the response observed in the HCV-infected patients is not merely due to the antiproliferative effect of IFN, but to the regression of clonal proliferation in response to anti-viral treatment with IFN, which is clearly associated with a virological response [142].

The management of extrahepatic manifestations is sometimes challenging due to their refractory nature, drug contraindications, toxicity, and adverse effects. In this case, the response of patient to the standard therapy with IFN-alpha plus RBV is poor and unsustained, often requiring additional immunosuppressants. Corticosteroids, cyclophosphamide, rituximab, and fludarabine have been introduced to refractory cases [143-146]. Rituximab is a chimeric monoclonal antibody against CD20 and has recently been used for HCV-associated EMC with favorable results [143, 144]. Rituximab is well tolerated with minimal toxicity usually limited to infusion periods. However, possible hepatitis reactivation and fatal infection are concerned. In fact, pure red cell aplasia due to parvovirus B [147] cytomegalovirus [148] and fatal varicella zoster infection [149] have been reported following rituximab. In this case, concurrent administration of anti-HCV agents including interferon-alpha plus ribavirin might be reasonable to suppress viral replication and to obtain possibly synergistic therapeutic effects against HCV-associated extrahepatic manifestations. Further studies on the safety and efficacy of rituximab are awaited. However, normally although rituximab temporarily eliminates normal B-lymphocytes, it is not generally associated with increased incidence of opportunistic infections [147]. Normal B lymphocytes re-emerge within weeks to months after administration of rituximab, and antibody production continues during B-lymphocytopenia because CD20-negative plasma cells are not eliminated. These data indicate that rituximab can be safely used in LPDs of HCV-positive patients.

Although, it is evident that larger therapeutical trials of anti-viral therapies are needed to determine their potential utility in HCV-infected patients with lymphoproliferative disorders, encouraging data have emerged from some of the above mentioned recent studies [4, 140, 141]. Multicentre controlled studies with pegylated interferon-alpha plus RBV are eagerly awaited.

FUTURE TREATMENT FOR HCV-RELATED CANCERS

Although HCC patients undergo medical and surgical treatment for primary tumor lesions, intrahepatic and extrahepatic recurrence frequently occurs, limiting patient survival. As previously described, the current treatments for HCC include surgical and non-surgical strategies. Unfortunately, at present, the five-year survival of individuals with HCC is low, mainly due to the late presentation of the disease as well as limited therapeutic options that are effective. In clinical practice, many patients do not qualify for, do not tolerate or do not respond to IFN-based therapy [150]. As a consequence, the number of patients presenting long-term sequelae of chronic hepatitis C, including HCC, is expected to further increase for the next 20–30 years.

Anyway, the considerable progress made using heterologous expression systems, functional cDNA clones, the replicon system, and, most recently, functional HCV pseudoparticles (Table **3**), has strongly contributed to development of novel anti-viral strategies. The development of effective combined treatments, including new medical agents, immunomodulators and gene therapy has been launched for treating HCV-related tumors and the results seem to be promising [151-153].

Table 3. ***In Vitro*** **and** ***In Vivo*** **Systems to Study HCV**

In Vitro
Transient cellular expression systems in cell lines and/or primary hepatocytes
Constitutive/inducible expression in stably transfected cell lines
Subgenomic/full-length replicon systems
Retroviral pseudoparticles
Chimeric viruses such as poliovirus-HCV
In Vivo
Transgenic mice
Immunodeficient mice/hepatocellular reconstitution models
Chimpanzee

Anticancer New Medical Agents

Antiangiogenic agents. The important vascularization that characterized HCC makes it an excellent candidate for the action of anti-angiogenic agents. This anti-cancer therapy has recently attracted an intense interest for its broad-spectrum of action, low toxicity, and the absence of drug resistance [154]. The most studied anti-angiogenic drugs are: thalidomide (phase II clinical trial), vascular endothelial growth factor (VEGF) antibody (phase I clinical trial), angiostatin (phase I clinical trial), endostatin (phase I clinical trial) and thrombospondin analogs (phase I clinical trial). Actually, however, adverse effects limit the use of these agents. In fact, in view of the significant neurological

toxicity observed using high dose thalidomide, encountered between the commonly cirrhotic HCC patients, this drug should be considered only in combination with other chemotherapy agents [155]. On the other hand, the transient expression of recombinant adenovirus-mediated human endostatin is a key problem for any therapeutic use. Conversely, the necessary multiple injection of this immunogenic transgene vector gives an immune response to the vector, causing a systemic toxicity. Indeed, in liver and kidney were observed hepatocytes and renal tubule degeneration and some degree of inflammatory reaction [156]. Furthermore, anti-angiogenic agents in combination with genetic immunotherapy (i.e. endostatin and IL (interleukin)-12) have been recently reported to exert a potent anti-tumor effect on hepatoma [157].

Anti-inflammatory agents, such as cyclo-oxygenase-2 (Cox-2) inhibitors (celecoxib, rofecoxib), interfere with the carcinogenic process. Several studies demonstrate that Cox-2 specific inhibitors have a significant anti-proliferative and pro-apoptotic effect on HCC cells, suggesting that selective block of Cox-2 may have preventive and therapeutic potential for human HCCs [158, 159]. Recently, Malka D *et al*. have reported a case of HCC, which has dramatically responded to celecoxib [160], giving new insights for its use in HCC treatment.

Novel means of delivering localized radiation, are 90-Yttrium (90Y) microspheres, Theraspheres, and Sirspheres for HCC treatment and ^{131}I-tositumomab and 90Y-ibritumomab tiuxetan for lymphomas. In a cohort of 65 patients, treatment with hepatic arterial 90Yttrium microspheres (Therasphere) appears to be a relatively safe and effective therapy for advanced-stage unresectable HCC [161].

Inhibitors of growth-factor-signaling and cell cycle enzymes. The mainly studied growth inhibitor agents are: Epidermal Growth Factor Receptor (EGFR) antagonists, inhibitors of MAPKs, Cdks (cyclin-dependent kinases), tyrosine kinases, PI3-kinase, phosphatase and tensin (PTEN), suramin and Raf kinase pathways.

BAY-43-9006 (Sorafenib, Onyx Pharmaceutical), an oral cytostatic Raf kinase inhibitor [162] is recommended for ongoing and future studies, because a recent phase I clinical study demonstrates that it provides some clinical benefits in patients with advanced refractory solid tumors [163].

EGFR-targeted agents (HER-1, O-13928 DD, Gefitinib etc.), which improve tyrosine kinase activity of EGFR, have shown promising anti-tumor activity in animal model of HCC [164, 165]. Finally, a class of drugs blocks the growth of tumor with minimal toxicity, inhibiting PI3K and Akt dependent growth factors, such as ABT-100 [166].

Anti-proliferative agents, include octreotide and arsenic trioxide. Regarding octreotide, although it is able to block proliferation and to induce apoptosis in HCC cells, a recent pilot study indicated that the beneficial response in terms of time to tumor progression and survival is limited [167]. Arsenic trioxide (Trisenox, Cell Therapeutics Europe) induces cell cycle arrest and apoptosis in hepatoma cells and inhibits the proliferation of tumor cells *in vitro* [168]. In a clinical trial this agent has presented some side effects (leukocytosis, nausea, abdominal pain, rash, fatigue, headache etc.), that do not appear to be permanent or irreversible.

Vitamins and derivatives. It has been indicated that vitamins (vitamin K2, vitamin E, vitamin A, vitamin D and their analogs) often act as specific antagonists of HCC tumor markers involved in control of cell growth (i.e. cdc25) [169], suggesting a role in preventing development of HCC. In addition, several studies demonstrate that combination of vitamin treatment with other surgical and non-surgical therapies ameliorates the clinical outcome in patients with inoperable HCC as well reduce recurrence in already operated patients [170-172].

Molecular Targets for Gene Therapy

The fundamental role of oncogenes and anti-oncogenes as important control elements of cell growth, differentiation, and apoptosis aroused an ever-increasing interest for these proteins as potential pharmaceutical targets for therapeutic intervention in cancer. Cancer gene therapy includes many options: silencing oncogenes, functional tumor suppressor genes, suicide gene/prodrug system, inhibitors of tumoral vascularization etc. One can consider gene therapeutic strategies aimed at inhibiting or inducing gene expression using various experimental systems, including, among others, antisense oligodeoxynucleotides, ribozymes, small interfering RNAs (siRNA) and oncolytic adenoviral vectors. Two latter molecular approaches have gained particular attention.

siRNA can specifically silence particular genes and may provide a powerful tool in genetic therapy for carcinoma [173, 174]. Unfortunately, resistance development is a potential obstacle also for siRNA-based therapy. HCV can develop resistance to prolonged treatment with siRNA through the accumulation of nucleotide point mutations within the siRNA target sequence [175, 176]. As expected, HCV replicons resistant to a given siRNA remain susceptible to siRNAs targeting different HCV RNA sequences, and the emergence of resistant replicons is diminished by the combination of two or more siRNAs. Thus, the use of two or more siRNAs targeting different sequences of the viral genome may provide a way to control the development of resistance. Moreover, interestingly, siRNAs that can specifically block cyclin E and survivin gene expression appear to inhibit growth in HCC cells [177, 178].

The effect of gene therapy strategies depends on a highly efficient neoplastic cell transduction resulting in intratumoral levels of the therapeutic protein able to induce tumor regression. This issue could be better reached with currently available oncolytic adenoviral vectors [179]. Apoptosis inductors (i.e. Smac and TRAIL), delivered by way of the oncolytic adenoviral vector, would provide a useful strategy for HCC eradication [180]. Effectively, NK4 (an hepatocyte growth factor (HGF)-antagonist and a broad angiogenic inhibitor), used in HCC gene therapy *via* a replication-deficient recombinant adenoviral vector, seems to be a promising strategy to treat HCC [181]. Furthermore, a strong anti-tumoral effect was observed in different HCC *in vitro* and *in vivo* models using oncolytic adenovirus based on the human telomerase reverse transcriptase and the E2F promoters [182, 183].

As already mentioned, adverse effects upon non-tumoral cirrhotic tissues often limit the use of anti-proliferative drugs. This problem could be overcome by having tumour cells transfected with any gene that renders them sensitive to prodrugs that are innocuous to non-transduced cells. Such genes should code for enzymes that convert a prodrug into a toxic metabolite. The best-characterized prodrug is thymidine kinase, that transforms ganciclovir into a toxic phosphorylated compound that inhibits both nuclear and mitochondrial DNA synthesis [184]. Thymidine kinase system for HCC treatment has shown efficacy in several studies [185-187]. To restrict prodrug gene expression to tumor tissue and avoid the damage of non-tumoral tissue, tissue-specific promoters can be used for intratumoral injection of the vector [188], such as that from alpha-fetoprotein (AFP) [189].

HCV Anti-Viral New Medical Agents

To prevent the onset of HCC, the best solution would be to eradicate the viral infection. Investigators have taken some different approaches to address this pressing medical need. Major research efforts have focused on the identification of agents that inhibit specific steps in the life cycle of the virus and drugs able to interfere with the host immune system. On the other hand, several efforts have been done in trying to develop an anti-HCV vaccine.

The 'HCV-specific drugs' have the capacity to block particular steps in HCV virus life cycle. They include molecules that inhibit HCV enzymes as well as agents, such as nucleic acid, that attack the viral RNA. The clinical success of HCV-specific drugs depends on their ability to suppress all viral variants as well as prevent the emergence of resistant viruses.

Drug design of molecules against HCV viral proteins. Each of the viral encoded replication enzymes, as well as viral receptors and the host immune system, has been studied in depth, because it might represent a target for anti-viral intervention. Specific inhibitors of the NS3 serine protease, as well as the NTPase/RNA helicase and the NS5B, are actually being developed by drug design as anti-viral agents; some of those are already in early phase clinical trials (Table **4**). It is likely that the combination of multiple drugs, possibly directed against viral as well against the host targets, will be necessary to bypass the HCV-related drug-resistance and efficaciously treat chronic HCV infected patients.

BILN 2061 (Ciluprevir) was the first HCV protease inhibitor to enter clinical trials [190]. To patients infected by genotype-1 HCV, BILN 2061 administered twice a day for two days, induced a dose-dependent decline of the viral load [190, 191]. Treatment with BILN 2061 was somewhat less effective on genotype-2 and -3 of HCV [192]. Unfortunately, the effect of BILN 2061 was transient, but the hope is that longer treatments with HCV protease inhibitors could lead to high rates of sustained viral response. Moreover, the clinical development of BILN 2061 was stopped because of the observation of cardiac toxicity in monkeys [192]. Additionally, against a future use of this drug, development of resistance to BILN 2061 was observed in the HCV replicon system [193, 194].

VX-950 is another inhibitor of NS3 serine protease that interacts covalently with the protease, but it develops viral resistance too [194]. Treatment with VX-950 induced a rapid decline of the viral load in patients infected by genotype-1 HCV at the end of two week therapy. However, this treatment was not sufficient to eradicate the virus, and viral RNA returned to baseline after stopping therapy.

Nucleoside analogs are transformed by the host cell to the corresponding nucleotides, which in turn inhibit synthesis of viral RNA as "chain terminators". In fact, they get incorporated by the viral polymerase in the nascent RNA molecule, inducing premature termination of the RNA synthesis. In particular, NM283 (Valopicitabine, Indenix Pharmaceutical/Novartis), a nucleoside analog of NS5B (Table **4**), when administered to genotype-1 HCV patients for, at least 2 weeks, induces a dose-dependent down-regulation of the viral load to less than 10% of the initial levels[3]. In all patients, viremia returned to pre-treatment levels after stopping therapy. This nucleotide analog was also given in combination with pegylated IFN-alpha. It caused a reduction in viral load of more than 10^5 fold, and HCV RNA was undetectable in most of the patients[4].

Non-nucleoside inhibitors (NNIs) of NS5B (Table **4**) seem to block the viral enzyme, hampering the conformational transition needed for initiation of RNA synthesis. Resistance to this class of inhibitor arises through a single mutation within the inhibitor-binding site [195].

Although, several studies are focusing their efforts on optimizing anti-viral therapy with anti-HCV nucleoside analogs and NNIs, actually, this class of drugs is characterized by diverse patterns of resistance. Hence, till now, there does not exist an exhaustive demonstration of their anti-viral activity.

Host Immunomodulators. A crucial question that future clinical studies need to address is whether combination therapy with solely HCV targeted drugs will be sufficient to cure patients or whether the stimulation of the host immune system by immunomodulators will be necessary to obtain a complete eradication of the virus.

HCV infection could be eradicated by agents that stimulate the host innate and adaptive immunity. With this purpose, synthetic agonists of Toll-like receptors (TLRs) 7 and 9 have recently demonstrated their potential in controlling HCV infection. TLRs are expressed by immune cells, which include macrophages, monocytes, dendritic and B cells [196]. They recognize the presence of exogenous microorganisms through the recognition of molecular patterns characteristic of pathogens such as bacteria, viruses and parasites [197]. The stimulation of TLRs initiates acute inflammatory responses by induction of anti-microbial genes and pro-inflammatory cytokines and chemokines. Preliminary data showed a statistically significant reduction in viral load, stimulating either TRL 9 or 7, during HCV infection, leaving a hope for future combined therapies.

[3] Abstract (n. 626) presented in the 40th Annual Meeting of the EASL, 2005; published in J Hepatol Vol. 42, supplement 2.

[4] Abstract (n. 93) presented in the 40th Annual Meeting of the EASL, 2005 ; published in J Hepatol Vol. 42, supplement 2.

Table 4. HCV-Specific Drugs

Compound	Target	Mechanism	Clinical trial phase	Company
BILN 2061 (Ciluprevir)	NS3-4A	Serine protease inhibitor	Phase II	Boehringer-Ingelheim
VX-950	NS3-4A	Serine protease covalent inhibitor	Phase Ib	Vertex/Mitsubishi
NM283 (Valopicitabine)	NS5B	Nucleoside analogue	Phase II	Idenix/Novartis
R803 HCV-086 HCV-796 JTK-109, JTK-003	NS5B	Non-nucleoside allosteric inhibitor	Phase II	Rigel ViroPharma/Wyeth Japan Tobacco

Vaccines. The generation of an effective HCV therapeutic vaccine is challenging, due to viral heterogeneity and the absence of adequate animal models or reliable tissue-culture systems for analysis and propagation of this pathogen.

Therapeutic vaccination with envelope protein E1 (InnoVac-C; Innogenetics NV) is the most advanced strategy and has reached phase II clinical trials [198]. Immunization with this vaccine candidate was well tolerated, elicited a significant *de novo* E1-specific T-cell response and increased the anti-E1 antibody levels in HCV chronically infected individuals. Improvement in liver histology was also detected in these individuals, although no significant reduction in viral load was observed [198].

It is now well established that cellular immunity is particularly relevant in the resolution of HCV infection [199]. DNA immunization can induce both humoral and cellular immune responses; therefore, it is an attractive approach for the development of an effective vaccine against HCV. DNA vaccines in the treatment of various diseases have progressed over the last few years from discovery to clinical trials [200-203]. Since the viral load is only partially down-regulated, using this kind of vaccines, combined use of accurate cytokines, chemokines or co-stimulatory molecules may be important to achieve a protective immune response.

Finally, considering the limitations imposed by the severity of the side effects associated with the anti-proliferative agents currently used or under investigation, a shift away from a single treatment to HCC to grant a privilege to combination therapy to cure HCC patients will be necessary to better therapeutically address liver cancer.

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