Review
The role of hepatitis C virus in the pathogenesis of hepatocellular carcinoma

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Hepatitis C virus (HCV) is a worldwide pandemic, chronically affecting over 170 million people worldwide. It is a major risk factor for the development of hepatocellular carcinoma (HCC) and there is increasing experimental evidence to suggest that the virus plays a direct role in neoplastic transformation. The purpose of this letter is to review the literature regarding two individual proteins of HCV, namely NS5A and core, and their role in the pathogenesis of HCC through perturbations of cellular pathways, in addition to their immunopathological effects of chronic inflammation. A systematic search of MEDLINE in addition to manual searches of citations in key papers was employed to identify relevant studies. There is overwhelming evidence suggesting the direct and indirect roles HCV plays in the pathogenesis of HCC. Recent progress in our understanding of the pathophysiology of HCV coupled with advances in in vitro models will ensure that positive strides are made in the treatment and management of this potentially fatal virus.

Key words: Hepatitis C, hepatocellular carcinoma, core, NS5A.

Introduction
Hepatitis C virus (HCV) was identified in 1989 and is now considered to be endemic worldwide, globally affecting at least 170 million people or 3% of the population. HCV is primarily spread parenterally and its mode of transmission includes mother to baby, blood–blood transmission and sexual transmission. The clinical picture of HCV infection is often an acute stage, followed by chronic infection in ~80% of people initially infected. Those in the acute stage may display mild flu-like symptoms, but are often asymptomatic. Those with chronic HCV infection may progress to cirrhosis of the liver and finally hepatocellular carcinoma (HCC). Current estimates suggest 2–5% of patients with liver cirrhosis due to HCV develop HCC annually.

Molecular biology
HCV is an enveloped virus with a positive-sense RNA genome of 9.6 kb. It is a member of the Flaviviridae family, of the genus Hepacivirus. The viral genome encodes for a single polyprotein that is subsequently cleaved into 10 mature proteins, with the structural proteins located near the 5' end of the polyprotein and the non-structural proteins located near the 3' end (Figure 1).

The envelope glycoproteins E1 and E2 are integral for cell recognition and binding. E1 is important in viral fusion with the target cell membrane. The core protein undergoes two cleavages to yield the mature protein. It forms the nucleocapsid, interacts with the glycoproteins E1 and E2 to form the envelope and can also affect the translational ability of the internal ribosome entry site. p7 is thought to be instrumental in the production of infectious viruses and may act as a virion protein, which is able to alter the permeability of the membrane.

NS2 and NS3 autocatalytically cleaves to release another serine proteinase situated at the N region of the NS3. NS3 then proceeds to cleave the rest of the non-structural proteins when complexed with its co-factor NS4A to release NS4B, NS5A and NS5B. The C terminal end of NS3 also serves as an RNA helicase to unwind double-stranded RNA during replication.

NS4B is involved in the replication complex of HCV by forming a membranous web which harbours the structural and non-structural proteins and provides the structural
support for the HCV replication complex. The function of NS5A is thought to be involved in interactions with the cell pathways and is a component in the replication complex, although its real function is unknown, and NS5B acts as the RNA-dependent RNA polymerase. All these non-structural proteins form a membranous complex through which RNA is replicated along the perinuclear membrane of the endoplasmic reticulum. Space constraints preclude a detailed analysis of the molecular biology of HCV and more detailed reviews can be found elsewhere in the literature.5

Cell culture systems and replication models for HCV

Until only recently, there has been a lack of a robust and reliable replication model enabling the HCV life cycle and its pathogenic role to be easily characterized. A detrimental consequence of this is that research into HCV has been difficult. There are many cell culture models which support the in vitro replication of HCV but an in-depth description of these lies beyond the scope of this project. Therefore, in order to provide a framework whereby the limitations of the studies in the reviewed literature can be realized, a very brief outline of experimental models will be described. More detailed reviews may be found elsewhere in the literature.6,7

Animal models used for the study of HCV have been limited by the narrow host range of the virus. Of these, chimpanzees are considered the only robust animal model. As with all animal models, infection is usually variable and transient, and susceptibility is low.8 In vitro models such as HuH7 and human hepatoblastoma cells (HepG2) tend to produce a low efficiency of HCV replication.9 Recently, the use of selected subgenomic HCV RNAs, known as replicons, has yielded promising results, with continuous autonomously replicating HCV RNAs in HuH7 cells, easily detected using northern and western blotting.10 However, in order to enhance replication, adaptive mutations in these replicons have occurred, which are seldom seen in nature. In addition, these mutations have led to a reduced infectivity in chimpanzees.11 Also, few cell lines are able to support the efficient replication of the replicons, with HuH7 being the most common. The subgenomic replicons lack structural protein-encoding genes, so these proteins cannot be studied. Although, genomic replicons have been created, viral assembly and release have not been demonstrated.

HCV, steatosis and fibrosis

Studies involving the use of transgenic mice have demonstrated that HCV and its proteins have the ability to induce fibrosis, either through direct interference with cell activation pathways or by indirect means, via the induction of steatosis.3,12

Fibrosis results from the deposition of extra-cellular matrix (ECM) material around the liver parenchyma. Stellate cells play an important role in liver fibrogenesis, depositing large amounts of ECM as a result of inflammation or hepatocyte damage. Other cells of the immune system such as Kupffer cells can generate reactive oxygen species (ROS), which stimulate stellate cell activity. ROS are produced naturally as a result of aerobic respiration. ROS, as well as having oncogenic properties through DNA damage, induces the production of TGFβ, which has been shown to have potent fibrogenic effects.13

Certain genotypes of HCV, in particular genotype 3, have been shown to cause an accumulation of lipids within hepatocytes, a pathology known as steatosis. Steatosis may then go on to cause the progression of fibrosis. Research has shown that HCV genotype 3 is directly involved in causing steatosis, supported by the fact that treatment of genotype 3 causes disappearance of steatosis.14 Core and NS5A have been hypothesized to be the HCV proteins involved in the formation of steatosis and may also be directly involved in liver fibrosis. Core has been demonstrated to decrease the activity of the microsomal triglyceride transfer protein (MTP). This protein is involved in the transfer of lipids within cells and its inhibition would reduce the formation of VLDLs as well as the secretions of ApoB and triglycerides (TG). This would then lead to accumulation and build-up of TGs in hepatocytes.15 The process by which this occurs is unknown, but one hypothesis indicates that HCV core could bind to lipids
which would then prevent its interaction of MTP with its target lipids. HCV core has also been demonstrated to bind to apo AII (a component of HDL), and this binding may help in the formation of its capsid.\(^{16}\) This binding prevents core from reducing VLDL levels but it causes secretions of the naked core into the blood stream, which may then play a role in immunological evasion.

Core may induce liver damage as it interacts with retinoid X receptor alpha, which is highly expressed in the liver and is normally involved in gene regulation and lipid metabolism. This interaction upregulates genes involved in lipid metabolism, although the mechanisms of how this occurs are unknown.\(^{17}\) HCV core has also been shown to induce oxidative stress in murine models through lipid peroxidation, that is to say, the degradation of lipids by oxidation.\(^{18}\) This is a result of its ability to induce the generation of ROS in the absence of any inflammation which is also implicated in ROS production. The importance of the absence of inflammation suggests that it is core itself which is responsible for ROS generation.

Perhaps, the chronic inflammatory process of HCV infection would lead to increased mutagenesis in the regenerating hepatocytes, leading to a multi-step process of mutations finally presenting as HCC. However, in auto-immune hepatitis, the occurrence of HCC is extremely rare, raising doubts as to whether inflammation alone is able to lead to such a high incidence of HCC in patients infected with HCV. This coupled with experimental evidence demonstrating the role of HCV in the development of HCC\(^{19}\) has implicated the direct role of HCV in HCC.

**HCV and hepatocellular carcinoma**

The pathogenesis of HCC from HCV infection is yet to be fully understood, with various viral protein–host cell interactions hypothesized to play a direct role in the development of HCC. Perturbations in the cell cycle, combined with upregulation of oncogenes and loss of tumour-suppressor gene functions, may combine to lead to HCC development; HCV proteins have been shown to interact with these cellular pathways. The natural history of HCV infection is progression to fibrosis and cirrhosis, leading to HCC in a significant proportion of the infected population. These viral protein–host cell interactions may play a role separate from cirrhosis in the development of HCC but they also play an integral role in the cause of chronic infection, leading to fibrosis and cirrhosis.

**HCV core**

HCV core has been shown to perturb and alter cell cycle growth at various stages, resulting in deregulation of mitosis (Figure 2). One study demonstrated that core is involved in interaction with the double-stranded RNA-activated protein kinase, PKR, which is involved in many cellular processes such as apoptosis and cell growth.\(^{20}\) PKR is activated by IFN and phosphorylates the eukaryotic initiation factor, eIF2\(\alpha\), limiting protein synthesis and so inhibiting cell and viral growth. PKR has also been shown to be involved in mediating several forms of stress-induced apoptosis.\(^{21}\) PKR also phosphorylates and deactivates I\(\kappa\)B (the cognate NF-\(\kappa\)B inhibitor). As well as mediating inflammation, NF-\(\kappa\)B protects against apoptosis.

Core may mediate G2/M phase arrest via a p53-independent manner.\(^{20}\) It has been shown that core induces the phosphorylation of PKR at threonine 446, which would alter PKR activity on different substrates as well as prevent apoptosis. In a previous study, mutations of PKR at residue 446 in mice have been shown to cause tumours.\(^{22}\) However, the results and conclusion of the study were limited with regard to the method employed and what is currently known about PKR pathways. Alisi et al.\(^{20}\) used hepatoma cells expressing core protein only but the disadvantage of this method is that core protein is not found independently in the cell during natural infections. Indeed, NS5A may interact with PKR\(^{23}\) and so may interfere with core. It would be interesting to see whether core and NS5A compete or complement each other with regard to PKR, if either of them influences PKR at all. Also, it has been suggested that core interferes with the PKR pathway to impair apoptosis, but no known pathways have yet been identified by which this may occur. Therefore, the conclusions drawn are as yet limited.

The functional consequences of the interaction of HCV with p53 and p73 (a related tumour-suppressor protein) has yet to be fully elucidated, but considering that cancer is a multi-step process that requires perturbations in negative and positive regulators of cell growth, and given the prominent role that p53/p73 has in cell proliferation, these pathways are thought to play influential roles in the pathogenesis of HCC.

The core protein is able to bind to p53, a key component in cell cycle arrest and apoptosis. By allowing HCV to modulate the cell cycle, it can either prevent apoptosis and allow the replication of its progeny or induce apoptosis to enable viral spread. The disruption of checkpoints in various stages of the cell cycle is one way that tumourgenesis can occur. Core may enhance p53 function by increasing the affinity of p53 to its DNA-binding site or by increasing its transcriptional activity without increasing p53 expression itself.\(^{24}\) However, although a small proportion of core is found in the nucleus, core is predominantly cytoplasmic\(^{5}\) and p53 is located within the nucleus, which may suggest that p53 enhancement may not be fully explained by these mechanisms. A separate study has suggested that core inhibits p53, but this was performed using core protein only and did not employ an appropriate control, as the endogenous p53 levels were not measured.\(^{25}\)
Core has also been shown to interact with the activity of a target gene of p53, p21\textsuperscript{WAF1/CIP1}. This protein normally translocates to the nucleus and inhibits cyclin-dependent kinase (CDK) complexes, resulting in cells failing to exit the G1 phase of their life cycle.\textsuperscript{26} Otsuka et al.\textsuperscript{24} demonstrated that p21\textsuperscript{WAF1/CIP1} activity is increased by p53 activation but Ray et al.\textsuperscript{27} concluded that p21\textsuperscript{WAF1/CIP1} activity is repressed by a p53-independent pathway. These differences may be reconciled by another study whereby p21\textsuperscript{WAF1/CIP1} activation depends on the form taken by core, i.e. either the innate or mature form.\textsuperscript{28} Full-length core (191 amino acids long) found in the cytoplasm increases the expression of p21\textsuperscript{WAF1/CIP1} by activating p53, whereas mature core found in the nucleus decreases p21\textsuperscript{WAF1/CIP1}. Subcellular localization of the mature form is regulated by the innate form. Therefore, the core protein function with regard to p21\textsuperscript{WAF1/CIP1} activation is dependent on its localization within the cell and this must be considered when investigating core—something not considered in previous studies.

A recent study by Fukutomi et al.\textsuperscript{29} has been able to demonstrate the ability of core to increase cell proliferation, using both transient transfection of the core protein and a full-length HCV replicon in Huh7 and Huh7.5 cells, respectively. Microarray analysis also revealed 372 genes (out of 12,500 analysed) to be transcriptionally affected by core. In particular, most oncogenes were upregulated in contrast to most tumour-suppressor genes which were downregulated. One of the genes found to be upregulated, wnt-1, is implicated in the oncogenic process, especially in HCC.\textsuperscript{4}

Although this study demonstrated these abilities of core, the mechanism by how they occur is yet to be identified. Indeed, the complex nature of cell-signalling pathways coupled with the multi-step process of hepatocarcinogenesis implies that a combination of sequential gene up/downregulation is required for HCC to occur. Therefore, the fact that these genes are affected does not necessarily imply how, if at all, they are involved in tumourigenesis. This study, however, does provide firm evidence of the ability of core to alter cellular pathways.

Core is involved in a multitude of host cell pathways, and space constraints prevent a detailed discussion of each pathway. For other postulated interactions of core with host cell pathways and their relevant references, please refer to Table 1.

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**Figure 2.** The complex nature of HCV core interactions with host cellular processes is demonstrated here. Red arrows indicate inhibition, black arrows indicate activation and purple arrows show activation or repression. Each group of pathways is colour-coded to allow easier visualization of interactions.
### Table 1. Postulated interactions of core with host cellular pathways

<table>
<thead>
<tr>
<th>Host cellular protein with which core interacts</th>
<th>Host cellular protein function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKR</td>
<td>Apoptosis and cell growth</td>
<td>20</td>
</tr>
<tr>
<td>p53 and p73</td>
<td>Tumour-suppressor protein</td>
<td>24</td>
</tr>
<tr>
<td>p21^{WAF1/CIP1}</td>
<td>Prevents exit of G1 phase of cell cycle</td>
<td>28</td>
</tr>
<tr>
<td>TNFα</td>
<td>Apoptosis</td>
<td>43, 44</td>
</tr>
<tr>
<td>NFκB</td>
<td>Anti-apoptotic, chemoattractant for immune cells</td>
<td>44, 45</td>
</tr>
<tr>
<td>LZIP</td>
<td>Tumour suppressor</td>
<td>46</td>
</tr>
<tr>
<td>hnRNP K</td>
<td>Stimulating the promoter of the oncogene c-myc; inhibiting the thymidine kinase promoter, which is important for G1/S transition</td>
<td>47</td>
</tr>
<tr>
<td>14-3-3-α</td>
<td>Ras/Raf/MAPK pathway</td>
<td>48</td>
</tr>
<tr>
<td>Bcl-xL</td>
<td>Anti-apoptotic</td>
<td>49</td>
</tr>
<tr>
<td>Bax</td>
<td>Pro-apoptotic</td>
<td>50</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Cell cycle arrest in the G1 phase; fibrogenesis; limiting the antiviral immune response</td>
<td>51</td>
</tr>
<tr>
<td>Cyclin E</td>
<td>Cell proliferation</td>
<td>52</td>
</tr>
<tr>
<td>MAPK pathway</td>
<td>Cell proliferation</td>
<td>53</td>
</tr>
<tr>
<td>Lymphotixin β receptor</td>
<td>Cell differentiation</td>
<td>54</td>
</tr>
<tr>
<td>Fas pathway</td>
<td>Apoptosis</td>
<td>60</td>
</tr>
</tbody>
</table>

**Figure 3.** The complexities of NSSA interactions with relevant host cellular pathways. Black arrows indicate activation, red arrows show repression and purple arrows show either activation or repression.
NS5A

The role of NS5A in cell transformation, differentiation and oncogenesis has been intensely studied and many cell-signalling pathways have been implicated. Indeed, the importance of the role of NS5A in HCC can be highlighted from a study where nude mice expressing NS5A all developed tumours.30 NS5A has been shown to interact with a multitude of proteins, but space constraints preclude the discussion of all of these. For an overview of NS5A interactions mentioned in what follows, please refer to Figure 3 and Table 2.

NS5A may cause the activation of NF-kB via a PKR pathway, leading to inflammation. It has been shown using yeasts and mammalian cell systems solely expressing NS5A that NS5A has a direct role in its ability to inhibit PKR.23 It has been suggested that a region within NS5A, known as the interferon sensitivity-determining region (ISDR), binds to PKR preventing it from dimerizing. This then disrupts PKR function and prevents phosphorylation of eIF2.

However, in a study involving cell lines expressing all the structural and non-structural HCV proteins, it was demonstrated that NS5A had no significant effect on PKR. The co-expression of other proteins may have localized NS5A to organelle membranes, thus reducing its potential to bind to PKR, as PKR is normally found throughout the cytoplasm.31 This provides an adequate explanation of the incongruent results between the two studies and demonstrates the importance of effective in vitro cell culture systems in correctly reflecting natural infection: by expressing a single HCV protein,23 an incorrect interpretation on the importance of NS5A and its ability to bind to PKR may have been realized.

A separate in vitro study using Huh7 cell lines harbouring HCV replicons32 seems to demonstrate NS5A does inhibit PKR pathway, supporting the initial data.23 However, mutations within the NS5A-coding region in order to enhance replication of HCV in vitro may not necessarily have reflected the true course of natural HCV infection. Indeed, these mutations altered the subcellular localization of the protein, which may impact on its interaction with PKR. Molecular epidemiological studies have further indicated the interaction of NS5A with PKR, where mutations within the ISDR correlated to response to IFNα treatment.33

This controversy surrounding the link between PKR and NS5A has demonstrated perfectly how, up until recently, a lack of both robust cell culture models and efficient HCV replication systems have impeded research in this subject, leading to conflicting results.

The direct binding and cytoplasmic co-localization of p53 as well as interaction of NS5A with the p53 co-activator hTAFII32, a key component of TFIIID has also been shown.34 Cytoplasmic sequestration of p53 could lead to the transcriptional repression of p53 target genes in a dose-dependent manner, and NS5A binding to hTAFII32 through an FXXφF (where X is any residue and φ a hydrophobic residue) motif may also play a vital role in abrogating the function of p53. NS5A may bind to the N-terminus of p53, which is a common activation site for other proteins such MDM2.35

This finding has been supported by another study, where p53 binding led to a reduction in p21WAF1 and consequently increased cell growth.36 However, in this latter study, it was suggested that binding to p53 is mediated by the N-terminal 150 residues of NS5A, whereas Lan et al.36 suggest it is mediated by the C-terminal 285 residues of NS5A. One possibility is that NS5A may bind to p53 via both the N and C terminus. However, the mechanism by which NS5A causes p53 cytoplasmic sequestration remains unknown. All these studies used the ectopic expression of NS5A which may not reflect natural infection, both in terms of NS5A expression levels and the potential cell interactions during virus morphogenesis.

In contrast to these two studies demonstrating the ability of NS5A to reduce p21WAF1 levels, Arima et al.37 showed that p21WAF1 may be increased as a result of NS5A interacting with Cdk1/2 cyclin complexes. It has been suggested that these conflicting results may have arisen due to the use of differing non-hepatic cell lines.38 Another explanation may be that NS5A may act on different parts of the cell pathway that influence p21WAF1, although the reasons as to why remain unknown.

### Table 2. Postulated interactions of NS5A with host cellular pathways

<table>
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<tr>
<th>Host cellular protein with which NS5A may interact</th>
<th>Host cellular protein function</th>
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</tr>
</thead>
<tbody>
<tr>
<td>PKR</td>
<td>Apoptosis and cell growth</td>
<td>23, 32</td>
</tr>
<tr>
<td>Growth factor receptor-bound protein 2 (Grb2)</td>
<td>Involvement in cell growth, differentiation and transformation</td>
<td>55</td>
</tr>
<tr>
<td>Interleukin 8</td>
<td>Polymorph chemotaxis and degranulation; inhibiting IFNα</td>
<td>56</td>
</tr>
<tr>
<td>PI3K</td>
<td>Anti-apoptotic</td>
<td>57</td>
</tr>
<tr>
<td>GSK-3β</td>
<td>Proto-oncogene</td>
<td>57</td>
</tr>
<tr>
<td>PS3</td>
<td>Apoptosis and suppress oncogenesis</td>
<td>34</td>
</tr>
<tr>
<td>p21WAF1/CIP1</td>
<td>Prevents exit of G1 phase of cell cycle</td>
<td>37</td>
</tr>
<tr>
<td>TNFα</td>
<td>Apoptotic protein</td>
<td>58</td>
</tr>
<tr>
<td>Human vesicle-associated protein</td>
<td>Vesicle transport</td>
<td>59</td>
</tr>
<tr>
<td>Bad</td>
<td>Proto-apoptotic protein</td>
<td>57</td>
</tr>
<tr>
<td>Bax</td>
<td>Apoptotic protein</td>
<td>50</td>
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</tbody>
</table>
Conclusion

Since the discovery of HCV nearly 20 years ago, great strides have been made in understanding the epidemiology, molecular biology and pathological nature of the virus. There is now an overwhelming weight of evidence suggesting direct and indirect roles of HCV in the pathogenesis of HCC, stemming from both in vitro and in vivo studies. HCV is able to induce immunopathological effects, generate ROS and cause steato-sis, fibrosis and cirrhosis. It is also able to exert direct oncogenic effects on the host cell through perturbations in cell signalling, secretory pathways and the cell cycle. However, many of the mechanisms of selected repression or induction of specific genes and pathways remain vague and difficult to elucidate.

The major obstacle to research in HCV has been a lack of a robust, reliable and efficient cell culture system. However, recently, a subgenomic replicon was derived from a viral isolate termed Japanese fulminant hepatitis 1 (JFH-1), a variant of HCV genotype 2a obtained from the serum of a Japanese patient. This replicon did not harbour any adaptive mutations yet retained its replicative efficiency and infectivity. Recent studies have subsequently led to the development of a full-length JFH-1 genome as well as chimeric full-length HCV genomes demonstrating efficient production of infectious HCV. New in vitro systems involving the use of JFH-1 will enable the study of the complete HCV life cycle as well as HCV interactions within the context of the whole genome.

One limitation of previous studies has been the individual expression (even potential over-expression) of single proteins, which do not reflect natural HCV infection. This problem has been further amplified by the variable use of truncated proteins among the different experiments, as well as the use of differing genotypes and subtypes. This may go part of the way to explaining the conflicting results of many studies.

However, the past two decades have not been without progress in our understanding of HCV. Core and NS5A have all been shown to interact with a multitude of proteins, impacting on cellular pathways which may contribute to tumourigenesis.

What does the future hold for HCV? Recent advances in cell culture models combined with a growing understanding of the pathological processes involved with HCV infection indicate a very positive stride towards effective treatment and management. Indeed, the ultimate objective to our understanding of HCV is to enhance and progress current antiviral therapies and potentially develop a vaccine against this worldwide pandemic. Only by first understanding HCV and its pathological role in the development of HCC can effective treatment be developed and delivered. Given the progress so far and the advent in new in vitro techniques to accelerate research, there is much hope for this in the not too distant future.

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