Review

HCV genetics and genotypes dictate future antiviral strategies

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Abstract

At the end of the 1980s, the hepatitis C virus (HCV) was cloned and formally identified as the cause of the majority of non-A and non-B hepatitis cases. Today, around 170 million people worldwide are infected with HCV, making it five times more common than infection with the human immunodeficiency virus (HIV). Several methods exist which mediate the spread of infection. One of the most common and efficient is sharing or re-using injecting equipment; studies have indicated that 80-90% of individuals in some populations of intravenous drug users test positive in serum HCV assays. Contracting HCV from infected blood transfusions was also a major cause of infection before screening tests were introduced in the early 1990s. Other possible, but less common, methods of infection transmission include mother-to-child during pregnancy, sexual contact and nosocomial acquisition (for example between surgical or dialysis patients). It appears that concurrent HIV-1 infection increases the risk of HCV transmission via the mother-to-child or sexual routes.

Introduction on clinical signs & symptoms

Acute infection with HCV usually only results in mild symptoms, and the majority of patients do not experience any signs at all (Lauer & Walker 2001). Symptoms that may be seen are non-specific – e.g. jaundice, nausea and a general feeling of being unwell (Lauer & Walker 2001) – and therefore cannot be used to conclusively diagnose HCV infection. The rationale for treatment of HCV infection in patients who are symptomatic at the acute infection stage is uncertain, as the few individuals who present with symptoms often recover spontaneously (i.e. in the absence of treatment) (Pearlman 2004).

This acute phase usually progresses to chronic infection, which often remains clinically silent (Lauer & Walker 2001) for many years until the development of severe liver damage (De Francesco et al. 2003). The period from infection to the detection of liver damage can last over 30 years (Lauer & Walker 2001), during which period the patient may unwittingly spread the virus to others as they are unaware of their infective status. HCV (Loukatou *et al.* 2015) infects

hepatocytes – which leads to the development of progressive fibrosis in over 50% of cases and cirrhosis in 15-20% of infected patients (De Francesco *et al.* 2003). Cirrhosis itself is a risk factor for hepatocellular carcinoma, the most serious consequence of HCV infection; liver cancer develops in 1-4% of cirrhotic HCV patients each year (Lauer & Walker 2001).

Many additional factors affect the prognosis of the HCV-positive patient. Alcohol consumption (Pearlman 2004), male sex (Lauer & Walker 2001) and infection at a later age (Lauer & Walker 2001) are all associated with an increased risk of cirrhosis (Lauer & Walker 2001). Concomitant hepatitis B or HIV infection also increases the rate of development and prevalence of cirrhosis. HIV co-infection has been shown to adversely affect treatment response rates and to increase treatment discontinuation (Pearlman 2004).

Testing for HCV

Today's methods for diagnosing HCV (Loukatou *et al.* 2015) have greatly increased accuracy over the 'first-generation' tests introduced after the identification of the HCV (Majid & Gretch 2002). Several types of tests are available for the various stages of HCV infection (Loukatou *et al.* 2014).

Diagnosis

Testing for HCV is usually undertaken in patients with known risk factors for infection, or who present with increased aminotransferase levels (Majid & Gretch 2002). An enzyme immunoassay (EIA) is used to diagnose 'seroconversion' caused by HCV infection (Majid & Gretch 2002). The EIA reacts with antibodies produced by the immune system in reaction to HCV (Loukatou *et al.* 2015) infection to produce a positive result (Majid & Gretch 2002). If a confirmatory test is required, a (more complex) polymerase chain reaction (PCR)-based technique is used (Majid & Gretch 2002). This is much more sensitive and may be required in, for example, immunosuppressed patients (Lauer & Walker 2001).

Establishing viral load prior to treatment

Quantitative assays are generally needed at this stage as the viral load before treatment begins can predict the chances of success (Boulaki *et al.* 2013). A low blood HCV level tends to favour an improved treatment response (Majid & Gretch 2002). Techniques are based on one of the following: a) complexation of viral genomic material with complementary oligonucleotides followed by binding to an amplifier molecule (branched deoxyribonucleic acid) (Majid & Gretch 2002), b) PCR technologies, c) generation of copy cDNA from viral RNA hybridised with a promoter region which is then used to generate multiple RNA copies which emit a 'flash signal' on testing (transcription-mediated amplification [TMA]) and, d) combination of PCR and TMA technologies to give nucleic acid sequence-based amplification (NASBA) (Majid & Gretch 2002). The most sensitive methods are PCR-based; bDNA technology is the least sensitive (Majid & Gretch 2002). TMA is as sensitive as the best PCR technologies but is only used qualitatively in clinics (Majid & Gretch 2002).

Monitoring response to treatment

HCV serum load is generally tested after 3 and 6 months of treatment (Majid & Gretch 2002). An undetectable viral load at 6 months gives a high chance of a successful treatment outcome (Majid & Gretch 2002). The same methods used to evaluate pre-treatment virus levels are used to monitor treatment response.

Diagnosing/Monitoring Liver Disease

Two main techniques are used in tracking the progress of liver disease in HCV-infected patients. Alanine aminotransferase levels are a non-invasive, low-cost way of diagnosing liver disease; however this can be an unreliable marker, as levels are prone to fluctuation and may remain within 'normal' limits even when liver damage is present (Lauer & Walker 2001). A liver biopsy is by far the most accurate determinant of current levels of hepatic damage and can also contribute to the assessment of a patient's prognosis; however the invasive nature of this procedure limits its use (Lauer & Walker 2001).

HCV Genetics

The HCV Genome

HCV is a member of the family flaviviridae, which also encompasses the dengue, yellow fever and hepatitis G viruses (Lauer & Walker 2001, Papageorgiou *et al.* 2014, 2016, Vlachakis & Champeris Tsaniras 2016, Vlachakis *et al.* 2012, 2013). The 9.6 kilobase genome shown above encodes over 3000 amino acids. The genome is initially translated by ribosomes into a single polyprotein (De Francesco *et al.* 2003) which is then processed into the ten functional protein products. Different areas of the genome exhibit differing rates of mutation (Lauer & Walker 2001). Two areas of the E2 protein are known as 'hypervariable', as they frequently mutate. In contrast, the 5'UTR is extremely highly conserved (around 90% sequence consistency) between HCV genotypes and subtypes, making it an ideal 'target' for diagnostic testing (Majid & Gretch 2002) and drug therapy.

Genotypes

By examining sequence similarities (Papangelopoulos *et al.* 2013), six distinct HCV genotypes can be identified – each with their own range of subtypes (Lauer & Walker 2001). There is over 30% genetic diversity between genotypes (Simmonds 2004), although much of this is localised to specific regions – such as the envelope proteins – while many essential enzymes remain almost unaltered between genotypes. This may be because envelope proteins constitute the portion of the

virus most likely to be recognised by the host immune system, and therefore mutations are selected for in a 'Darwinian' process as they allow the virus to evade host immune responses. Enzymes, on the other hand, cannot easily be dramatically altered as this could prevent them from functioning. Between subtypes, a lesser (20-25%) degree of genetic diversity is observed, and much of this can be accounted for by changes in the base sequence which result in synonymous coding (i.e. the sequence changes but still codes for the same amino acids) (Simmonds 2004). Genotypes 1a and 1b are the most common in the West (Simmonds 2004), with genotypes 2 (Lauer & Walker 2001) and 3 (Simmonds 2004) seen over the same geographical area but less frequently; other genotypes are almost never seen here (Lauer & Walker 2001).

Genotype 3 has also been present over the whole of Asia for many years now (Pybus *et al.* 2005). In Egypt, a country which has the highest proportion of HCV infection in the world, genotype 4 predominates (Lauer & Walker 2001), whilst genotype 5 is frequently seen in South Africa (Simmonds 2004) (although genotypes 1 and 2 are common in the rest of Africa) (Pybus *et al.* 2005). Genotype 6, although previously only seen in Southeast Asia, has now also spread to Australia (Simmonds 2004).

Knowledge of the genotype (and subtype) of an HCV infection is useful in predicting treatment responses. Infection with strains 2 or 3 is known to be more amenable to treatment, while genotype 1 infection is more likely to require prolonged therapy and has lower successful treatment rate (Lauer & Walker 2001).

HCV Therapy - Current Treatments & Future Targets

Current Treatments

Due to the combination of a high number of infected persons and the poor success rates of current treatments, there is an urgent need for new, more effective HCV treatments (Loukatou *et al.* 2015). Although several areas have been identified for the development of new HCV therapies, Ribavirin together with pegylated interferon-α (PEG IFN-α; the 2a and 2b forms are both used) is the current treatment of choice for HCV infection (Pearlman 2004). Ribavirin, a guanosine analogue, is the only nucleoside analogue to have been identified as being useful in HCV treatment (Gordon & Keller 2005). PEG IFN-α acts both as an antiviral (by inhibiting replication) and immunomodulating (by enhancing the host immune response) agent (Gordon & Keller 2005). Previously, 'standard' IFN-α was used but attachment of polyethylene glycol residues was found to increase both its activity and half-life (Pearlman 2004), therefore allowing a more effective treatment to be given less frequently.

Problems are often encountered when using the therapy above, not least of which is the fact that - even with optimal treatment regimes - response rates remain at a little over half of all patients treated. Even where treatment is successful, up to 50% of patients will relapse (i.e. the infection returns) (Walker 1999). Other problems include the high cost of treatment, the fact that PEG IFN-α can only be given by injection, the lower success rates with infections caused by genotypes other than HCV genotype 2 or 3 and several side -effects (Lauer & Walker 2001).

Viral Life Cycle-Based Future Targets

Due to the combination of a high number of infected persons and the poor success rates of current treatments, there is an urgent need for new, more effective HCV treatments. Although several areas have been identified for the development of new HCV therapies, research directed at viral life cycle targets has the greatest possibility for producing a true cure for HCV. This is because, unlike other research areas such as antifibrotic agents (Pearlman 2004), a drug which blocks an essential step in the virus life cycle will prevent replication and therefore 'kill' the virus and allow the body to clear the infection. In addition, drugs aimed at virus-specific enzymes have a lower potential for adverse effects as (in theory) they should not affect host enzymes. Viral life-cycle targets have been the focus of most research and development (R&D) efforts that have been disclosed to date. Here, only those targets which have already been the subjecs (Walker & Hong 2002) of published drug discovery efforts (Kandil *et al.* 2009, Walker 1999) will be discussed.

It should be noted that investigations into possible treatments have been severely limited by the lack of suitable small-animal or tissue culture models for HCV infection, even though bovine viral diarrhoea virus has been used in cell culture as a substitute for HCV during screening (Buckwold *et al.* 2003). Currently, the only widely accepted animal model available is the chimpanzee, which has too many costs and ethical issues to be routinely used for identification of possible candidate drugs (Gordon & Keller 2005). However, new models have been and are still being developed which are revolutionising the area of anti-HCV drug development and testing (Vlachakis *et al.* 2013). A successful and fairly widely used subgenomic replicon system - where the virus genome has the areas coding for structural elements removed and different genes for replication control and marker regions added (Papangelopoulos *et al.* 2013) - was developed for use as a cell culture system by Lohmann *et al.* in 1999. Small animal models have also been the focus of several research teams, resulting in the production of transgenic mice with livers containing viable chimeric human hepatic tissue (Mercer *et al.* 2001), although this approach - together with similar reports on HCV infection of tree shrews and transgenic mice - appears to have received less attention than cell culture models (Moradpour *et al.* 2002).

Structural envelop glycoproteins E1 and E2

The structural proteins comprise the HCV viral capsid encoded by the core proteins and the envelope glycoproteins E1 and E2. The envelop glycoproteins are located on the surface of virion and represent a multifunctional complex of proteins, which operate a number of significant biological functions. Based on descriptions bearing, they are heavily glycosylated with complex sugar moieties and represent critical determinants for HCV entry into permissive cells and facilitating receptor binding. Moreover, the envelope structural glycoproteins E1 and E2 mediate the fusion procedure and entry into host cells membrane. The glycoproteins also contribute to virion assembly, bind host lipoproteins and apolipoproteins, and are the primary targets of the humoral immune response. Although these critical functions must be maintained, HCV envelop glycoproteins E1 and E2 are the targets of host adaptive immune response and must be changed in order to escape antigens detection, which is frequently mutate in viral replication. Until now, many studies have been performed in order to understand all aspects of HCV glycoproteins biology, including functionality, structure, genetic diversity and anti-genicity, and to use it as a potential vaccination strategies against HCV virus.

Internal ribosomal entry sites (IRES)

Contained within the 5'-UTR of the HCV genome, the IRES allows binding of the viral genome to host ribosomes, which enables protein translation to be initiated (Majid & Gretch 2002). Drug discovery efforts to date have revealed antisense agents (which bind to the RNA sequence to prevent it from interacting with the ribosome), some small molecule inhibitors which may interact with ribosomal binding sites within the IRES, and ribozymes (Papageorgiou *et al.* 2014) which contain antisense portions for targeting and catalytic sequences for genome destruction (Walker 1999). One of the most well-defined classes of small molecule inhibitors is the phenazines (Wang *et al.* 2000), although current testing does not appear to have extended beyond *in vitro* evaluation. Several antisense oligonucleotides have been developed and tested (McCaffrey *et al.* 2003), and the ribozyme agent Heptazyme has entered clinical trials - although its development has been halted after severe toxicity was seen in primates (Gordon & Keller 2005). Although this is a setback for ribozyme drugs / IRES targeting (Loukatou *et al.* 2014), this remains an important target which may produce useful anti-HCV drugs in the future (Papageorgiou *et al.* 2014).

NS5B (non-stractural protein) Rna-Dependent Rna polymerase

This enzyme has been identified as a major target in the search for new anti-HCV drugs as it is both essential for replication (Chan *et al.* 2004) and has no host equivalent in non-infected cells. The genetic similarities observed between this RNA-dependent RNA polymerase (RdRp) and other related polymerases has aided its characterisation, and crystal structures (Bressanelli *et al.* 1999) have now revealed that the HCV RdRp has a 'classic' palm/fingers/thumb structure (Bressanelli *et al.* 1999, Chan *et al.* 2004, Lesburg *et al.* 1999, Walker & Hong 2002). The enzyme also has a large hydrophobic carboxy terminal (Bressanelli *et al.* 1999); this may mean that the enzyme is membrane-bound *in vivo* (Papageorgiou *et al.* 2013).

The sequence of events catalysed by this enzyme (i.e. potential target processes for drug-based inhibition) is thought to run in the following order: template docking & nucleotide binding, initiation, elongation, termination andt emplate release (Bressanelli *et al.* 1999).

Compounds so far reported as having anti-HCV RdRp activity can be easily divided into two classes: nucleoside and non-nucleoside inhibitors (Bressanelli *et al.* 1999). Nucleoside inhibitors have previously proved very successful in the treatment of viral infections such as HIV but, of those in clinical use, only ribavirin has been found to show anti-HCV activity. Novel anti-HCV nucleoside analogues have been identified and are generally thought to act either by preventing chain elongation or by causing problems with viral replication following incorporation into HCV RNA (Bressanelli *et al.* 1999). Non-nucleoside analogues, which may result in increased selectivity (nucleoside analogues could affect endogenous enzymes which utilise nucleic acid residues) of varying structures and potency have also been identified (Bressanelli *et al.* 1999, McCaffrey *et al.* 2003, McKercher *et al.* 2004). However, none of the compounds so far discovered appear to have entered clinical testing (Vlachakis *et al.* 2015).

NS3-4A (non-stractural protein protease)

The amino terminal (Vlachakis *et al.* 2014) end 1/3rd of the NS3 protein has a serine protease function (Moradpour *et al.* 2002), which requires the NS4A protein as a cofactor to increase stability and activity (Gordon & Keller 2005). The structure of NS3, both alone (Barbato *et al.* 1999, Love *et al.* 1996) and in complex with NS4A (McCoy *et al.* 2001, Yan *et al.* 1998) has been fully elucidated by X-ray crystallography and NMR spectroscopy. This revealed that NS3 portion forms two six-stranded β barrels connected by short loops (Love *et al.* 1996) and preceded by a 28 amino acid hydrophobic region (amino terminal) (Barbato *et al.* 1999). A zinc binding site is formed by one histidine and three cysteine residues (Love *et al.* 1996). Interacting with the NS4A protein allows the N -terminal 28 amino acids to form an α helix and β strand (in the absence of NS4A, no structural features are seen) (Barbato *et al.* 1999) and also causes the NS3 D1 β strand to interact with a second region of the complex to take on a structural support role (Barbato *et al.* 1999). This high level of structural information

(Vlachakis *et al.* 2014), together with the essential nature of the enzyme (it is required for NS4B, NS5A and NS5B formation) has made it an attractive target (Moradpour *et al.* 2002). However, the nature of the enzyme active site means that progress has been slow to date, as the site is a) unlike other members of the chymotrypsin superfamily (and therefore cannot be targeted by known inhibitors of this family of enzymes) (Gordon & Keller 2005), b) is wide and exposed, with few features which can be exploited for drug binding (Walker 1999) and c) unusually requires a cysteine residue or analogue in the P1 residue binding pocket (Walker 1999)

Despite this, several inhibitors of the NS3-4A serine protease have been designed, which can be loosely assigned to 3 groups (Gordon & Keller 2005). The first, and perhaps most obvious, are the product analogues (Gordon & Keller 2005). Initially, larger peptide mimics, such as hexapeptides (Gordon & Keller 2005, Johansson *et al.* 2003), were developed although it was subsequently discovered that smaller di- and tripeptides and their analogues were also effective (Gordon & Keller 2005, Johansson *et al.* 2003, Nizi *et al.* 2004). The second group of compounds are small molecules which inhibit protease activity via non-competitive mechanisms (Gordon & Keller 2005). The final group consists of large RNA polymers (Gordon & Keller 2005). To date, the most successful compound is BILN 2061 (developed by Boehringer Ingelheim), a macrocyclic peptidomimetic developed from the N-terminal cleavage products of reactions catalysed by the serine protease, which has entered clinical trials (Lamarre *et al.* 2003).

NS3-4A (non-stractural protein helicase)

The carboxy terminal two thirds of the NS3 enzyme constitutes an RNA helicase/nucleoside triphosphatase (NTPase) function (Kim *et al.* 1995) which has also been a major target of drug discovery efforts. *In vivo* (Spyropoulos *et al.* 2012), the enzyme is used to separate negative-stranded RNA (synthesised after infection of a cell) from the positive-stranded RNA (found packaged in infective virions) with which it would automatically have formed a double-stranded complex (Gordon & Keller 2005). This enables the virus to use cellular machinery to synthesise more positive-stranded RNA – using the negative-stranded form as a template – for inclusion in infective virus particles (Gordon & Keller 2005). The motion of unwinding double-strand RNA as performed by NS3 helicase appears to proceed in steps of 18 kilobases (kb) along the genome. One part of the enzyme moves 18kb along the double-stranded RNA and stops, after which a second part follows behind and unwinds the double helix. The enzyme's action then 'pauses' before repeating the process on the next section of RNA (Bianco 2004). The energy for this comes from the breakdown of nucleoside triphosphates (usually adenosine triphosphate) (Bianco 2004) by the enzyme's NTPase functionality. It is interesting to note that HCV helicase, unlike many similar enzymes, exhibits poor substrate specificity (Vlachakis 2007) and is capable of unwinding RNA/RNA, DNA/DNA and RNA/DNA duplexes (Gordon & Keller 2005). The NTPase function has a similarly low specificity requirement and will hydrolyse all endogenous forms of NTPs as well as synthetic acyclic NTPs and nucleoside analogues (Borowski *et al.* 2002).

Conclusion

The crystal structure of HCV helicase (Loukatou *et al.* 2014) has been fully elucidated, both as the enzyme alone (Cho *et al.* 1998, Yao *et al.* 1997) and in complex with a single-stranded DNA oligonucleotide (Kim *et al.* 1998). Three domains have been observed, together with binding clefts for RNA and NTPs (Cho *et al.* 1998) domains 1 and 2 show great similarity both in their structure (Vangelatos *et al.* 2009) and in interactions with genetic material; both contain α helices either side of a six-stranded parallel β sheet (domain 1 also has a seventh, antiparallel strand) (Kim *et al.* 1998). In contrast, domain 3 is largely composed of α helices with two antiparallel β strands (Kim *et al.* 1998). Domains 1 and 3 are closely associated via packaging of various α helices, while domain 2 is more loosely bound to both (Spyropoulos *et al.* 2012). This allows some limited movement of domain 2, and leaves a single large cleft at the domain 1/domain 2 and domain 2/domain 3 interfaces (Kim *et al.* 1998).

This is proposed to be the binding site for RNA *in vivo* (Cho *et al.* 1998), with the mobility of domain 2 thought to be involved in allowing unwinding (Yao *et al.* 1997). A phosphate binding loop (Spyropoulos *et al.* 2012), similar to those seen in related ATPases is present in the amino terminal region of domain 1 which suggests that the NTPase function is located here (Kim *et al.* 1998).

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