Hepatitis C virus: Discovery to future prospects

The 2020 Nobel prize in Physiology or Medicine has been awarded to three scientists, Harvey J. Alter (National Institute of Health), Michael Houghton (University of Alberta), and Charles M. Rice (Rockefeller University) 'for the discovery of hepatitis C virus'. Hepatitis C virus was one of the major causes of blood transfusion associated hepatitis which progressed to hepatocellular carcinoma (HCC) in almost 5% of the cases. The number of hepatitis cases post transfusion reduced extensively after discovery of hepatitis B virus and development of blood tests for its detection prior to transfusion. This won the 1976 Nobel Prize in Physiology or Medicine to Baruch S. Blumberg. However, even after testing for HBV, the incidences of transfusion associated hepatitis continued, which led to the search for a different etiological agent and the discovery of hepatitis C virus. Development of testing kits for HCV and establishment of *in vitro* HCV replication setup led to the detailed analysis of viral proteins and the regulation of host machinery by the virus. This paved the way for the development of effective anti-virals, potent to completely cure the patient of viral RNA. We still do not have a vaccine against HCV and there still remain certain important aspects of HCV to be understood for complete eradication of the virus from human population.

Historical discovery of hepatitis C virus

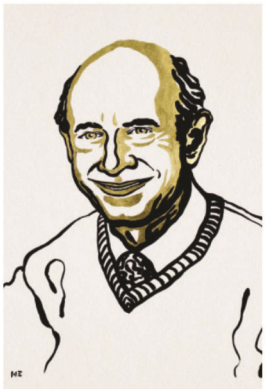
Hepatitis is a condition of inflammation of liver due to several different reasons which can progress to fibrosis, cirrhosis and finally to hepatocellular carcinoma (HCC). Its initial reports date back to 3000 BC when Sumerians reported the first description of hepatitis. They referred liver as 'the home for soul' and jaundice (a prominent symptom of hepatitis) was reported as an attack on the liver by the devil named 'Ahhazu'. Later, Hippocrates described jaundice after observing the fulminant epidemic of jaundice and associated death within two weeks. He described 'The bile contained in the liver is full of phlegm and blood, and erupts... After such an eruption, the patient soon raves, becomes angry, talks nonsense and barks like a dog'. The contagious nature of hepatitis was first reported by Pope Zachary in 8th century where he suggested isolation of patients suffering from jaundice. Thereafter, the jaundice epidemic broke in military men and civilians in 17–18th century. It was named as *Jauniesse des camps* by the French, *Soldaten gelbschut* by the German and *Icterus* by the clinicians, all referring to yellowness of skin developed as the major symptom. The next major milestone in study of hepatitis was the accidental discovery of hepatitis B antigen by Baruch Blumberg while studying the genetics of susceptibility of diseases. He identified an atypical antigen from a haemophiliac patient of Australian aborigine and named it 'The Australian antigen' which was found to be hepatitis B virus in later studies[1]. Further studies led to the discovery of other hepatitis viruses classified as *infectious* which can be transmitted enterically (identified as hepatitis A in 1973) and *serum derived* which are transmitted parenterally, mostly through blood (identified as hepatitis B).

Observation of non-A non-B viral hepatitis

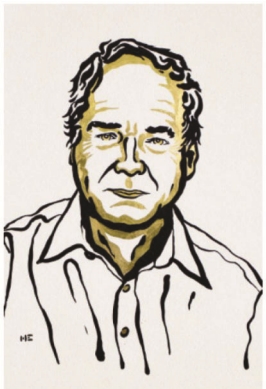
In 1970s, the advent of serological tests for hepatitis A and B led to the observation of a novel hepatitis in blood transfusion-associated patients that did not have serological markers for either of the viruses. This was then termed as non-A, non-B viral hepatitis (NANBH). Almost 10% of the blood transfusion patients developed NANBH which had the propensity to progress to liver cirrhosis in 20% of the cases. Harvey J. Alter was among the first to report the development of hepatitis in patients, post-transfusion of blood from HBV and HAV negative donors[2,3]. This pointed towards the presence of a novel, previously unidentified infectious agent, being transmitted through plasma[4]. Upon further analysis and research, he showed for the first time that the plasma from NANBH patients could induce acute hepatitis in Chimpanzees. Such hepatitis could be induced from plasma of both, acute and chronic NANBH patients, illustrating the infectivity of the agent for long periods in the infected individuals[5]. Extensive efforts were made to characterize this new virus. Major progress in the study of progression of liver diseases was made by the development of Chimpanzee model, but still NANBH specific antigen, antibody or virus could not be isolated until late 1980s.

Identification of infectious agent, hepatitis C virus

Molecular approaches began to identify the virus-specific cDNAs for NANBH-infected liver. After various tiring and failed attempts by different groups, Michael Houghton's lab was finally able to identify the specific clone responsible for NANBH[6]. Total RNA and DNA were isolated from infected chimpanzee plasma, which was used to generate a cDNA library to be checked for its reactivity with the serum antibodies from NANBH-infected patient. While many of the clones were identified as host



Harvey J. Alter



Michael Houghton

Charles M. Rice

autoantigens, after screening of millions of clones, a clone labelled 5-1-1 was identified to be derived from NANBH viral genome. After careful examination of the aetiology of clone and the subsequent overlapping clones, NANBH was identified to be 10 kb long positive-stranded RNA virus and termed as hepatitis C virus (HCV). This and the adjacent clones were then used to develop an enzyme immunoassay which laid the foundation for the first-generation blood screening test for HCV to prevent the blood transfusion-associated spread of HCV[7]. Development of these testing kits and associated prevention of post-transfusion hepatitis provided the proof of HCV being a major cause of parenteral NANBH across the world. The viral genome was then extensively studied and characterized.

Establishment of efficient infection model

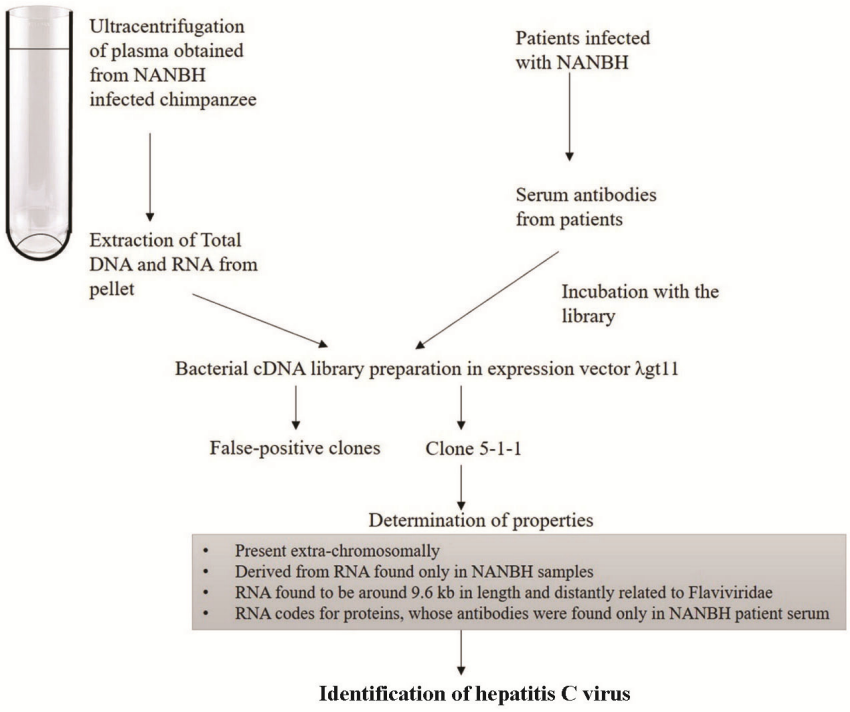
Even after the discovery of hepatitis C virus, verification of its ability to cause hepatitis alone required isolation of replicative virus. While the viral ORFs had been described and were being studied, the infectious HCV genome was not characterized until 1996 when the functional role of HCV 5′UTR and 3′UTR was established[8]. Different genetic variations in the viral sequences were analysed and a variant of the virus was then generated through genetic engineering. This variant, comprising viral 3′UTR and additional sequences at 5′ end and devoid of certain variations thought to be inhibiting the virus replication, was generated by Charles M. Rice and others. Transcription from this clone provided infectious RNA, capable of inducing hepatitis in chimpanzee after direct inoculation in the liver, establishing the potential of HCV alone to cause hepatitis and its involvement in transfusion associated NANBH[9]. Later, in 2005, a cell culture model for HCV replication was also established using a clone derived from a patient with fulminant hepatitis and named JFH1 (Japanese fulminant hepatitis)[10].

The information was used for design of novel targets against different viral proteins which served as a cure for HCV.

Genome and life cycle

According to WHO, 71 million people have chronic hepatitis C infection worldwide and approximately 399,000 people die each year from hepatitis C, mostly from cirrhosis and HCC. HCV genomic RNA is 9.6 kb in length and encodes for a poly-protein precursor chain of approximately 3011 amino acid residues. 5′ Untranslated region (UTR) and 3′UTR flank both sides of the ORF encoding for the polypeptide and are essential for translation and replication of the virus. The HCV 5′UTR is 341 nucleotides (nts) in length and is organized into 4 highly structural domains (domain I-IV). The domain II-IV along with 30 nts downstream of initiator AUG coding sequence forms internal ribosome entry site (IRES). IRES is a structural motif where translation machinery is recruited to the viral RNA since canonical cap-dependent translation cannot initiate on the uncapped viral RNA. The polyprotein generated is further cleaved into 10 proteins by host and viral proteases. Out of the 10 proteins, there are 4 structural proteins, namely, E1, E2, core and p7; and 6 non-structural proteins, namely, NS2, NS3, NS4A, NS4B, NS5A and NS5B[11]. Core is the nucleocapsid protein which oligomerises to coat the viral genetic material, forming a viral particle. Core is also involved in other cellular functions like viral RNA translation, replication, activation of various signalling pathways involved in metabolism of lipids, and cellular transformation. The viral nucleocapsid acquires the envelope coated by viral E1 and E2 glycoproteins. These are essential for viral entry into the cells because of their interaction with the surface receptors. p7 is the integral membrane protein which acts as a viroporin and helps in establishment of higher pH for viral assembly by dissipating the proton concentration through the pores created in the vesicles. Viral non-structural proteins are responsible for viral replication, formation of membranous web for HCV replication, viral assembly, release and various other cellular functions.

The life cycle of HCV is entirely cytoplasmic. On entering the host cell, first of all the virus uses host machinery to initiate IRES mediated translation for synthesizing the structural and non-structural proteins. The translated proteins are essential for viral replication, for example: NS5B is an RNA-dependent RNA polymerase which synthesizes the negative strand RNA using the positive strand viral genome as the template.



Schematic for the identification of hepatitis C virus by Michael Houghton's group[21].

Once enough proteins are synthesized, there is a switch from translation to replication. A negative-strand RNA is synthesized on the positive-strand RNA genome, yielding a double stranded RNA (dsRNA) intermediate. Further, using the negative strand as a template, more and more positive-strand RNA molecules are synthesized which are translated to form more viral proteins. Positive-strand RNAs are further packaged in the viral capsid for the release of new virion molecules. Multiple host factors are involved at each step of viral life cycle and have been discussed extensively in the cited review[12]. The interplay between these host factors and the viral RNA and proteins decides the outcome of viral infection and the viral pathogenesis. These host–viral interaction sites provide novel candidates for development of effective therapeutic agents.

What is still left?

Although there has been extensive study and effective anti-virals targeted against various HCV proteins, it is yet far from being eradicated. Some factors that contribute to this are the emergence of drug resistant variants, unsuccessful candidate vaccine trials and unavailability of an appropriate model system to study the steps of disease progression.

Lack of an appropriate model organism

Relevant model organisms are an absolute necessity for study of any disease biology and pathogenesis. It will enable the close and appropriate study of HCV-biology, mechanisms of host–viral interactions, which will allow the development of novel anti-virals and vaccine candidates.

Chimpanzee is the only primate, other than humans, which is susceptible to HCV infection. It has extensively been used for HCV studies and has yielded many important insights into the virus establishment, immune response by the host. It has been extremely important in pre-clinical testing of different regimes of anti-viral drugs. The intravenous injection of either tissue culture derived, or clinically isolated virus can induce acute hepatitis in chimpanzee which can progress to chronic infection, but chronic infection does not progress to fibrosis, cirrhosis and hepatocellular carcinoma in them. Further, the associated practical, financial and ethical constraints have limited the usage of the system and have encouraged the need for development of smaller animal model system. Since mice are not susceptible to HCV infection, various attempts have been made to immunocompromise or to humanize these mice for expression and functioning of human hepatocytes in the immunodeficient mice. Various different animal models developed for HCV studies have been reviewed extensively in the cited review[13].

The establishment of virus infection requires receptor for viral entry and the appropriate host factors for viral replication and translation in cells for production of progeny viral particles. Many such factors have already been characterized. The viral entry is dependent on presence of CD81, claudin, occluding receptors[14] and certain critical host factors like human La protein have been shown to guide this host tropism[15]. In light of these observations and the advent of Nobel-winning CRISPR-Cas9 editing tools, humanization of these host factors using CRISPR-Cas system might prove to be a huge step forward in development of the HCV disease model in mice.

Vaccine

While HCV RNA can be cured from most of the infected individuals, the response to anti-viral therapy depends on the HCV genotypic variations as well as host genetic determinants. Many drug-resistant cases have been encountered, owing to the error prone replication by HCV RNA-dependent RNA polymerase. Some cases of severe side effects of Direct Acting Antivirals (DAAs) have also been reported. Therefore, there is a need for development of effective vaccination strategy against the HCV infection. The presence of neutralizing antibodies in the serum samples of patients who cleared the infection has given a hope for the development of both, prophylactic and therapeutic vaccines.

Recombinant viral protein vaccine consisting of E1/E2 was among one of the first candidates used for vaccine trials in chimpanzee models. Further, neutralizing antibodies were identified against epitope in E2 protein that provided complete protection in chimeric mice models but the extensive glycosylation and variations in the viral sequences guide the escape from the antibody-mediated protection. Therapeutic vaccines based on different viral structural and non-structural proteins have also been designed. Further whole inactivated virus vaccine has limited usage because of limited production in cell culture system. Further different attempts are extensively discussed in the review by Naderi *et al*.[16] and Bailey *et al*.[17].

We have also been working on HCV vaccine strategy against the viral genotypes more prevalent in the Indian subcontinent. We have developed several platforms for HCV vaccine, including DNA vaccine, virus like particles (VLPs), recombinant adenoviruses and necrotic cells, all of which generate robust immune responses in mice[18]. Apart from the VLPs, these platforms were designed to result in necrosis of cells targeted by the vaccine and predicted to result in cross presentation of the specific HCV immunogen. The DNA construct, recombinant adenoviruses and necrotic cells are primarily designed to elicit cell mediated immunity whereas the virus-like particles primarily elicit HCV cross neutralizing antibody.

Even though there have been different approaches for vaccine development, they have not successfully completed the pre-clinical trials. Absence of an appropriate disease model is one of the major factors limiting the success rate of field trials.

Progression to HCC even after HCV clearance

Hepatocellular carcinoma is the extreme limit of HCV pathogenesis. Many HCV proteins have been shown to exhibit direct carcinogenic effect. DAAs are being used to effectively inhibit HCV life cycle in the infected individuals. While DAAs reduce the risk of development of HCC, there are cases where the patients progress to HCC even after viral clearance by DAA treatment. This suggests that certain carcinogenic cues or pathways are already activated upon viral infection, which are not cleared even after viral clearance. These cues can be some proteins, miRNAs, lncRNAs or epigenetic changes. It has been shown that in an HCC patient, the part of liver that has progressed to HCC shows very low HCV replication compared to the adjacent non-cancerous infected part[19]. This

points towards the presence of certain 'malignant factors' which could either be secreted by infected cells that leads to transformation of adjacent cells or is present in the infected cells keeping the viral copy number under check.

There are some hypotheses that the DAA treatment could even increase the risk to HCC development[20]. This is because of the balancing functions of immune system in tackling the virus and preventing cancer progression. DAA treatment disturbs this balance by rapid HCV clearance which reduces the immune surveillance in the infected areas and hence provide the escape for progression to HCC.

Further, HCV infection has a drastic effect on epigenetic regulation by mediating DNA and histone tail modifications such as H3K27ac. HCV associated epigenetic changes have been shown to be associated with higher risk for HCC progression. These epigenetic changes were shown to be present even after DAA mediated HCV clearance from tissue samples of chronic-infected HCV patients. After the epigenetic changes have been made, a regulation on gene expression has already been exposed, which then becomes independent of the presence of stimulating virus signal.

Such changes have a potential to be exploited as prognosis markers for development to HCC and as a potential drug target to be explored in combination with DAAs. Epigenetic changes in the infected individuals could also serve as an avenue for the design of personalized medicines for prevention of disease progression.

Concluding remarks

This year Nobel prize is a much deserving one for the extensive work of last 30 years from discovery of the pathogen to development of effective blood screening test kits and antivirals, encouraging scientists around the world to deal with such viral infections with a positive attitude towards tackling it, especially now in the time of COVID-19 pandemic by an RNA virus.

We have anti-virals against HCV, but there still remains a lot of unsolved mystery including relapse, progression to HCC and unavailability of vaccine. The recognition of the importance of HCV study by Nobel prize would encourage the research community working on HCV biology and disease progression to continue with enthusiasm towards a greater aim of better human health.

1. Blumberg, B. S., Alter, H. J. and Visnich, S., *JAMA*, 1965, **191**, 541–546.
2. Alter, H. J., Holland, P. V., Purcell, R. H., Lander, J. J., Feinstone, S. M., Morrow, A. G. and Schmidt, P. J., *Ann. Internal Med.*, 1972, **77**, 691–699.
3. Feinstone, S. M., Kapikian, A. Z., Purcell, R. H., Alter, H. J. and Holland, P. V., *New Eng. J. Med.*, 1975, **292**, 767–770.
4. Alter, H. J., Holland, P. V., Morrow, A. G., Purcell, R. H., Feinstone, S. M. and Moritsugu, Y., *Lancet*, 1975, **2**, 838–841.
5. Alter, H. J., Purcell, R. H., Holland, P. V. and Popper, H., *Lancet*, 1978, **1**, 459–463.
6. Choo, Q. L., Kuo, G., Weiner, A. J., Overby, L. R., Bradley, D. W. and Houghton, M., *Science*, 1989, **244**, 359–362.
7. Kuo, G. *et al*., *Science*, 1989, **244**, 362–364.
8. Kolykhalov, A. A., Feinstone, S. M. and Rice, C. M., *J. Virol.*, 1996, **70**, 3363–3371.
9. Kolykhalov, A. A., Agapov, E. V., Blight, K. J., Mihalik, K., Feinstone, S. M. and Rice, C. M., *Science*, 1997, **277**, 570–574.
10. Lindenbach, B. D. *et al.*, *Science*, 2005, **309**, 623–626.
11. Scheel, T. K. and Rice, C. M., *Nat. Med.*, 2013, **19**, 837–849.
12. Sharma, G., Raheja, H. and Das, S., *IUBMB Life*, 2018, **70**, 41–49.
13. Burm, R., Collignon, L., Mesalam, A. A. and Meuleman, P., *Front Immunol.*, 2018, **9**, 1032–1032.
14. Lindenbach, B. D. and Rice, C. M., *Nat. Rev. Microbiol.*, 2013, **11**, 688–700.
15. Kumar, A., Manna, A. K., Ray, U., Mullick, R., Basu, G., Das, S. and Roy, S., *J. Virol.*, 2014, **88**, 4319–4327.
16. Naderi, M., Gholipour, N., Zolfaghari, M. R., Moradi Binabaj, M., Yegane Moghadam, A. and Motalleb, G., *Int. J. Mol. Cell Med.*, 2014, **3**, 207–215.
17. Bailey, J. R., Barnes, E. and Cox, A. L., *Gastroenterology*, 2019, **156**, 418–430.
18. Kumar, A. *et al.*, *Vaccine*, 2016, **34**, 1115–1125.
19. Harouaka, D. *et al.*, *Proc. Natl. Acad. Sci.*, 2016, **113**, 1375–1380.
20. Rinaldi, L. *et al.*, *Cancers* (*Basel*), 2020, **12**, 1351.
21. Houghton, M., *Liver Int.*, 2009, **29**(Suppl. 1), 82–88.

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