Pathology Features and Molecular Genetic Mechanisms of Hepatocellular Carcinoma Development in Patients with Hepatitis C Associated Liver Cirrhosis

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Abstract

Hepatitis C virus (HCV) is a single stranded RNA virus belonging to the family of Flaviviridae. The hepatitis C virus has unique ability to cause persistent infection in susceptible host. The chronicity rate is 75-85% after acute infection and 20-30% of HCV infected patients will develop cirrhosis, end-stage liver disease or hepatocellular carcinoma (HCC). Post transfusion HCV has been virtually eliminated by screening of donor blood after 1992 and injection drug use now appears to be the most common remaining risk factor for HCV infection. Chronic infection of HCV is a major risk of hepatocellular carcinoma and the pathogenesis of HCC in chronic HCV infection is due to chronic inflammation for long period of time which leads to fibrosis, cirrhosis and carcinoma formation. Pathology features of chronic hepatitis C, cirrhosis and hepatocellular carcinoma were described. Molecular genetic mechanisms involved in the development of hepatitis C associated cirrhosis and hepatocellular carcinoma were reviewed. Understanding the mechanisms of initiation and progression of HCC will provide principles for early diagnosis, treatment, and prevention.

Keywords: Hepatitis C; Cirrhosis; Hepatocellular carcinoma

Introduction: Overview of Hepatitis C

Hepatitis C virus is a single stranded RNA virus that constitutes 9379 nucleotides in the genome. There is a single open reading frame (ORF) with non-coding RNA regions at its 5' and 3' ends. The ORF codes for about 3000 amino acids which is then cleaved into series of smaller proteins by viral and host proteases. These smaller proteins contain structural proteins (the core protein and two envelope proteins E1 and E2) and non-structural proteins, including proteases, helicase and RNA-dependent RNA polymerase which form part of replication machinery [1,2].

Cleavage of structural proteins from the large polyprotein is catalyzed by host signal peptidase while cleavage in the nonstructural region requires HCV-encoded non-structural protease. The 5' and 3' non-coding regions of the viral genome are highly conserved among different genotypes. They are important for translation of viral proteins and replication of the virus [3]. Different from hepatitis B virus, HCV cannot integrate into host liver cell genome and its role in carcinogenesis appears to be that of a chronic inflammation, necrosis, irregular regeneration of fibrotic tissue and hepatocytes, which causes cirrhosis and malignant transformation. The development of this transformation usually takes more than two decades but this process maybe faster in patients who have co-morbidity.

The host for HCV is very stringent and the virus only replicates in human and certain non-human primates such as chimpanzees and tamarins. HCV replicates through an RNA-dependent RNA polymerase and is very prone to mutation. There are high variability of nucleotide sequences among different genotypes (30-50%) and subtypes (15-30%). These features hampered the development of vaccine and new therapeutic agents. Currently, six major genotypes were identified. Genotype 1, 2, 3 are found in developed Western countries and genotype 1 account for the majority of HCV infection in US (71%). Genotype 4 is endemic to Egypt and the Middle East. Genotype 5 is confined to Southern Africa but recent studies indicated that it can be found worldwide. Genotype 6 was found in south-east Asia [4]. Patients infected with HCV genotype 1 do not respond well to interferon based therapies.

HCV is transmitted through transfusion of blood or blood products, injection drug use, needle stick, maternal-infant transmission or sexual intercourse [5]. Post-transfusion HCV has been virtually eliminated by screening of donor blood in most developed countries but chronic infection remains prevalent in blood donors in some geographic region. The chronicity of HCV infection is very high and 75-80% of infected patients will have indolent course [6,7]. The natural course of the disease could be affected by other host (age, gender, race, genetic variants) or environmental factors. For example, in children the chronicity is 50%, in young women it is estimated to be 55% but could be as high as 90% in African-American patients [8]. 20% of acutely infected patients undergo spontaneous recovery while 20-30% is progressive and will have severe histologic outcome such as cirrhosis, end-stage liver disease or HCC.

Chronic infection of hepatitis C occurs in 3% of world population (approximately 170 million people) with highest frequency occurs in south-east Asia and Europe [9]. According to National health and nutrition examination survey, HCV infection is common in the United States. 3.9 million Americans were infected and 2.7 million had ongoing virus replication in 1988-1994 [10]. While this may be underestimated due to asymptomatic patients and unreported cases, the number of infected patients is increasing as well. The morbidity and cost of HCV infection is significant. It is estimated that 50% of liver transplant and 36% of patients on the transplant waiting list [11] are due to hepatitis C infection. Chronic HCV increases risk of cirrhosis by 34 folds and HCC by 5-20 folds [12,13]. HCV related HCC accounts for 50% of all HCC cases in the United States, 50% in south-east Asia and 80% in Japan [14].

Pathology features of Chronic Hepatitis C and Molecular Mechanisms of Liver Fibrosis

Pathology features of chronic hepatitis C are inflammation, hepatocytes regeneration and fibro genesis. Prominent infiltration of lymphocytes, variable number of plasma cells, scattered macrophages

and eosinophils can be found in portal region. Steatosis is usually macrovesicular, which may be associated with severe necroinflammatory activity. Periportalnecroses evoke reaction of inflammatory cells to secrete cytokines and chemokines. These soluble factors will stimulate matrix producing cells such as fibroblasts, neutrophils and macrophages to produce degrading enzymes. Fibrosis develops after repeated and persistent injury which leads to derangement of the architecture, portal hypertension and produces irreversible rearrangement of the circulation such as cirrhosis. Fibrosis is not only the result of necrosis, collapse and scar formation but also the result of derangements in the synthesis and degradation of matrix by injured mesenchymal cells that synthesize the various components of the matrix in the liver [15]. Collagen type I, III and IV are abundant in portal triad, sinusoid, and fibrous septa of hepatocytes and basement membrane of arteries, veins and bile duct. Collagen types V-VII are large collagens that function as anchoring structures. The increased production of glycoproteins, such as laminin, fibronectin, entactin, undulin and elastin, correlates with the degree of fibrosis and facilitate cross linkage of the fibers. Hepatic stellate cells are mainly involved when hepatocellular damage is limited or concentrated within the liver lobe. Figure 1 showed typical pathology features of chronic hepatitis C, cirrhosis and hepatocellular carcinoma. Cirhosis is major risk factor for HCC. Understanding the molecular mechanisms of how cirrhosis develop to HCC will provide clue for treatment and cancer prevention. It has been reported that transforming growth factor beta (TGF-β) is the mediator of fibrogenesis and platelet-derived growth factor (PDGF) is the major inducer of hepatic stellate cell proliferation which suggested that various TGF-β and PDGF inhibitors maybe promising reagents for theraputics [15-17]. Portal myofibroblasts and fibroblasts also provide predominant contribution when the damage is located in the portal tracts [18].
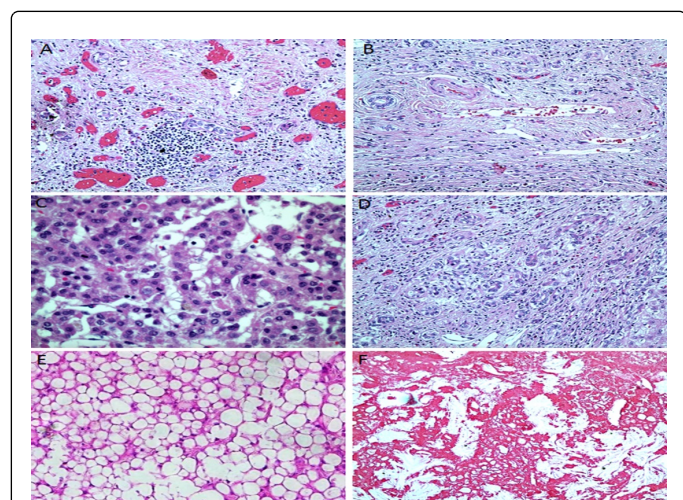
Liver biopsy is the gold standard for diagnosis and staging of fibrosis. There is no accurate and effective way to monitor progression of hepatitis C and fibrosis by non-invasive method. History, physical exam and laboratory tests, such as hepatic panel, are relatively non-specific and could not correlate well with liver biopsy. More than one third of patients with chronic hepatitis C had normal alanine aminotransferase (ALT) level although decreased platelet count and prolonged prothrombin time have better accuracy [19]. In current practice, serum ALT levels, grade of inflammation activity and stage of fibrosis are the main predictors of disease progression. Pathology evaluation of fibrosis activities range from absent, mild, moderate, extensive to cirrhosis and special staining such as Masson trichrome, reticulin, Verhoeff's elastic and orcein stains can aid visualizing collagen or elastic fibers [20]. Differential diagnosis includes Wilson's disease, primary biliary cirrhosis, primary sclerosing cholangitis, autoimmune hepatitis and α1-antitrypsin deficiency. Figure 2 showed special stains in hepatitis C associated fibrotic liver tissue for diagnosis and differential diagnosis. Several scoring systems have been applied in clinical pathology. These include Knodell index [21,22], Ishak and Metavir [23]. Although sampling error, observer variation and different biopsy specimen size are potential problems, these semiquantitative grading indexes provided good correlation between pathology and clinical findings and liver biopsy remains the best method to evaluate liver fibrosis in order to determine the prognosis and indication for therapy. In untreated patients, regular ALT measurements and repeat liver biopsy are carried out to access the progression of fibrosis.
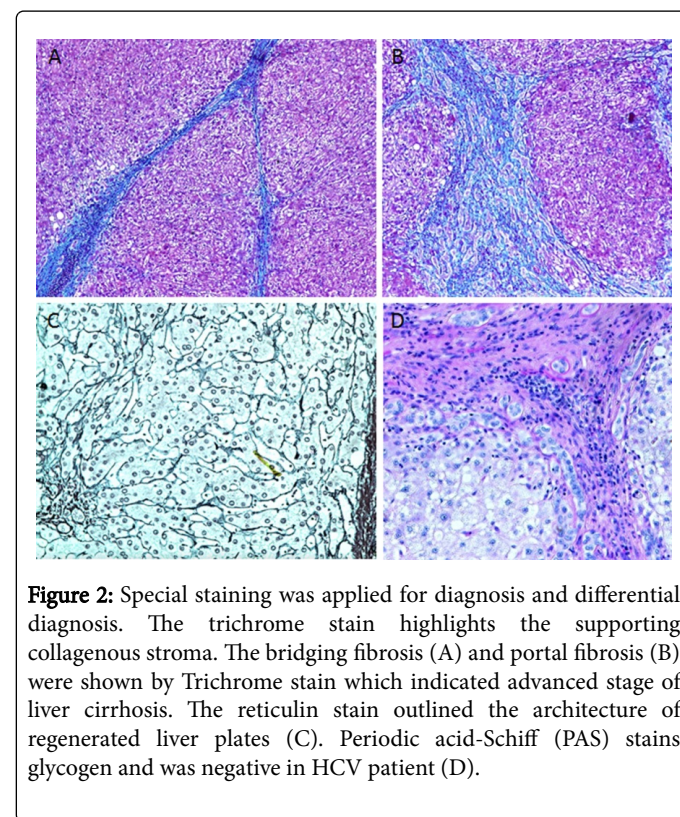

Figure 1: Pathology of chronic hepatitis C, cirrhosis and hepatocellular carcinoma in a patient. Lymphosytes infiltrate and lymphoid aggregates were presented in portal triad indicating the chronicity of HCV infection (A). There was extensive generation of fibrotic tissue that distorted the portal triad, suggesting liver cirrhosis (B). Irregular regeneration of hepatic cells due to chronic inflammation was shown in panel C. Atypical glandular tissue (D) and steatosis (E) were significant. Piecemeal necrosis is one of typical features of chronic hepatitis (F).

Figure 2: Special staining was applied for diagnosis and differential diagnosis. The trichrome stain highlights the supporting collagenous stroma. The bridging fibrosis (A) and portal fibrosis (B) were shown by Trichrome stain which indicated advanced stage of liver cirrhosis. The reticulin stain outlined the architecture of regenerated liver plates (C). Periodic acid-Schiff (PAS) stains glycogen and was negative in HCV patient (D).

Pathology features and molecular mechanisms of development of cirrhosis and HCC

Cirrhosis, a pathological condition defined by deranged hepatic architecture and parenchymal nodular regeneration resulting from progressive fibrosis, is the end stage of chronic liver disease. Cirrhosis is classified as micronodular (nodules<3 mm) or macronodular (nodules>3 mm) based on the nodule's size. Microscopically, fibrous bands surround the regenerative nodules containing thickened liver cell plate are presented. Inflammatory infiltration can be visualized in fibrous septa. Combination of special stains is applied to distinguish hepatic necrosis with regenerative nodules from cirrhosis in all cases [20]. Clinical manifestations range from asymptomatic to hepatic failure. Ascites, splenomegaly and esophageal varices are consequences of portal hypertension. The prognosis depends on the underlying etiology, availability of effective treatment and severity of liver injury. Hepatic transplantation is indicated in many cases and is the only definitive treatment.

Cirrhosis is the most common predisposing condition towards HCC. Once cirrhosis is established, the risk of HCC development is increased and the annual risk is estimated to be 1-6%. The mechanisms of carcinogenesis of HCV are likely to be the chronic inflammation and hepatocellular injury which induce malignant transformation of the hepatocytes. Various HCV proteins are oncogenic. The core protein is involved in cell signaling, transcription activation, apoptosis, lipid metabolism, transformation [24]. It induces HCC in transgenic mouse model [25]. E2 protein can interact with immune system and inhibit T and NK cells to promote malignant cell proliferation and survival. NS3 protein has protease, RNA helicase and NTPase activity which promote carcinogenesis by interact with p21 and p53 [26,27]. Host and environmental factors, such as older age, male, heavy alcohol intake (>50g/d), co-infection with HIV or HBV, increase the risk of HCC development. Figure 3 summarize the molecular mechanisms of development of HCV associated HCC.
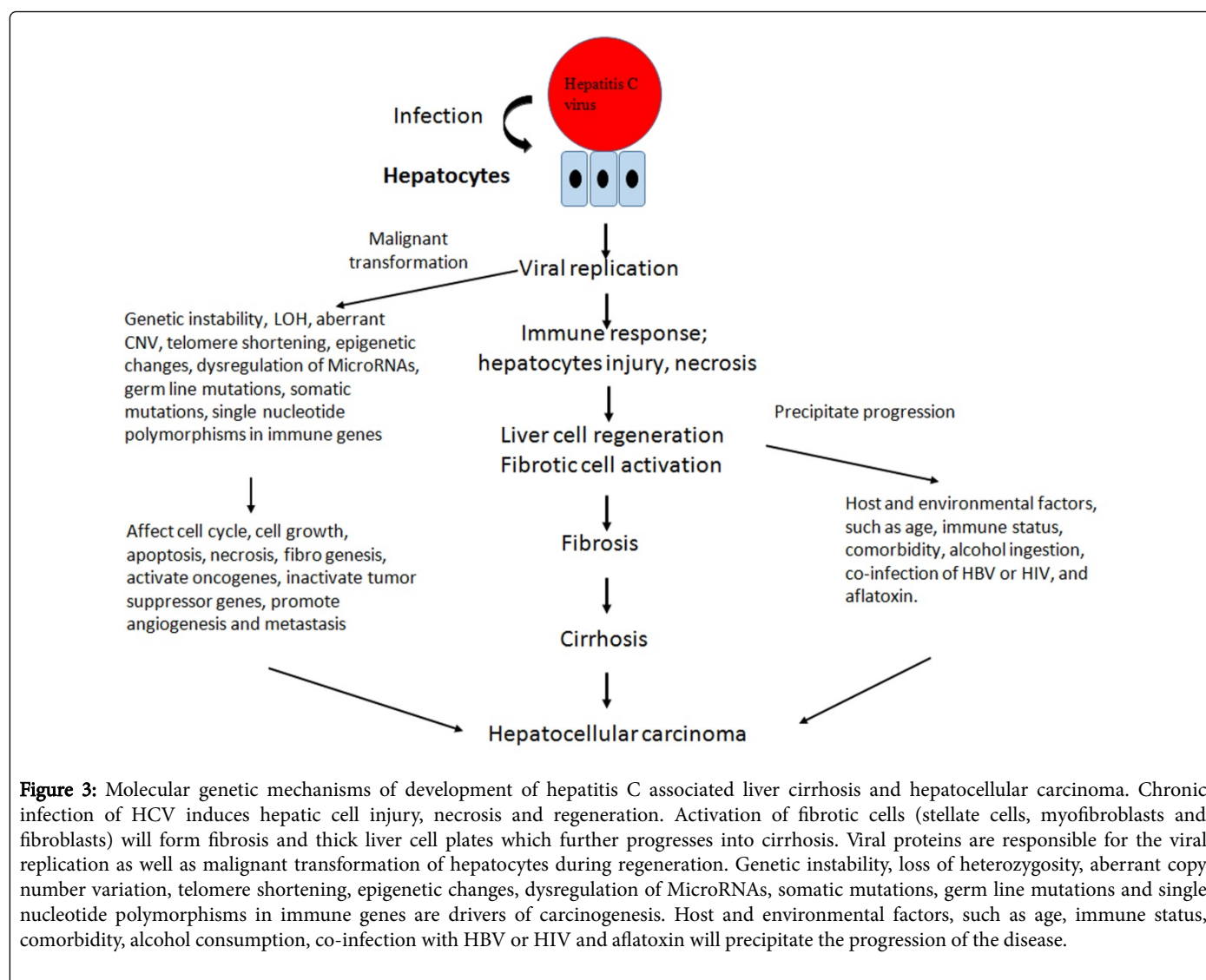

Figure 3: Molecular genetic mechanisms of development of hepatitis C associated liver cirrhosis and hepatocellular carcinoma. Chronic infection of HCV induces hepatic cell injury, necrosis and regeneration. Activation of fibrotic cells (stellate cells, myofibroblasts and fibroblasts) will form fibrosis and thick liver cell plates which further progresses into cirrhosis. Viral proteins are responsible for the viral replication as well as malignant transformation of hepatocytes during regeneration. Genetic instability, loss of heterozygosity, aberrant copy number variation, telomere shortening, epigenetic changes, dysregulation of MicroRNAs, somatic mutations, germ line mutations and single nucleotide polymorphisms in immune genes are drivers of carcinogenesis. Host and environmental factors, such as age, immune status, comorbidity, alcohol consumption, co-infection with HBV or HIV and aflatoxin will precipitate the progression of the disease.

Pathology features and outcome of HCC

In gross inspection, single large mass with or without satellite nodules can be found. The tumor is soft and bile stained. Histology of the tumor usually shows wide range of differentiation of hepatocytes and trabecular architechture with thickened cell plates lined by endothelial cells. Most tumors are moderately differentiated and atypical glandular structures can be visualized.

HCC has high mortality and poor prognosis. The median survival for resectable HCC is up to 45 months while for unresectable tumors is less than 6 months. Surgical mortality rate is about 8.8% and significant adverse prognostic indicators for hepatic resection of tumor include elevated alkaline phosphatase value, tumor size >2cm, presence of satellite lesions and vascular invasion [28].

Genetics of HCC

Since hepatitis B virus propagate by integration of viral DNA into host genome, chromosomal aberrations and genome instability are more common in HBV than in HCV-related HCC. Mechanisms of progression from HCV to HCC are not well known. It is postulated HCV proteins may concur indirectly to the genetic instability of infected cells through suppression of DNA repair mechanisms, induction of DNA breaks, enhancement of mutation frequency and chromosome rearrangements [29]. Telomere length was shorter in patients with chronic active HCV and in patients in remission which may cause genetic instability, increased aneuploidy and malignant transformations [30,31]. Loss of heterozygosity (LOH) analyses has revealed several chromosomal loci harboring potential tumor suppressors, such as TP53 and IGF2R, are clinically significant in patients with primary hepatocellular carcinoma [32-34]. Copy number aberrations that harbor oncogenes and tumor suppressor genes were reported by several studies. These studies provided information for the identification of oncogenic drivers [35-38]. Methylation or hypermethylation of tumor suppressor genes were common findings in HCC. Methylation of the sense strand of the adenomatous polyposis coli (APC) tumor-suppressor gene was detected predominantly in HCC instead of in normal liver and other non-HCC disease liver tissue [39]. Germ line mutations in APC gene were identified in three children who had hepatoblastoma, indicating APC gene mutation is associated with hepatic carcinogenesis [40]. The tumor suppressor gene p16INK4A negatively regulate cell cycle and is mainly inactivated by an epigenetic change involving promoter hypermethylation in hepatocarcinogenesis. High frequency of somatic p16INK4A gene alterations occurred in HCC while germ line mutations were also observed in a subset of HCC patients [41-44]. Role of MicroRNAs in carcinogenesis has been extensively studied and dysregulation of microRNA could affect multiple signaling pathways that promote cancer development and metastasis. For example, miR-26a acted as tumor suppressor targeting hepatocyte growth factor (HGF)-MET and vascular endothelial growth factor receptor (VEGFR) pathways. Down regulation of miR26a was observed in HCC which could induce tumor migration and invasion [45]. miR-122 in particular, is highly enriched in liver and has been shown to interact with HCV [46].

Somatic mutations in catenin beta-1 (CTNNB1), phosphoinositide-3-kinase-catalytic-alpha (PIK3CA) and TP53 were identified in HCV induced HCC [47]. Mutations in TP53 affect downstream cell cycle-related genes and cell proliferation-related genes. Mutant p53 tumors may have higher malignant potentials than those with wild type p53. CTNNB1 mutations were present in 33.3% of HCV-related HCC [38]. Among HCV-related HCC, TP53 and CTNNB1 mutations were similarly distributed in HCC from Asia, America, and Europe, suggesting the absence of an exogenous genotoxic factor diversely distributed in different geographic regions. PIK3CA is an effector of the phosphatase and tensin homolog (PTEN)–AKT pathway that affects cell proliferation, apoptosis and angiogenesis. However, controversial reports have been published on the presence of somatic mutations in the exon 9 of PIK3CA gene. Novel inactivating mutations in AT-rich interaction domain-containing protein 2 (ARID2) (also called BRAF200) were found in HCV-associated HCC. Notably, 18.2% of individuals with HCV-associated HCC in the United States and Europe harbored ARID2 inactivation mutations, suggesting that ARID2 is a tumor suppressor gene that is relatively commonly mutated in this tumor subtype [48]. Hepatocyte growth factor receptor (HGFR), also called MET, is an oncogene in the tyrosine kinase family. Somatic mutations in MET were identified in childhood hepatocellular carcinoma [49]. Targeted activation of human MET oncogene to adult liver in transgenic mice caused slowly progressive hepatocarcinogenesis, indicating activation of MET could be a driver of carcinogenesis in liver [50].

As 20% of acutely infected patients undergo spontaneous recovery while 20-30% patients will have progressive disease and severe histologic outcome such as cirrhosis, end-stage liver disease or HCC, this indicated genes involved in the immune response may contribute to the ability to clear the virus. Single nucleotide polymorphism (SNP) rs12979860 located upstream of the IL28B gene could affect outcome of HCV infection in a natural history setting. The variant was genotyped in HCV cohorts comprised of individuals who spontaneously cleared the virus (n=388) or had persistent infection (n=620). Results showed the C/C genotype strongly enhances resolution of HCV infection among individuals of both European and African ancestry. This is the strongest and most significant genetic effect associated with natural (spontaneous) clearance of HCV. The data implicated a primary role for IL28B in resolution of HCV infection [51]. Genes encoding the inhibitory NK cell receptor killer cell immunoglobulin-like receptor, 2 domains, long cytoplasmic tail 3 (KIR2DL3) and its human leukocyte antigen C group 1 (HLA-C1) ligand directly influence resolution of hepatitis C virus (HCV) infection. This effect was observed in Caucasians and African Americans with expected low infectious doses of HCV but not in those with high-dose exposure, in whom the innate immune response is likely overwhelmed. The data suggest that inhibitory NK cell interactions are important in determining antiviral immunity and that diminished inhibitory responses confer protection against HCV [52].

Genotypes not only affect natural history of the disease, but also affect response to treatment. A genetic polymorphism near IL28B is associated with an approximately two fold change in response to treatment among patients of European ancestry and African-Americans, indicating genotypes can be used for regimen selection for patients [53]. Figure 3 showed molecular and genetic mechanisms of hepatitis C induced fibrosis/cirrhosis and hepatocellular carcinoma formation.

Summary

It is important to screen general populations for HCV infection and routine screening of chronic hepatitis C should begin with liver biopsy in patients with positive history and follow up with serum AFP and abdominal ultrasound in severe cases [54]. For HCV positive patients, the algorithm for the management is described in Figure 4.
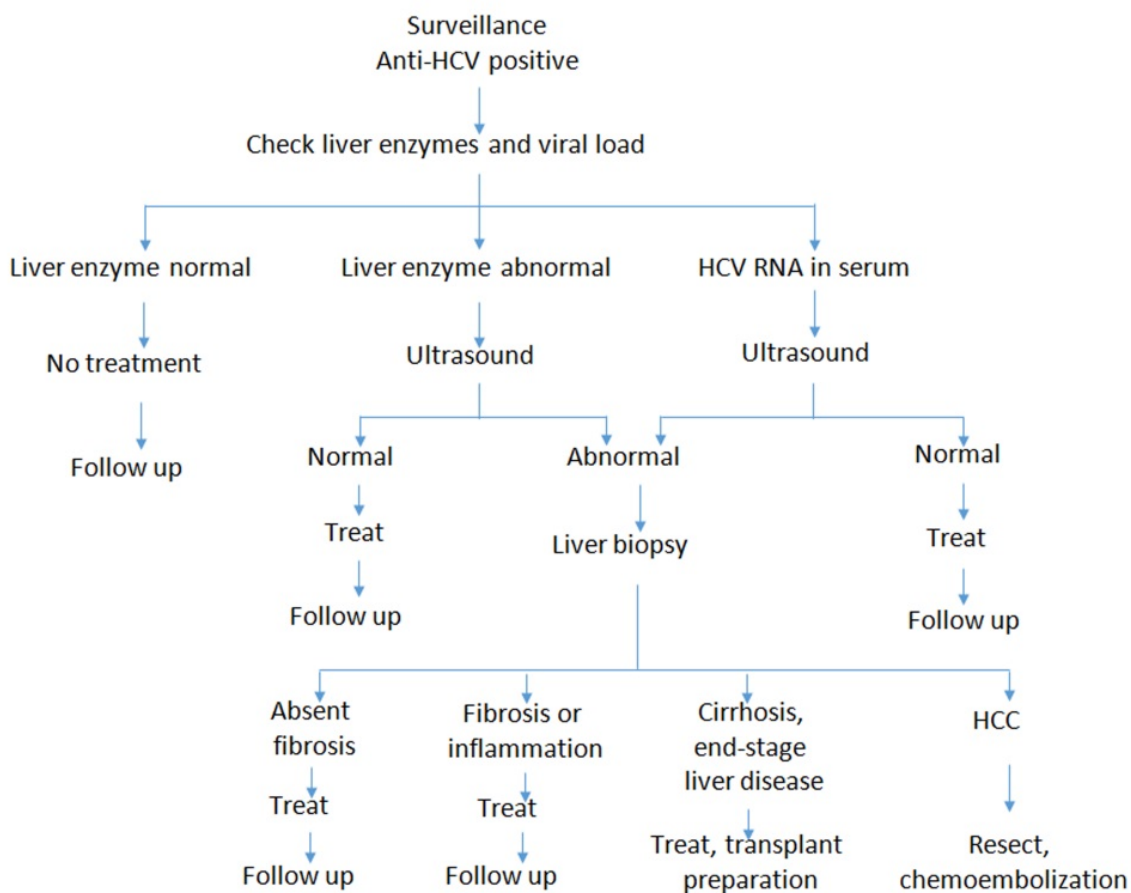
Figure 4: Algorithm of management of hepatitis C positive patients after surveillance. Individuals who have elevated liver enzymes, detectable hepatitis C virus RNA in the serum or abnormal liver biopsy, such as moderate degrees of inflammation or fibrosis, should be treated. Current therapy for genotype 1 infection is a combination of interferon, ribavirin and a protease inhibitor or nucleotide polymerase inhibitor sofosbuvir. Dual therapy with interferon and ribavirin or sofosbuvir with ribavirin is applied for treatment of genotype 2 and 3. Patients with genotype 4 should be treated with combination of sofosbuvir, interferon and ribavirin (http://www.who.int/hiv/pub/hepatitis/hepatitis-c-guidelines/en/). HCV RNA level should be checked after 12 weeks and therapy will be continued if HCV RNA become undetectable or >2 logs below baseline. Viral RNA level should be checked again after stopping therapy for sustained virological response.

	Chronic hepatitis C	Cirrhosis	HCC
Pathology features	Lymphocytes infiltration and lymphoid aggregates in portal region; piecemeal necrosis; progressive fibrosis; steatosis	Fibrous scars bridging portal tracts to each other or to central veins; fibrosis surround regenerating nodules of hepatocytes, liver cell plate thickened (Masson trichrome stain and reticulin stain for visualization of collagen and reticulin); variable inflammatory infiltration	Increased nuclear to cytoplasm ratio; necrosis; steatosis
Differential diagnosis	Hepatitis B, autoimmune hepatitis, PBC*, PSC*, Wilson's disease; α1-antitrypsin deficiency	Focal nodular hyperplasia; hemochromatosis; Wilson's disease; α1-antitrypsin deficiency; congenital hepatic fibrosis	Low grade dysplastic nodule; high grade dysplastic nodule

Table 1: Pathology Features and Differential Diagnosis of Chronic Hepatitis C, Cirrhosis and HCC*

Currently, vaccination against hepatitis C is not available and development of efficient hepatitis C vaccine is hampered by the variety of viral genome. Liver biopsy remains to be the gold standard for the evaluation of the chronic hepatitis C and cirrhosis. Typical pathology features of chronic hepatitis C, cirrhosis and hepatocellular carcinoma are portal inflammation, periportal fibrosis, irregular regeneration of hepatocytes, pseudo glandular formation, necrosis and steatosis. Table 1 summarized pathology features and differential diagnosis of chronic

hepatitis C, cirrhosis and HCC. Hepatocellualr carcinoma usually presents late and resection is seldom possible. Due to the high mortality rate, surveillance of HCC should be conducted in high risk patient population. It has been reported that HCC diagnosed by regular screening have a significantly lower serum AFP level, smaller tumor size, less bilobar disease, less portal vein infiltration and less distant metastasis compared with the symptomatic subjects. As a result, more patients are amenable to surgical resection [55]. Molecular mechanisms of how HCV cause liver fibrosis and HCC are not clear. Growth factors such as TGF-beta and PDGF are involved in liver regeneration and fibrosis. Genetic instability, telomere shortening, loss of heterozygosity, copy number aberrations, epigenetic changes, somatic mutations and germ line mutations were identified as genetic factors that promote disease progression and carcinogenesis. Further investigation will be beneficial for early detection and improve survival.

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