Online Submissions: wjg.wjgnet.com
wjg@wjgnet.com
doi:10.3748/wjg.15.5080

World J Gastroenterol 2009 October 28; 15(40): 5080-5085
World Journal of Gastroenterology ISSN 1007-9327

BRIEF ARTICLE

Hepatitis C virus genotype 3a infection and hepatocellular carcinoma: Pakistan experience

Muhammad Idrees, Shazia Rafique, Irshad-ur Rehman, Haji Akbar, Muhammad Zubair Yousaf, Sadia Butt, Zunaira Awan, Sobia Manzoor, Madiha Akram, Mahwish Aftab, Bushra Khubaib, Sheikh Riazuddin

Muhammad Idrees, Shazia Rafique, Irshad-ur Rehman, Haji Akbar, Muhammad Zubair Yousaf, Sadia Butt, Zunaira Awan, Sobia Manzoor, Madiha Akram, Mahwish Aftab, Bushra Khubaib, Division of Molecular Virology & Molecular Diagnostics, National Centre of Excellence in Molecular Biology, University of the Punjab Lahore, 87-West Canal Bank Road Thokar Niaz Baig, Lahore-53700, Pakistan
Sheikh Riazuddin, National Centre of Excellence in Molecular Biology, University of the Punjab Lahore, 87-West Canal Bank Road Thokar Niaz Baig, Lahore-53700, Pakistan
Author contributions: Idrees M and Riazuddin S designed the study; Idrees M performed all the experimental work under the supervision of Riazuddin S and wrote the manuscript; Riazuddin S also provided vital reagents and analytical tools and was involved in editing the manuscript; Rafique S, Rehman I, Akbar H, Yousaf MZ, Butt S, Awan Z, Manzoor S, Akram M, Aftab M and Khubaib B participated in data analysis.
Supported by Ministry of Science & Technology Government of Pakistan Scientific Project Grant
Correspondence to: Dr. Muhammad Idrees, Division of Molecular Virology & Molecular Diagnostics, National Centre of Excellence in Molecular Biology, University of the Punjab Lahore, 87-West Canal Bank Road Thokar Niaz Baig, Lahore-53700, Pakistan. idreeskhan@cemb.edu.pk
Telephone: +92-42-5293144 Fax: +92-42-5421316
Received: June 1, 2009 Revised: September 10, 2009
Accepted: September 17, 2009
Published online: October 28, 2009

Abstract

AIM: To assess the association between chronic hepatitis C virus (HCV) infection and hepatocellular carcinoma (HCC) in Pakistan, and the genotype distribution among these HCC patients.

METHODS: One hundred and sixty-one subjects with HCC were included in this study. Liver biopsy was performed on 145 of the patients; sixteen were excluded because they failed to fulfill the inclusion criteria. Qualitative polymerase chain reaction (PCR) was performed for hepatitis B virus and HCV. Samples positive for HCV RNA were genotyped using genotype-specific PCR and confirmed by HCV 5′ noncoding region sequencing analysis.

RESULTS: Chronic HCV infection was identified a major risk factor (63.44% of tested HCC patients) for the development of HCC. The time from HCV infection to appearance of cancer was 10-50 years. In the HCC patient population, broader distributions of genotypes were present with genotype 3a as the predominant genotype. Using the type-specific genotyping method, we found HCV genotype 3a in 40.96%, 3b in 15.66%, 1a in 9.63%, and 1b in 2.40% of HCC tissue samples. About 28% of cases were found with mixed genotypes. Two cases were unable to be genotyped because of low viral load. Sixty-six percent of treated patients with cirrhosis had an end of treatment response, but unfortunately they relapsed quickly when the treatment was discontinued, and HCC developed during a median 3.8 years.

CONCLUSION: There was a strong association between chronic HCV infection and HCC in Pakistan, and between HCV genotype 3a and HCC.

Key words: Hepatocellular carcinoma; Hepatitis C; Genotyping; Etiology; Prevalence

Peer reviewers: Chifumi Sato, Professor, Department of Analytical Health Science, Tokyo Medical and Dental University, Graduate School of Health Sciences, 1-5-45 Yushima, Bunkyoku, Tokyo 113-8519, Japan; Henry LY Chan, MD, Associate Professor, Department of Medicine and Therapeutics, The Chinese University of Hong Kong, Hong Kong, China

Idrees M, Rafique S, Rehman I, Akbar H, Yousaf MZ, Butt S, Awan Z, Manzoor S, Akram M, Aftab M, Khubaib B, Riazuddin S. Hepatitis C virus genotype 3a infection and hepatocellular carcinoma: Pakistan experience. *World J Gastroenterol* 2009; 15(40): 5080-5085 Available from: URL: http://www.wjgnet.com/1007-9327/15/5080.asp DOI: http://dx.doi.org/10.3748/wjg.15.5080

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the commonest cancers in the world, with an estimated incidence of 5000000 to 10000000 new cases every year[1]. Hepatitis C virus (HCV) infection, hepatitis B virus (HBV) infection, alcoholic liver disease, and non-alcoholic fatty liver disease are the major causes of cirrhosis in patients with HCC[2,3].

Chronic HCV infection frequently leads to liver cirrhosis and is associated with an elevated risk for progression into HCC[4,5]. Epidemiological surveys have identified HCV in 10%-80% of HCC patients reported in different populations[5,6]. HCV has also been reported to be the major cause of HCC in Japan[7], Italy[6] and Spain[8], but is less important in South Africa[9] and Taiwan[10]. Association of HCV infection with HCC has also been well documented in the United States[11].

Etiology, clinical features, and survival of HCC vary considerably in different populations[12]. In Pakistan, HCC is a leading cause of death and accounts for 60%-90% of all primary liver malignancies[13]. Some studies have shown hepatitis B surface antigen (HBsAg) positivity in 60% of patients with HCC[14,15]. However, some other studies have reported positivity for HCV infection in up to 80% of patients with HCC[16]. It is believed that HCV infection is a major etiological factor for HCC[17], however, not all patients with HCV infection develop HCC. A number of host factors such as male sex, older age at infection, long disease duration, excessive alcohol consumption, and high liver iron overload have been reported to influence disease progression[18,19]. Several additional studies have noted variables such as chronic co-infection with HBV and human immunodeficiency virus (HIV)[20], obesity and steatosis[21], type 2 diabetes[22], and asymptomatic cryoglobulinemia[17,18,23]. In addition to these host factors, several viral factors such as genotype and peripheral viral load have also been reported to influence disease progression[24]. Some studies have identified that cirrhotic patients infected with HCV type 1b carry a significantly higher risk of developing HCC compared to those infected with other HCV types[25,26]. However, the results of other studies[27,28] are in disagreement with these studies, and demonstrate no association of a particular HCV genotype with the development of HCC.

No such studies on the association or otherwise of HCV genotype with the development of HCC are available from Pakistan. Therefore, this study was performed to: (1) study various risk factors for the development of HCC; (2) investigate the prevalence of HCV in patients with HCC; and (3) evaluate if there is any association between particular HCV genotypes and HCC.

MATERIALS AND METHODS

Patients

For initial examination, 161 subjects with chronic hepatitis managed as end-stage liver disease patients at various hospitals of Punjab and North West Frontier Province of Pakistan were enrolled. All these patients underwent ultrasound-guided liver biopsy. Of these 161 subjects, 145 satisfied the inclusion criteria such as: HCC was confirmed by liver imaging (ultrasonography and computed tomography); histologically confirmed HCC; chronic liver disease of any etiology, with ascites and encephalopathy. Sixteen patients were excluded from the study because they failed to fulfill the study criteria (14 subjects) or were unwilling to participate in the study (two subjects). The study was started in March 2001 and ended in April 2009. The clinical records of these patients were examined to identify the etiology of HCC. Documentation of the histology of liver tissue surrounding the cancer, together with possible sources of transmission and duration of blood-borne infectious hepatitis, was made. The time of transmission of HCV infection was calculated from the time of first major/minor surgery or first blood transfusion; only these patients were used to calculate the range/median duration of infection. Serum samples were collected and stored at -20℃, at the time of diagnosis of HCC. All the liver biopsies were transported in liquid nitrogen and stored at -70℃. Liver function tests such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase and bilirubin levels of all the samples were estimated using an auto-analyzer (Hitachi, Tokyo, Japan). Serum α-fetoprotein (AFP) concentration was determined by solid-phase, two-site chemiluminescent immunometric commercial diagnostic assay, using an Immulite-100 automated immunoassay system (Diagnostic Products, Los Angeles, CA, USA). From all the subjects, written informed consent was obtained. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee and Institutional Review Board of the Centre.

ELISAs for HBsAg, anti-HBsAg, anti-hepatitis B core antigen (HBc), anti-HCV and anti-HIV 1 and 2

All the patients were screened for HBsAg, anti-HBsAg, anti-HBc, anti-HCV and anti-HIV 1 and 2 using third-generation ELISA kits (DRG Instruments, Germany) as described by the manufacturer.

HBV qualitative polymerase chain reaction (PCR)

Hepatitis B viral DNA was extracted from 200 μL of stored serum and 2-5 mg of liver tissue of each of the patients using Gentra DNA Isolation Kit (PUREGENE, USA). Qualitative detection of serum HBV DNA was done by PCR amplification of the surface antigen gene using specific forward (5'AGAACATCGCATCAGGAC TC-3'; nt: 159-178) and reverse (5'CATAGGTATCTTG CGAAAGC-3'; 642-623) primers. One microliter of the first-round products was re-amplified using nested PCR with internal forward (5'AGGACCCCTGCTCGTGTT AC-3'; 181-200) and reverse (5'AGATGATGGGATG-GGAATAC-3'; nt: 619-600) primers. The amplified products were detected on agarose gel electrophoresis after staining with ethidium bromide and visualization on a UV transilluminator.

Qualitative and quantitative detection of HCV RNA

HCV qualitative RT-PCR was carried out as described previously[29]. HCV RNA was quantified using a SmartCycler II Real-time PCR (Cepheid, USA), using HCV RNA quantitative kits (Sacace Biotechnologies, Italy) according to the kit protocol.

Table 1 Characteristics, biochemistry and etiology of HCC patients (n = 145) n (%)

Risk factor	Value
Age ± SD (yr)	58 ± 11
Male	107 (73.79)
ALT	61 (42.1)
AST	61 (42.1)
Alkaline phosphatase	145 (100)
Bilirubin	145 (100)
AFP elevation	125 (86.2)
Cirrhosis present	98 (67.58)
HBsAg-positive (alone)	18 (12.41)
Anti-HBc-positive (alone)	2 (1.37)
Anti-HBs-positive	10 (6.89)
HBV-DNA PCR-positive (alone)	26 (17.93)
Anti-HCV-positive (alone)	92 (63.44)
HCV-RNA PCR-positive	83 (57.24)
HBV- and HCV-positive	19 (13.10)
No known etiology	6 (4.13)

HCC: Hepatocellular carcinoma; ALT: Alanine aminotransferase; AST: Aminotransferase; AFP: α-fetoprotein; HCV: Hepatitis C virus; HBV: Hepatitis B virus.

HCV genotyping

Core HCV genotyping were performed as described previously[30] for all HCV-RNA-positive sera and tissues. Genotypes were confirmed by HCV 5' noncoding region (5' NCR) sequencing using ABI PRISM 3100 Genetic Analyzer (Applied Biosystem Inc., Foster City, CA, USA) in both directions. Sequences of isolates were aligned with representative sequences for each major genotype and subtype selected from the GenBank database with the help of the Multalign program. The phylogenetic analysis of HCV isolates was performed with MEGA 3.0 software[31], and phylogenetic trees were constructed by the neighbor-joining method, using the bootstrap-resampling test from the MEGA program (1000 bootstrap replications).

HCV treatment

The medical records of the HCV-related HCC patients showed that a total 21 patients had been treated previously for HCV infection. These treated patients had received 3 MU recombinant interferon-α three times weekly, subcutaneously, and ribavirin (10 mg/kg per day) for a total of 24 wk.

Statistical analysis

The data were analyzed and summary statistical analysis was carried out using SPSS for Windows version 10.0. The results for all variables were given in the form of averages (SD). The χ^2/Fisher's exact test and independent sample t test were used for categorical/continuous variables.

RESULTS

Characteristics and biochemistry of HCC patients

Patient demographics and biochemical and clinical data are shown in Table 1. HCC patients were older (58 ± 11 years), were predominantly male (73.8%), and had no history of chronic alcoholism. Data collection was incomplete for one aspect, namely, exact duration of illness. The time of HCV transmission was calculated from the time of first major/minor surgery or first blood transfusion, which might not have been the exact date of virus acquisition. All the patients with HCC had raised levels of serum bilirubin (> 1.0 mg/dL) and alkaline phosphatase (> 300 U/L). ALT and AST levels were abnormal (ALT > 40 IU/mL, AST > 35 IU/mL) only in 42% of patients. AFP level was elevated (> 15 IU/mL) in 86.2% of patients with HCC. Cirrhosis was present in 67.6% of HCC patients. All the patients were found to be negative for anti-HIV.

Etiology of HCC

Out of the 145 patients with HCC, HCV antibodies were present in 92 (63.4%) serum samples. Two patients were found to be tissue-positive by PCR but no anti-HCV antibodies were present. Eighty-one patients were found to be tissue- positive by PCR out of 92 anti-HCV-positive patients (88.04%). Of these patients with HCC caused by HCV, 68 were male and 13 were female. The mean age was 55 ± 10 years for HCV-related HCC. HCV RNA was detected in the serum of all these 81 tissue-positive patients. All the patients with HCV-related HCC had a history of chronic HCV infection. The peripheral HCV RNA loads were as low as 10000 copies to as high as 3.7×10^8 copies/mL. No significance difference was found between the viral loads in serum and tissues of the same patients. Twenty-eight cases were caused by HBV, of whom 18 (19.31%) also had markers for current HBV infection (HBsAg-positive), and two patients (1.37%) had markers for past infection (HBsAg-negative; anti-HBsAg-positive; anti-HBc positive). The age was 65 ± 12 years for HBV-associated HCC patients. Nineteen (13.1%) of the HCC cases had markers for HCV and HBV. Out of these 19 cases with dual infection, two were HBV-DNA-positive and HBsAg-negative. In 6 (4.13%) cases, the etiology of liver cancer could not be determined from the medical records or serology. All these six HCC patients with unknown etiology were younger than the HCV-related HCC patients (45-50 years).

Distribution of HCV genotypes in HCC patients

Table 2 shows the results of HCV genotyping. A total of 83 tissue samples (81 positive for HCV RNA and anti-HCV, and two positive for HCV RNA and negative for anti-HCV) were used for HCV genotyping. Using the type-specific genotyping method, we found HCV genotype 3a in 40.96%, 3b in 15.66%, 1a in 9.63%, and 1b in 2.40% of HCC tissue samples. Twenty-four tissues (28.91%) were found with mixed genotypes. Of the 24 mixed genotypes, 10 were infected with genotypes 3a and 3b, eight with 1a and 3a, and six with 1a and 3b. Two tissue samples were found to be untypable as no genotype was detected. Both of the untypable patients had no cirrhosis and had a low viral load (< 10^4 IU/mL). The genotyping results for all single genotypes were confirmed by sequencing. The sequence data of the sequences were submitted to GenBank. The Accession

Table 2 Results of HCV genotype determination in HCC patients (n = 83)[1]

HCV genotype	No. of HCC cases	Percentage
1a	8	9.63
1b	2	2.40
3a	34	40.96
3b	13	15.66
3a + 3b	10	12.48
1a + 3a	8	9.63
1a + 3b	6	7.22
NT	2	2.40

[1]Eighty-one were positive for tissue/serum RNA by PCR and positive for serum anti-HCV, and two were positive for tissue/serum RNA by PCR but negative for serum anti-HCV. Eleven patients with HCC caused by HCV were not genotyped, as these were anti-HCV-positive by ELISA, but were HCV-RNA-negative, thus they could not be genotyped utilizing the molecular genotyping method. NT: Not typed.

Numbers provided for our nucleotide sequences by GenBank are EF173955-EF174011.

Anti-viral treatment history

The medical records of the patients showed that 21 of 94 patients with HCV-associated HCC had received previous standard interferon therapy for a total of 24 wk. Of these treated patients, 13 were male and eight were female. Cirrhosis was present in all of these 21 treated patients. Twenty of these patients had genotype 3a (12 male and eight female) and one 3b (male). Fourteen (66.7%) of these patients (eight male and six female; all with genotype 3a) had an end of treatment response but relapsed after discontinuation of treatment, with no sustained viral response.

DISCUSSION

Several viral and host factors have been studied extensively since the identification of HCV infection as a major risk factor for the development of HCC[4,9,17]. Among the viral factors, the presence of some HCV genotypes adds to the list of risk factors for HCC. In the present study, the etiology of 145 patients with HCC was assessed with special emphasis on HCV genotype. More than 73% of the enrolled patients with HCC were male. It has been reported already that men have a higher liver cancer rate than women, with a ratio between 2:1 and 4:1[32]. The reasons for the higher proportion of male patients with HCC might be the possibility that more men are infected with HBV and HCV, consume alcohol, smoke, have increased iron stores, higher body mass index, and a possible involvement of male sex hormones in the onset of HCC[33]. Most patients (96.5%) in the current study were elderly and their ages ranged from 58 to 68 years. They were possibly infected on receiving injections or major/minor surgery at a median time of 20 years previously. Our observation of late onset of HCC is in agreement with earlier reports from other parts of the world where the transition from acute infection to cirrhosis and detection of HCC took 20-30 years[34]. It is important to mention here that, in recent years, with the increasing incidence of HCC, the age of patients with HCC has been decreasing among persons aged 45-60 years[2]. AFP elevation was observed in the present study in about 86% of patients with HCC. Cirrhosis was present in > 67% of HCC cases studied. Previous studies have shown that cirrhosis underlies HCC in > 80% of affected individuals[35,36]. Therefore, any agent that leads to cirrhosis should be seen as a risk factor for the development of HCC. It has also been reported that the risk among those with cirrhosis increases in parallel with the impairment of liver function, and in subjects with increased AFP concentration[35].

Thirty-two percent of our patients with HCC were without liver cirrhosis, which showed that infection with HCV and HBV could be correlated with the emergence of HCC, even in the absence of liver cirrhosis. It has been established that the mechanism for the development of HCC in HBV-related cases is associated with the integration of HBV DNA into hepatocytes[37]. However, such a mechanism has not been established for HCV, because to date, integration of HCV RNA into cellular DNA has not been reported, even when there has been evidence for the direct involvement of HCV in oncogenicity. According to recent reports, the possible risks are involvement of various viral proteins such as core, NS3 and NS4 in the induction of liver cell proliferation, by interfering directly with the major cellular transduction networks[38,39].

Several major findings have emerged from the current study. The first finding is the identification of chronic HCV infection as a major risk factor for the development of HCC in Pakistan, because anti-HCV was observed in > 63% of patients with HCC. The overall anti-HCV prevalence rate is 14%-15% and HBV carrier rate is 2%-3% in the general population of Pakistan[29,40]. Overall, our data are consistent with the results of studies already reported from high-risk areas for HCC such as Japan, Italy and Spain, where majority of reported HCC cases are HCV-related[6-8]. It is clear from the present study and from others that the greatest proportional increases have occurred recently in HCV-related HCC worldwide, and that HBV-related HCC has been stable and at its lowest rate[16]. The rate of HCV-related HCC is likely to continue to increase, and it is estimated that this increase will peak around the year 2010, not only in North America and Europe[41], but also in the rest of the world including Pakistan. Presently, the annual incidence of HCV-related HCC ranges between 2% and 8%[38].

The second major finding of the current study is the evidence that links HBV with HCC, with about 19% of cases caused by HBV. This link was expected and is unquestionable, as has been reported previously[40]. Co-infection with HBV was also found as an additional etiological factor for HCC in the current study, which supports other published studies[42,43]. In two HCV-RNA-positive patients, HBV DNA was detected even in the absence of serological markers for HBV in serum. Previously, it has also been reported that the rate of

occult infection in such patients can be as high as 63%[44]. It has been reported that the implementation of HBV vaccination has resulted in a significant decrease in the incidence of HBV-related HCC[45]. In 4.13% cases in the present study, the etiology of liver cancer could not be determined from medical records or from serology and molecular biology. All these patients were non-drinking males, but were chain smokers.

Another more interesting and somewhat surprising finding in the present study was the observation that HCV genotype 3a was the predominant genotype in 41% of HCC cases. This suggests that genotype 3a is a major risk factor associated with the development of HCC compared with other HCV genotypes. However, the question whether HCV genotype plays a role in the development of liver cirrhosis and HCC is still debatable. Previously, the effect of HCV genotype 1b has been scrutinized as a risk factor for HCC[25,26]. However, some other studies have revealed no preferential role of individual HCV genotypes in HCC[27,28]. Although in our study HCV genotype 3a was predominant in HCV-related HCC, this genotype has been reported previously to induce a high sustained response, and has been less responsible for severe disease as compared to genotypes 1a, 1b and 4[46]. It seems that the high percentage of HCC in patients with HCV genotype 3a might result from the fact that genotype 3a can equally cause increased oncogenicity, as can other genotypes such as 1a and 1b. Finally, patients with cirrhosis had no sustained response rates and treatment did not reduce the incidence of HCC. However, more studies with a large number of cirrhotic patients, along with adequate controls, are required to confirm this observation of the current study.

In conclusion, HCC was found mostly in patients with chronic HCV infection and with liver cirrhosis in Pakistan. There also seemed to be a strong association between chronic HCV infection with genotype 3a and HCC, as the high prevalence of genotype 3a in the HCC population reflected increased oncogenicity. Treatment did not stop the development of HCC. However, studies with larger numbers of patients could confirm that HCV genotypes vary in their propensity to produce clinically significant liver disease.

ACKNOWLEDGMENTS

We thank all the clinicians and patients for their cooperation in the study. We thank Dr. Saleem Kamili, Chief, Reference Laboratory, Division of Viral Hepatitis CDC for critically reading the manuscript. This project was partially supported by the Ministry of Science & Technology, Government of Pakistan.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is currently one of the fastest growing causes of cancer-related deaths worldwide. The geographical prevalence varies considerably from country to country and Pakistan is a high-risk area for the disease. A strong association has been established between chronic hepatitis C virus (HCV) infection and hepatocarcinogenesis. A specific HCV genotype could play a role in the development of HCC.

Research frontiers

In Pakistan, HCC is a leading cause of death and accounts for 60%-90% of all primary liver malignancies. Positivity for HCV infection is up to 80% in HCC. Are HCV genotypes playing a role in the development of liver cirrhosis and HCC? This question remains debatable. Previously, the effect of HCV genotype 1b has been scrutinized as a risk factor for the development of HCC. However, some other studies have revealed no preferential role of individual HCV genotypes in HCC. Therefore, the current study was carried out to assess if there was any association between chronic hepatitis infection with various HCV genotypes and HCC.

Innovations and breakthroughs

In the current study, the authors identified various risk factors for the development of HCC and particularly investigated the prevalence of HCV in patients with HCC. They further assessed the association between chronic hepatitis infection with various HCV genotypes and HCC, and found a strong association between chronic HCV infection with genotype 3a and HCC. Previously, the effect of HCV genotype 1b has been scrutinized as a risk factor for HCC.

Peer review

This paper describes the relationship between HCV genotype 3a infection and HCC development. Although there have been many studies on the difference among HCV genotypes in hepatocarcinogenesis, there has not been a sufficient number of reports on genotype 3a. Therefore, this paper deals with an interesting issue.

REFERENCES

1 **Moradpour D**, Wands JR. The molecular pathogenesis of hepatocellular carcinoma. *J Viral Hepat* 1994; **1**: 17-31
2 **El-Serag HB**. Hepatocellular carcinoma: recent trends in the United States. *Gastroenterology* 2004; **127**: S27-S34
3 **Llovet JM**, Burroughs A, Bruix J. Hepatocellular carcinoma. *Lancet* 2003; **362**: 1907-1917
4 **Saito I**, Miyamura T, Ohbayashi A, Harada H, Katayama T, Kikuchi S, Watanabe Y, Koi S, Onji M, Ohta Y. Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. *Proc Natl Acad Sci USA* 1990; **87**: 6547-6549
5 **Kayali Z**, Tan S, Shinkunas L, Voigt M, LaBrecque DR, Stapleton JT, Brown KE, Schmidt WN. Risk factors for hepatitis C fibrosis: a prospective study of United States veterans compared with nonveterans. *J Viral Hepat* 2007; **14**: 11-21
6 **Colombo M**, Kuo G, Choo QL, Donato MF, Del Ninno E, Tommasini MA, Dioguardi N, Houghton M. Prevalence of antibodies to hepatitis C virus in Italian patients with hepatocellular carcinoma. *Lancet* 1989; **2**: 1006-1008
7 **Kiyosawa K**, Sodeyama T, Tanaka E, Gibo Y, Yoshizawa K, Nakano Y, Furuta S, Akahane Y, Nishioka K, Purcell RH. Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. *Hepatology* 1990; **12**: 671-675
8 **Bruix J**, Barrera JM, Calvet X, Ercilla G, Costa J, Sanchez-Tapias JM, Ventura M, Vall M, Bruguera M, Bru C. Prevalence of antibodies to hepatitis C virus in Spanish patients with hepatocellular carcinoma and hepatic cirrhosis. *Lancet* 1989; **2**: 1004-1006
9 **Kew MC**, Houghton M, Choo QL, Kuo G. Hepatitis C virus antibodies in southern African blacks with hepatocellular carcinoma. *Lancet* 1990; **335**: 873-874
10 **Chen DS**, Kuo GC, Sung JL, Lai MY, Sheu JC, Chen PJ, Yang PM, Hsu HM, Chang MH, Chen CJ. Hepatitis C virus infection in an area hyperendemic for hepatitis B and chronic liver disease: the Taiwan experience. *J Infect Dis* 1990; **162**: 817-822
11 **El-Serag HB**. Hepatocellular carcinoma and hepatitis C in

the United States. *Hepatology* 2002; **36**: S74-S83
12 **Omata M**, Dan Y, Daniele B, Plentz R, Rudolph KL, Manns M, Piratvisuth T, Chen DS, Tateishi R, Chutaputti A. Clinical features, etiology, and survival of hepatocellular carcinoma among different countries. *J Gastroenterol Hepatol* 2002; **17** Suppl: S40-S49
13 **Ogunbiyi JO**. Hepatocellular carcinoma in the developing world. *Semin Oncol* 2001; **28**: 179-187
14 **Taseer IH**, Malik IH, Mustafa G, Arshad M, Zafar MH, Shabbir I, Khan MT, Hashmi N, Narjis S, Khan MI. Association of Primary Hepatocellular Carcinoma with Hepatitis B Virus. *Biomedica* 1996; **12**: 79-81
15 **Malik IA**, Ahmad N, Butt SA, Tariq WUZ, Muzaffar M, Bukhtiari N. The role of hepatitis B and C viruses in the etiology of hepatocellular carcinoma in Northern Pakistan. *J Coll Phy Surg Pak* 1995; **5**: 26-28
16 **Rehman AU**, Murad S. Hepatocellular Carcinoma: A retrospective analysis of 118 cases. *J Coll Physicians Surg Pak Feb* 2002; **12**: 108-109
17 **Di Bisceglie AM**. Hepatitis C and hepatocellular carcinoma. *Hepatology* 1997; **26**: 34S-38S
18 **Poynard T**, Bedossa P, Opolon P. Natural history of liver fibrosis progression in patients with chronic hepatitis C. The OBSVIRC, METAVIR, CLINIVIR, and DOSVIRC groups. *Lancet* 1997; **349**: 825-832
19 **Smith BC**, Gorve J, Guzail MA, Day CP, Daly AK, Burt AD, Bassendine MF. Heterozygosity for hereditary hemochromatosis is associated with more fibrosis in chronic hepatitis C. *Hepatology* 1998; **27**: 1695-1699
20 **Benhamou Y**, Bochet M, Di Martino V, Charlotte F, Azria F, Coutellier A, Vidaud M, Bricaire F, Opolon P, Katlama C, Poynard T. Liver fibrosis progression in human immunodeficiency virus and hepatitis C virus coinfected patients. The Multivirc Group. *Hepatology* 1999; **30**: 1054-1058
21 **Poynard T**, McHutchison J, Davis GL, Esteban-Mur R, Goodman Z, Bedossa P, Albrecht J. Impact of interferon alfa-2b and ribavirin on progression of liver fibrosis in patients with chronic hepatitis C. *Hepatology* 2000; **32**: 1131-1137
22 **Mason AL**, Lau JY, Hoang N, Qian K, Alexander GJ, Xu L, Guo L, Jacob S, Regenstein FG, Zimmerman R, Everhart JE, Wasserfall C, Maclaren NK, Perrillo RP. Association of diabetes mellitus and chronic hepatitis C virus infection. *Hepatology* 1999; **29**: 328-333
23 **Kayali Z**, Buckwold VE, Zimmerman B, Schmidt WN. Hepatitis C, cryoglobulinemia, and cirrhosis: a meta-analysis. *Hepatology* 2002; **36**: 978-985
24 **Poynard T**, Ratziu V, Benhamou Y, Opolon P, Cacoub P, Bedossa P. Natural history of HCV infection. *Baillieres Best Pract Res Clin Gastroenterol* 2000; **14**: 211-228
25 **Stankovic-Djordjevic D**, Djordjevic N, Tasic G, Dinic M, Karanikolic A, Pesic M. Hepatitis C virus genotypes and the development of hepatocellular carcinoma. *J Dig Dis* 2007; **8**: 42-47
26 **Raimondi S**, Bruno S, Mondelli MU, Maisonneuve P. Hepatitis C virus genotype 1b as a risk factor for hepatocellular carcinoma development: a meta-analysis. *J Hepatol* 2009; **50**: 1142-1154
27 **Nousbaum JB**, Pol S, Nalpas B, Landais P, Berthelot P, Bréchot C. Hepatitis C virus type 1b (II) infection in France and Italy. Collaborative Study Group. *Ann Intern Med* 1995; **122**: 161-168
28 **Ryu SH**, Fan X, Xu Y, Elbaz T, Zekri AR, Abdelaziz AO, Di Bisceglie AM. Lack of association between genotypes and subtypes of HCV and occurrence of hepatocellular carcinoma in Egypt. *J Med Virol* 2009; **81**: 844-847
29 **Idrees M**, Lal A, Naseem M, Khalid M. High prevalence of hepatitis C virus infection in the largest province of Pakistan. *J Dig Dis* 2008; **9**: 95-103
30 **Idrees M**. Development of an improved genotyping assay for the detection of hepatitis C virus genotypes and subtypes in Pakistan. *J Virol Methods* 2008; **150**: 50-56
31 **Kumar S**, Tamura K, Jakobsen IB, Nei M. MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics* 2001; **17**: 1244-1245
32 **McGlynn KA**, Tsao L, Hsing AW, Devesa SS, Fraumeni JF Jr. International trends and patterns of primary liver cancer. *Int J Cancer* 2001; **94**: 290-296
33 **Yu MW**, Yang YC, Yang SY, Cheng SW, Liaw YF, Lin SM, Chen CJ. Hormonal markers and hepatitis B virus-related hepatocellular carcinoma risk: a nested case-control study among men. *J Natl Cancer Inst* 2001; **93**: 1644-1651
34 **López-Labrador FX**, Ampurdanés S, Forns X, Castells A, Sáiz JC, Costa J, Bruix J, Sánchez Tapias JM, Jiménez de Anta MT, Rodés J. Hepatitis C virus (HCV) genotypes in Spanish patients with HCV infection: relationship between HCV genotype 1b, cirrhosis and hepatocellular carcinoma. *J Hepatol* 1997; **27**: 959-965
35 **Bruix J**, Sherman M, Llovet JM, Beaugrand M, Lencioni R, Burroughs AK, Christensen E, Pagliaro L, Colombo M, Rodés J. Clinical management of hepatocellular carcinoma. Conclusions of the Barcelona-2000 EASL conference. European Association for the Study of the Liver. *J Hepatol* 2001; **35**: 421-430
36 **Fattovich G**, Stroffolini T, Zagni I, Donato F. Hepatocellular carcinoma in cirrhosis: incidence and risk factors. *Gastroenterology* 2004; **127**: S35-S50
37 **Shafritz DA**, Shouval D, Sherman HI, Hadziyannis SJ, Kew MC. Integration of hepatitis B virus DNA into the genome of liver cells in chronic liver disease and hepatocellular carcinoma. Studies in percutaneous liver biopsies and post-mortem tissue specimens. *N Engl J Med* 1981; **305**: 1067-1073
38 **Ishido S**, Hotta H. Complex formation of the nonstructural protein 3 of hepatitis C virus with the p53 tumor suppressor. *FEBS Lett* 1998; **438**: 258-262
39 **Qadri I**, Iwahashi M, Simon F. Hepatitis C virus NS5A protein binds TBP and p53, inhibiting their DNA binding and p53 interactions with TBP and ERCC3. *Biochim Biophys Acta* 2002; **1592**: 193-204
40 **André F**. Hepatitis B epidemiology in Asia, the Middle East and Africa. *Vaccine* 2000; **18** Suppl 1: S20-S22
41 **Wong JB**, McQuillan GM, McHutchison JG, Poynard T. Estimating future hepatitis C morbidity, mortality, and costs in the United States. *Am J Public Health* 2000; **90**: 1562-1569
42 **Bréchot C**. Pathogenesis of hepatitis B virus-related hepatocellular carcinoma: old and new paradigms. *Gastroenterology* 2004; **127**: S56-S61
43 **Kew MC**, Yu MC, Kedda MA, Coppin A, Sarkin A, Hodkinson J. The relative roles of hepatitis B and C viruses in the etiology of hepatocellular carcinoma in southern African blacks. *Gastroenterology* 1997; **112**: 184-187
44 **Pollicino T**, Squadrito G, Cerenzia G, Cacciola I, Raffa G, Craxi A, Farinati F, Missale G, Smedile A, Tiribelli C, Villa E, Raimondo G. Hepatitis B virus maintains its pro-oncogenic properties in the case of occult HBV infection. *Gastroenterology* 2004; **126**: 102-110
45 **Chang MH**, Chen CJ, Lai MS, Hsu HM, Wu TC, Kong MS, Liang DC, Shau WY, Chen DS. Universal hepatitis B vaccination in Taiwan and the incidence of hepatocellular carcinoma in children. Taiwan Childhood Hepatoma Study Group. *N Engl J Med* 1997; **336**: 1855-1859
46 **Manns MP**, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling M, Albrecht JK. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; **358**: 958-965

S- Editor Tian L **L- Editor** Kerr C **E- Editor** Zheng XM