EDITORIAL

Persistent occult hepatitis B virus infection: Experimental findings and clinical implications

Patricia M Mulrooney-Cousins, Tomasz I Michalak

Patricia M Mulrooney-Cousins, Tomasz I Michalak, Molecular Virology and Hepatology Research, Division of BioMedical Science, Faculty of Medicine, Health Sciences Centre, Memorial University, St. John's, NL A1B 3V6, Canada

Supported by operating research grants from the Canadian Institutes of Health Research, Canada and the Canada Research Chair Program, and the Canada Foundation for Innovation

Correspondence to: Tomasz I Michalak, MD, PhD, Molecular Virology and Hepatology Research, Faculty of Medicine, Health Science Centre, Memorial University, 300 Prince Philip Drive, St. John's, NL A1B 3V6, Canada. timich@mun.ca

Telephone: +1-709-7777301 Fax: +1-709-7778279

Received: July 24, 2007 Revised: September 5, 2007

<http://www.wjgnet.com/1007-9327/13/5682.asp>

INTRODUCTION

Despite the availability of effective prophylactic vaccines, hepatitis caused by hepatitis B virus (HBV) remains one of the most troublesome infectious diseases responsible for significant morbidity and mortality in the world. HBV-induced chronic hepatitis B (CHB), defined as a prolonged liver inflammation accompanied by HBV surface antigen (HBsAg) in serum for longer than 6 mo, develops in up to 10% of individuals infected as adults and in about 85% of individuals exposed in the neonatal period and early childhood^[1,2]. Consequently, up to 400 million people worldwide are afflicted with serum HBsAg-positive chronic infection, which in high endemic areas is mainly acquired by vertical virus transmission^[3]. Furthermore, it is estimated that as much as one third of the world population have been exposed to HBV^[4]. Many of these individuals may unknowingly carry the virus. It is assumed that, due to low virus loads in many of the cases, HBV may not be identifiable by commonly used serological assays based on detection of HBsAg and that the infection is often asymptomatic or has non-specific symptoms. This serologically and clinically unapparent infection makes the true global incidence of HBV hard to estimate. Consequently, the pathogenic significance of HBV might be much greater than that currently assumed.

OCCULT HBV INFECTION

The clinical data and the findings from animal models accumulated in the past ten years strongly argue that persistent, serologically silent, i.e., serum HBsAg-negative, HBV infection is a natural consequence of resolved acute hepatitis B, but it may also occur following an asymptomatic exposure to HBV, and may have serious pathological and epidemiological repercussions. Another important aspect of infections with HBV and other related viruses, that has been known for a long time but remained controversial and/or not fully realized, is the fact that the immune system, in addition to the liver, is the site of active hepadnaviral replication and long-term persistence.

Occult HBV infection is defined as the existence of HBV DNA in the serum, cells of the lymphatic (immune) system, and/or hepatic tissue in the absence of serum HBsAg. Most frequently, occult HBV infection follows

Abstract

Hepatitis B virus (HBV) is a highly pathogenic virus that causes chronic liver diseases in millions of people globally. In addition to a symptomatic, serologically evident infection, occult persistent HBV carriage has been identified since nucleic acid amplification assays of enhanced sensitivity became introduced for detection of hepadnaviral genomes and their replicative intermediates. Current evidence indicates that occult HBV infection is a common and long-term consequence of resolution of acute hepatitis B. This form of residual infection is termed as secondary occult infection (SOI). The data from the woodchuck model of HBV infection indicate that exposure to small amounts of hepadnavirus can also cause primary occult infection (POI) where virus genome, but no serological markers of exposure to virus, are detectable, and the liver may not be involved. However, virus replicates at low levels in the lymphatic system in both these forms. We briefly summarize the current understanding of the nature and characteristics of occult hepadnaviral persistence as well as of its documented and expected pathological consequences.

© 2007 WJG. All rights reserved.

Key words: Hepatitis B virus; Hepadnaviruses; Woodchuck hepatitis virus; Hepadnaviral hepatitis; Occult viral persistence; Hepadnavirus lymphotropism; Primary occult infection; Secondary occult infection; Virus reactivation

Mulrooney-Cousins PM, Michalak TI. Persistent occult hepatitis B virus infection: Experimental findings and clinical implications. *World J Gastroenterol* 2007; 13(43): 5682-5686

resolution of acute hepatitis and continues indefinitely after clearance of HBsAg and biochemical improvement in liver function^[5-9]. For this reason, this form of infection is termed as residual or secondary occult infection or SOI, in opposite to primary occult infection or POI, which will be discussed later. Serological testing in SOI normally reveals the presence of antibodies to HBV core antigen (anti-HBc), which are now recognized not only as a valuable marker of prior exposure to HBV but also as an indicator suggesting actually progressing occult HBV infection. Nonetheless, recent estimates suggest that up to 20% of individuals with occult HBV carriage evidenced by HBV DNA detection could be nonreactive for anti-HBc or any other serological indicator of exposure to HBV^[7]. It also is of note that the detection of naturally acquired antibodies to HBsAg (anti-HBs) does not exclude the existence of occult infection^[5-7,9].

DETECTION OF OCCULT HEPADNAVIRAL PERSISTENCE

One of the main current challenges in diagnosis of occult HBV infection is the relatively low sensitivity of HBV DNA detection assays. The present assays are based on different approaches to amplification of HBV DNA by polymerase chain reaction (PCR) and their lowest detection limits range between 200 and 1000 virus genome copies, which are also referred to as virus genome equivalents (vge) per mL of plasma or serum. Although these assays are adequate to detect prodromal and symptomatic HBV infections and to monitor the progress of antiviral therapy, they are yet not sufficiently sensitive to identify occult infection in the majority of the individuals affected, where serum loads are usually below 100 vge per mL, as well as to determine whether virus was indeed completely eradicated following antiviral treatment^[9]. In contrast to the clinical assays, the current research tests, which in our laboratory apply nested PCR and detection of resulting amplicons by nucleic acid hybridization (NAH), i.e., PCR/NAH, identify hepadnaviral DNA at loads below 10 vge/mL^[10,11]. Furthermore, by testing of serial samples of serum and peripheral blood mononuclear cells (PBMC) and, when feasible, liver biopsies, and by amplifying different non-overlapping regions of the virus genome, detection of occult infection is greatly enhanced^[9]. Application of these ultrasensitive assays to investigation of clinical materials and animal models, particularly the woodchuck model of hepatitis B, led to identification in the last decade of occult hepadnavirus infection as a new, distinctive entity and to delineation of its unique characteristics. However, due to yet limited population-based studies and relatively low sensitivity of the assays approved for clinical testing, the full impact of this infection remains to be determined. Nonetheless, with the recent introduction of clinical HBV nucleic acid testing (HBV NAT) in many parts of the world, significant progress in this area is expected.

EXPERIMENTAL OCCULT HBV INFECTION IN THE WOODCHUCK MODEL

Many of the characteristics of occult HBV infection have

been originally identified in the woodchuck model of hepatitis B^[5]. Woodchuck hepatitis virus (WHV) infecting the Eastern North American woodchuck (*Marmota monax*) is accepted as the most adequate, natural model of human HBV infection, hepatitis B and HBV-induced hepatocellular carcinoma (HCC). WHV closely resembles HBV in the structure, biology, and in the patterns and outcomes of the liver diseases induced. Much of the basic understanding of the natural course, requirements for transmission, molecular and immunological properties of occult hepadnaviral carriage, and its long-term pathological ramifications is owed to the woodchuck-WHV experimental system^[10-15].

Occult WHV infection was first identified as a residual, life-long persistent infection in woodchucks who apparently completely resolved experimental acute hepatitis and developed anti-WHs (antibodies to WHV surface antigen, WHsAg)^[10]. Analysis of sequential sera, PBMC and liver biopsies collected during the lifespan of these animals, as well as autopsy lymphatic and hepatic tissues, using sensitive PCR/NAH assays, identified WHV DNA in all three compartments, i.e., serum, lymphoid cells and the liver. In addition, the WHV replicating intermediates, covalently closed circular DNA (cccDNA), and virus mRNA were consistently found in the cells of the lymphatic system and the liver. Anti-WHc (equivalent of anti-HBc) persisted in all animals for life. This form of WHV infection became later termed as SOI^[5,14]. In addition and unexpectedly, a low grade, intermittent liver inflammation was diagnosed throughout the remaining life in the majority of the woodchucks who apparently completely resolved acute hepatitis^[10]. Not less importantly, one fifth of the animals developed HCC in 3-5 years after recovery and despite anti-WHs presence^[10]. The same rate of HCC development was reported in woodchucks recovered from acute hepatitis which were examined by other investigators^[16]. Most recently, it has also been shown that residual WHV in animals with SOI can be reactivated by treatment with an immunosuppressive agent, Cyclosporin A, leading to transiently serum WHsAg-positive infection^[17]. Taken together, the data accumulated indicate that despite resolution of acute hepatitis, the replication of WHV continues for life, albeit at very low levels, both in the liver and the lymphatic system, that limited inflammatory process continues in hepatic tissue, and that the silently persisting virus may retain its oncogenic potency. This implies that although the immune system is able to resolve acute hepatitis and can keep persistently propagating hepadnavirus under relative control, it is unable to eradicate the virus completely^[10,14]. The same conclusion was recently reached when bicistronic WHV core-gamma interferon (IFN) DNA vaccine was found to be able to prevent WHV hepatitis, but could not mount sterile immunity and prevent establishment of occult infection^[18]. Furthermore, it became apparent that the detection of anti-WHc in the absence of other serological indicators of infection is a reliable indicator of occult WHV persistence^[15]. This state, similarly as strong HBV-specific cytotoxic T cell (CTL) and T helper lymphocyte responses detectable years after resolution of acute hepatitis B^[19,20], is likely a consequence of sustained restimulation of the immune system with a

viral protein produced during low-level virion assembly^[15]. The high degree of compatibility between WHV and HBV infections and the data from studies on otherwise healthy anti-HBc-positive individuals^[21,22] suggest that the occurrence of isolated anti-HBc could also be of value in identifying occult HBV persistence.

The virus recovered from woodchucks with SOI remains infectious. It was observed that WHV harvested from PBMC isolated during SOI and *ex vivo* stimulated with lipopolysaccharide (LPS) induced classical acute WHV hepatitis in virus-naïve animals^[10]. Furthermore, woodchuck dams with SOI were found to transmit virus to offspring, however, the infection induced frequently displays characteristics different than those of SOI^[11]. Thus, WHV DNA and WHV cccDNA was harboured only in the lymphatic system, but not in the liver, and in the absence of serological markers of infection, including anti-WHc antibodies. This form of hepadnaviral infection was later named as primary occult infection or POI^[14]. Interestingly, POI, in contrast to SOI, is not associated with protection from reinfection and hepatitis after challenge with liver pathogenic doses of WHV^[11]. In subsequent studies, the same profile of occult infection was experimentally induced in adult animals by intravenous injections with WHV doses lower than 1000 virions^[14]. At the same time, it was also established that WHV quantities higher than 1000 virions, in addition to infecting the lymphatic system, consistently cause classical acute hepatitis. This revealed that doses greater than 1000 virions are liver pathogenic in woodchucks^[14]. From these data it was inferred that the threshold level of WHV required to infect lymphoid cells is 100 to 1000-fold lower than that to infect hepatocytes. WHV sequence variation was not responsible for the induction of these two contrasting forms of infection^[14].

At the present time, POI is the entity which has not yet been convincingly identified in humans. However, the detection of occult HBV DNA-positive, anti-HBc-negative infection may suggest its existence. The persistence of trace amounts of replicating virus, especially at the extrahepatic locations, could have an impact in terms of unforeseen virus transmission and induction of disorders which are not yet considered to be related to hepadnaviral infection. Small quantities of the virus carried across the placenta may induce POI, similarly to CHB developing in neonates born to mothers with serum HBsAg-positive infection.

OCCULT HEPADNAVIRAL INFECTION AND THE HOST'S IMMUNE SYSTEM

Since the cells of the immune system are clearly the site of WHV propagation, independent of whether infection is symptomatic or occult, it became of interest to enumerate the cells carrying the virus in different forms of experimentally induced infection. By applying *in situ* PCR combined with flow cytometric quantification of cells containing amplified WHV core gene sequences, the proportion of WHV DNA-positive circulating lymphoid cells was found to range from 3.4% to 20.4% (mean 9.6%) in serum WHsAg-positive hepatitis, as compared to 1.1%

to 14.6% (mean 4.8%) in occult WHV infection^[13]. There was no difference between SOI and POI in the numbers of infected cells.

In terms of WHV-specific T cell responses, recent studies have shown that animals with POI display weak but evident, persistent and multispecific virus-specific T cell proliferative reactivity and that this cellular response does not provide protection against challenge with liver pathogenic doses of WHV^[23] (Gujar *et al*, manuscript in preparation). The existence of active peripheral WHV-specific T cell response and innate immune cell activation in the livers of animals with SOI have also been established^[12] (Gujar *et al*, manuscript in preparation). Overall, the findings in experimental SOI in woodchucks are closely compatible with those reported for humans convalescent from acute hepatitis B^[19,20].

PRIMARY VERSUS SECONDARY OCCULT WHV INFECTION

In summary, experimental occult hepadnaviral infection occurs in two distinctive forms, as primary and secondary infection. POI is induced upon exposure to WHV doses at or below 1000 vge and engages the lymphatic system, although the infection with time may also spread to the liver^[11,14]. Animals with POI carry virus in serum and lymphoid cells at levels which are comparable with those occurring in SOI and mount virus-specific T cell proliferative response, but they do not have serological (immunovirological) markers of infection and they are not protected from challenge with liver pathogenic doses of the virus. On the other hand, in SOI, a low-level replication of the virus progresses in both the liver and the lymphatic system, anti-WHc and frequently anti-WHs are detectable, and the animals are protected from hepatitis when re-exposed to liver pathogenic doses of WHV. Moreover, while SOI can be accompanied by low grade, protracted liver inflammation and HCC may finally develop, the liver is entirely normal in POI and a possible development of hepatoma has not yet been assessed.

IMPLICATIONS OF OCCULT HEPADNAVIRAL PERSISTENCE

Given the high degree of similarity between WHV and HBV and infections induced by these viruses, it is important to recognize the impact of silent HBV persistence in terms of its overall incidence, infectivity and pathogenic consequences. The previous clinical and research focus on the liver as essentially the only reservoir of HBV has somehow restricted characterization of extrahepatic sites of hepadnaviral replication and its potential pathological consequences. Recent studies are bringing occult HBV infection and the virus' lymphotropic nature to the forefront. Some of the cardinal works on occult HBV infection were accomplished by examining lymphoid cells of patients with resolved acute hepatitis B where HBV DNA sequences were found^[19,24,25]. Furthermore, immunological studies showed that vigorous CTL and T cell proliferative

responses specific against HBV antigens persisted for years after recovery^[19,20]. Many case reports also indicate that immunosuppression caused by chemotherapy^[26,27], immunomodulatory agents^[28], or immune deficiencies, such as HIV infection^[29] or hematological malignancies^[30], can reactivate occult infection. As in woodchucks with SOI, mild necroinflammation has been documented in liver samples obtained many years after recovery from acute hepatitis B^[31,32]. Liver fibrosis and cirrhosis of unknown origin has now been explained by occult HBV infection in many retrospective studies^[33,34]. There also is strong evidence for the risk of HCC development in patients with occult HBV^[35-38] and this risk is further elevated in alcoholics^[39] and in patients with other liver ailments, like hepatitis C^[40]. Interestingly, convincing evidence points to the conservation of the HBV genome sequence in occult infection, negating the possibility that faulty HBsAg production accounts for all incidences of occult infection^[32,41]. On the other hand, studies demonstrating the passage of HBV in non-liver cells, such as stem cells^[42,43] and bone marrow^[44], and by blood donations collected from individuals with occult HBV^[45] are increasingly frequent in the literature. Recent estimates suggest that transient HBV DNA levels of up to 10⁴ vge/mL can be found in sera from apparently healthy individuals with occult infection, which evade detection by current clinical assays, especially when testing is done on single serum or plasma samples^[9,46].

CONCLUSION

The seriousness of the consequences of occult HBV infection is yet not fully recognized. Accumulated evidence indicate that occult HBV can be both a source of virus contamination in blood and organ donations, as well as the reservoir from which full blown hepatitis can arise. The oncogenic potency of occult HBV persistence becomes progressively evident. This silent infection can also affect the progression and outcomes of other viral diseases, particularly hepatitis C. It can be expected that while the burden of CHB will decrease with time due to introduction of more effective antiviral therapies, occult HBV infection could become a main concern. Understanding in its entirety of the nature and consequences of this form of HBV persistence should be of a high priority and this quest has already been initiated.

REFERENCES

- 1 **Ganem D**, Prince AM. Hepatitis B virus infection--natural history and clinical consequences. *N Engl J Med* 2004; **350**: 1118-1129
- 2 **Michalak TI**. Immunology of hepatitis B virus. In: Colacino JM, Heinz BA. *Hepatitis Prevention and Treatment*. Basel, Switzerland: Birkhäuser Verlag, 2004: 87-105
- 3 **Lok AS**, McMahon BJ. Chronic hepatitis B. *Hepatology* 2001; **34**: 1225-1241
- 4 **Lavanchy D**. Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. *J Viral Hepat* 2004; **11**: 97-107
- 5 **Michalak TI**. Occult persistence and lymphotropism of hepadnaviral infection: insights from the woodchuck viral hepatitis model. *Immunol Rev* 2000; **174**: 98-111
- 6 **Bréchet C**, Thiers V, Kremsdorf D, Nalpas B, Pol S, Paterlini-Bréchet P. Persistent hepatitis B virus infection in subjects without hepatitis B surface antigen: clinically significant or purely "occult"? *Hepatology* 2001; **34**: 194-203
- 7 **Torbenson M**, Thomas DL. Occult hepatitis B. *Lancet Infect Dis* 2002; **2**: 479-486
- 8 **Raimondo G**, Pollicino T, Cacciola I, Squadrito G. Occult hepatitis B virus infection. *J Hepatol* 2007; **46**: 160-170
- 9 **Michalak TI**, Pham TNQ, Mulrooney-Cousins PM. Molecular diagnosis of occult hepatitis C and hepatitis B virus infections. *Future Virol* 2007; **2**: 451-465
- 10 **Michalak TI**, Pardoe IU, Coffin CS, Churchill ND, Freake DS, Smith P, Trelegan CL. Occult lifelong persistence of infectious hepadnavirus and residual liver inflammation in woodchucks convalescent from acute viral hepatitis. *Hepatology* 1999; **29**: 928-938
- 11 **Coffin CS**, Michalak TI. Persistence of infectious hepadnavirus in the offspring of woodchuck mothers recovered from viral hepatitis. *J Clin Invest* 1999; **104**: 203-212
- 12 **Hodgson PD**, Michalak TI. Augmented hepatic interferon gamma expression and T-cell influx characterize acute hepatitis progressing to recovery and residual lifelong virus persistence in experimental adult woodchuck hepatitis virus infection. *Hepatology* 2001; **34**: 1049-1059
- 13 **Mulrooney PM**, Michalak TI. Quantitative detection of hepadnavirus-infected lymphoid cells by in situ PCR combined with flow cytometry: implications for the study of occult virus persistence. *J Virol* 2003; **77**: 970-979
- 14 **Michalak TI**, Mulrooney PM, Coffin CS. Low doses of hepadnavirus induce infection of the lymphatic system that does not engage the liver. *J Virol* 2004; **78**: 1730-1738
- 15 **Coffin CS**, Pham TN, Mulrooney PM, Churchill ND, Michalak TI. Persistence of isolated antibodies to woodchuck hepatitis virus core antigen is indicative of occult infection. *Hepatology* 2004; **40**: 1053-1061
- 16 **Korba BE**, Wells FV, Baldwin B, Cote PJ, Tennant BC, Popper H, Gerin JL. Hepatocellular carcinoma in woodchuck hepatitis virus-infected woodchucks: presence of viral DNA in tumor tissue from chronic carriers and animals serologically recovered from acute infections. *Hepatology* 1989; **9**: 461-470
- 17 **Menne S**, Cote PJ, Butler SD, Toshkov IA, Gerin JL, Tennant BC. Immunosuppression reactivates viral replication long after resolution of woodchuck hepatitis virus infection. *Hepatology* 2007; **45**: 614-622
- 18 **Wang J**, Gujar SA, Cova L, Michalak TI. Bicistronic woodchuck hepatitis virus core and gamma interferon DNA vaccine can protect from hepatitis but does not elicit sterilizing antiviral immunity. *J Virol* 2007; **81**: 903-916
- 19 **Rehermann B**, Ferrari C, Pasquinelli C, Chisari FV. The hepatitis B virus persists for decades after patients' recovery from acute viral hepatitis despite active maintenance of a cytotoxic T-lymphocyte response. *Nat Med* 1996; **2**: 1104-1108
- 20 **Penna A**, Artini M, Cavalli A, Levrero M, Bertolotti A, Pilli M, Chisari FV, Rehermann B, Del Prete G, Fiaccadori F, Ferrari C. Long-lasting memory T cell responses following self-limited acute hepatitis B. *J Clin Invest* 1996; **98**: 1185-1194
- 21 **Hoofnagle JH**, Seeff LB, Bales ZB, Zimmerman HJ. Type B hepatitis after transfusion with blood containing antibody to hepatitis B core antigen. *N Engl J Med* 1978; **298**: 1379-1383
- 22 **Marusawa H**, Uemoto S, Hijikata M, Ueda Y, Tanaka K, Shimotohno K, Chiba T. Latent hepatitis B virus infection in healthy individuals with antibodies to hepatitis B core antigen. *Hepatology* 2000; **31**: 488-495
- 23 **Gujar SA**, Michalak TI. Primary occult hepadnavirus infection induces both virus-specific and generalized T cell responses in the absence of virus-specific humoral immunity. *Hepatology* 2006; **44**: 536A
- 24 **Michalak TI**, Pasquinelli C, Guilhot S, Chisari FV. Hepatitis B virus persistence after recovery from acute viral hepatitis. *J Clin Invest* 1994; **93**: 230-239
- 25 **Murakami Y**, Minami M, Daimon Y, Okanoue T. Hepatitis B virus DNA in liver, serum, and peripheral blood mononuclear cells after the clearance of serum hepatitis B virus surface

- antigen. *J Med Virol* 2004; **72**: 203-214
- 26 **Lok AS**, Liang RH, Chiu EK, Wong KL, Chan TK, Todd D. Reactivation of hepatitis B virus replication in patients receiving cytotoxic therapy. Report of a prospective study. *Gastroenterology* 1991; **100**: 182-188
 - 27 **Hui CK**, Cheung WW, Zhang HY, Au WY, Yueng YH, Leung AY, Leung N, Luk JM, Lie AK, Kwong YL, Liang R, Lau GK. Kinetics and risk of de novo hepatitis B infection in HBsAg-negative patients undergoing cytotoxic chemotherapy. *Gastroenterology* 2006; **131**: 59-68
 - 28 **Madonia S**, Orlando A, Scimeca D, Olivo M, Rossi F, Cottone M. Occult hepatitis B and infliximab-induced HBV reactivation. *Inflamm Bowel Dis* 2007; **13**: 508-509
 - 29 **Chamorro AJ**, Casado JL, Bellido D, Moreno S. Reactivation of hepatitis B in an HIV-infected patient with antibodies against hepatitis B core antigen as the only serological marker. *Eur J Clin Microbiol Infect Dis* 2005; **24**: 492-494
 - 30 **Lalazar G**, Rund D, Shouval D. Screening, prevention and treatment of viral hepatitis B reactivation in patients with haematological malignancies. *Br J Haematol* 2007; **136**: 699-712
 - 31 **Yuki N**, Nagaoka T, Yamashiro M, Mochizuki K, Kaneko A, Yamamoto K, Omura M, Hikiji K, Kato M. Long-term histologic and virologic outcomes of acute self-limited hepatitis B. *Hepatology* 2003; **37**: 1172-1179
 - 32 **Bläckberg J**, Kidd-Ljunggren K. Occult hepatitis B virus after acute self-limited infection persisting for 30 years without sequence variation. *J Hepatol* 2000; **33**: 992-997
 - 33 **Bréchet C**. Pathogenesis of hepatitis B virus-related hepatocellular carcinoma: old and new paradigms. *Gastroenterology* 2004; **127**: S56-S61
 - 34 **Ikeda K**, Marusawa H, Osaki Y, Nakamura T, Kitajima N, Yamashita Y, Kudo M, Sato T, Chiba T. Antibody to hepatitis B core antigen and risk for hepatitis C-related hepatocellular carcinoma: a prospective study. *Ann Intern Med* 2007; **146**: 649-656
 - 35 **Paterlini P**, Gerken G, Nakajima E, Terre S, D'Errico A, Grigioni W, Nalpas B, Franco D, Wands J, Kew M. Polymerase chain reaction to detect hepatitis B virus DNA and RNA sequences in primary liver cancers from patients negative for hepatitis B surface antigen. *N Engl J Med* 1990; **323**: 80-85
 - 36 **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
 - 37 **Donato F**, Gelatti U, Limina RM, Fattovich G. Southern Europe as an example of interaction between various environmental factors: a systematic review of the epidemiologic evidence. *Oncogene* 2006; **25**: 3756-3770
 - 38 **Huo TI**, Wu JC, Lee PC, Chau GY, Lui WY, Tsay SH, Ting LT, Chang FY, Lee SD. Sero-clearance of hepatitis B surface antigen in chronic carriers does not necessarily imply a good prognosis. *Hepatology* 1998; **28**: 231-236
 - 39 **Yotsuyanagi H**, Hashidume K, Suzuki M, Maeyama S, Takayama T, Uchikoshi T. Role of hepatitis B virus in hepatocarcinogenesis in alcoholics. *Alcohol Clin Exp Res* 2004; **28**: 181S-185S
 - 40 **Chemin I**, Trépo C. Clinical impact of occult HBV infections. *J Clin Virol* 2005; **34** Suppl 1: S15-S21
 - 41 **Pollicino T**, Raffa G, Costantino L, Lisa A, Campello C, Squadrito G, Levrero M, Raimondo G. Molecular and functional analysis of occult hepatitis B virus isolates from patients with hepatocellular carcinoma. *Hepatology* 2007; **45**: 277-285
 - 42 **Knöhl A**, Boehm S, Hahn J, Holler E, Jilg W. Long-term surveillance of haematopoietic stem cell recipients with resolved hepatitis B: high risk of viral reactivation even in a recipient with a vaccinated donor. *J Viral Hepat* 2007; **14**: 478-483
 - 43 **Seth P**, Alrajhi AA, Kagevi I, Chaudhary MA, Colcol E, Sahovic E, Aljurf M, Gyger M. Hepatitis B virus reactivation with clinical flare in allogeneic stem cell transplants with chronic graft-versus-host disease. *Bone Marrow Transplant* 2002; **30**: 189-194
 - 44 **Iwai K**, Tashima M, Itoh M, Okazaki T, Yamamoto K, Ohno H, Marusawa H, Ueda Y, Nakamura T, Chiba T, Uchiyama T. Fulminant hepatitis B following bone marrow transplantation in an HBsAg-negative, HBsAb-positive recipient; reactivation of dormant virus during the immunosuppressive period. *Bone Marrow Transplant* 2000; **25**: 105-108
 - 45 **Liu CJ**, Lo SC, Kao JH, Tseng PT, Lai MY, Ni YH, Yeh SH, Chen PJ, Chen DS. Transmission of occult hepatitis B virus by transfusion to adult and pediatric recipients in Taiwan. *J Hepatol* 2006; **44**: 39-46
 - 46 **Allain JP**. Occult hepatitis B virus infection. *Transfus Clin Biol* 2004; **11**: 18-25

S- Editor Liu Y L- Editor Rippe RA E- Editor Lu W