report showed that the core promoter mutations of A1762T/G1764A do not affect expression of the X gene or impair its stimulatory effect on viral genome replication (Hussain et al., 2009). The effect of the mutant X protein on the viral localization remains unclear and has to be elucidated in the future study.

We also performed HBcAg immunohistochemical examination of the liver tissue samples, and it was confirmed that HBcAg was retained in the hepatocytes of fulminant hepatitis patients, from whom the fulminant HBV strain was isolated. In these samples, HBcAg was observed in the cytoplasm besides the nucleus. The different distribution pattern of HBcAg between in vitro and in vivo might be due to many differences of conditions such as the characteristics of cells and the absence/presence of immune system. Generally, it is thought that HBV-infected hepatocytes are targeted by immune system including T cells (Chisari, 1997), and that the immune response is strongly induced in fulminant hepatitis patients. The retained HBcAg in the cells could induce such immune response. Alternatively, it is speculated that the retained viral proteins might have direct cytopathic effects. Ning and Shih (2004) reported that cells showing the nucleolar localization of HBcAg were often apoptotic, suggesting that the presence of HBcAg in the nucleus may perturb cytokinesis. It was also suggested that the large surface protein or X protein of HBV induced apoptosis (Chirillo et al., 1997; Foo et al., 2002). A strain of HBV that was associated with a fatal outbreak of fulminant hepatitis showed enhanced replication and induced apoptosis in primary Tupaia hepatocytes (Baumert et al., 2005). Interestingly, Sugiyama et al. (2006) showed that the endoplasmic reticulum stress, which was evaluated by the Grp78 promoter activity in genotype A to D HBVs obtained from HBeAg positive patients, was the highest in genotype B2. If the endoplasmic reticulum stress is enhanced further by the retained intracellular viral proteins including HBcAg, apoptosis or inflammation might be promoted, resulting in fulminant hepatitis. Further studies are needed to clarify whether such retention is caused by other fulminant HBV strains.

In conclusion, the fulminant HBV strain that was isolated from consecutive fulminant hepatitis patients retained the core particles and the core particle-associated HBV DNA in the cells. The mutations of A1762T/G1764A, G1862T, and G1896A might work together for the retention. These findings may have important implications for understanding the mechanism leading to fulminant hepatitis.

Materials and methods

Construction of plasmids

Using serum of one of the consecutive patients with fulminant hepatitis B (FH-2) in Japan (Nagasaki et al., 2008), total DNA was extracted with QIAamp DNA Blood Mini Kit (QIAGEN GmbH, Hilden, Germany) and subjected to nested polymerase chain reaction (PCR) for two overlapping fragments; the amplified fragments were nt 1051–3215/1–327 (2492 nt; fragment A) and nt 180–1953 (1774 nt; fragment B). PCR was performed with high fidelity polymerase, PrimeSTAR HS DNA polymerase (TaKaRa Bio, Inc., Shiga, Japan). The amplification products were cloned into pUC118 vectors, and digested with XbaI. The fragments A and B were ligated, and finally, a plasmid containing 1.3-fold HBV genome (nt 1051–3215/1–1953) was constructed and named pBFH2 (Fig. 1A).

QuikChange II-E Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used to introduce nucleotide substitutions into pBFH2. Each mutation found in the core promoter and precore regions, A1762T/G1764A, G1862T, and G1896A (Fig. 1B), was converted into wild-type nucleotides, and to construct plasmids with combined nucleotide substitutions, these converted plasmids were used next as templates. As a result, seven variant constructs were generated from pBFH2, and all constructs were sequenced to confirm the nucleotide substitutions. There were two copies of the core promoter and precore regions in the plasmids, and the mutations in both copies were converted by the site-directed mutagenesis.

Cell culture and transfection

Human hepatoma HepG2 or Huh7 cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% bovine serum at 37 °C and 5% CO_2. For the assay of HBV replication, six-well plates were seeded with 5×10^5 HepG2 or Huh7 cells each. On the next day, 1.5 μg of plasmid DNA was transfected to these cells using TransIT LT-1 Transfection Reagent (Mirus, Madison, WI), and the culture supernatant and cells were collected 3 days later. The transfection efficiency was evaluated by Great EscAPe SEAP Reporter System 3 (Clontech, Mountain View, CA), in which 10 ng/ml of a reporter plasmid expressing secreted alkaline phosphatase (SEAP) was cotransfected. Experiments were performed at least in triplicate.

Detection of intracellular replicative intermediates of HBV

The core particle-associated HBV DNA in the cells was isolated as described previously (Abdelhamed et al., 2002) with slight modifications. Three days after transfection the cells were washed with phosphate-buffered saline (PBS) and lysed in 400 μl of lysis buffer (50 mM Tris–HCl, 1 mM EDTA, 1% Nonidet P-40) per well. The lysed cells were centrifuged at 14,000 rpm for 5 min and the supernatant was collected. To remove unprotected DNA, 10 units of DNase I was added to 160 μl of the supernatant, followed by incubation at 37 °C for 1 h. The reaction was stopped by EDTA, and total DNA was extracted with a QIAamp DNA Blood Mini Kit. After ethanol-precipitation, it was analyzed by Southern blot analysis using a full-length HBV DNA probe labeled with PCR DIG Probe Synthesis Kit (Roche Diagnostics). The signal of HBV DNA was analyzed with the LAS-1000 image analyzer (Fuji Photo Film, Tokyo, Japan) and quantified by densitometry with ImageJ 1.39u (The National Institutes of Health, Bethesda, MD).

Quantification of extracellular HBV DNA, HBsAg, and HBeAg

To digest the input plasmid DNA in the culture supernatant, 5 μl of the supernatant was treated with 5 units of DNase I (TaKaRa Bio, Inc.) at 37 °C for 1 h, and the reaction was stopped with EDTA. Then, total DNA was extracted with a QIAamp DNA Blood Mini Kit, and 10 μl of 200 μl DNA solution was subjected to real-time PCR using a LightCycler system (Roche Diagnostics, Mannheim, Germany) as described previously (Jardi et al., 2001). HBsAg and HBeAg in 50 μl of the culture supernatant were assayed by enzyme-linked immunosorbent assay (ELISA), using an HBsAg ELISA kit (Hope Laboratories, Belmont, CA) and ELISA kit for HBeAg (BioChain Institute, Inc., Hayward CA), respectively.

Confocal fluorescence microscopy

At 48 h post-transfection, the culture slides were washed in PBS and the cells were fixed in ethanol for 10 min at room temperature (RT). After fixation, the cells were washed and incubated in blocking solution, 10% (v/v) goat serum prepared in PBS, for 30 min at RT. The cells were incubated with a diluted (1:500) rabbit polyclonal anti-HBcAg antibody (Dako, Glostrup, Denmark) as the primary antibody for 1 h at RT, washed in PBS, and incubated with Alexa Fluor 546 goat anti-rabbit IgG (Molecular Probes, Eugene, OR) as the second antibody for 1 h at RT. At the same time, the F-actin and nucleus were stained with Alexa Fluor 488 phalloidin (Molecular Probes) and TO-PRO-3 iodide (Molecular Probes), respectively. Images were captured by confocal microscopy (Nikon, Tokyo, Japan) with EZ-C1 software.

Immunohistochemistry

Tissue samples were obtained from three of five consecutive cases of fulminant hepatitis B previously reported by us (Nagasaki et al., 2008). As controls, samples from three acute hepatitis B patients and a chronic hepatitis B patient were evaluated. For negative control, a sample from a nonalcoholic steatohepatitis patient was also used. Each tissue was preserved for routine pathological evaluation using paraffin-embedded samples. For HBcAg immunohistochemical examination, after treatment with antigen retrieval solution (Dako) and quenching endogenous peroxidase activity by methanol–peroxide solution, paraffin-embedded liver sections (2 μm) were incubated with a diluted (1:700) rabbit polyclonal anti-HBcAg antibody (Dako) at 4 °C overnight. After rinsing with PBS, Histofine Simple Stain MAX PO (M) (Nichirei, Tokyo, Japan) was added for 1 h at RT. Nuclear counterstaining was performed using hematoxylin for light microscopy after detecting reactions with VECTOR NovaRED (Vector Laboratories, Inc., Burlingame, CA). These liver specimens were observed with a digitalized light microscope BZ-8000 (Keyence, Osaka, Japan).

Statistical analysis

Statistical analyses were performed using Mann–Whitney U test for comparison of continuous variables between two groups. Differences were considered to be statistically significant when $P<0.05$.

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REVIEW

Prevention of hepatocellular carcinoma complicating chronic hepatitis C

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Abstract

Chronic hepatitis C virus (HCV) infection accounts for most cases of hepatocellular carcinoma (HCC) in Japan and is the second major cause in many other countries. Development of HCC takes a considerable time after onset of HCV infection, between 20–40 years in most cases, and usually develops after cirrhosis is established. Although only a minority of HCV infections reach this stage, the high prevalence of chronic HCV infection in many countries (1–3%) is such that HCC related to HCV infection poses a significant public health issue 20–50 years after the onset of HCV epidemics. Due to advances in testing, and accessibility of clean, disposable medical apparatus including syringes and needles, and particularly screening of donor blood for anti-HCV and by nucleic acid testing, new cases of HCV infection have decreased in most countries, except for continued transmission by injection drug users (IDU). A key difference between HBV and HCV infection is that HCV can be eradicated by effective antiviral treatment. Sustained eradication of HCV reverses hepatic fibrosis, thereby preventing progression to cirrhosis and risk of HCC. Further, it has been well demonstrated that interferon-based antiviral therapy suppresses development of HCC in high-risk patients, particularly when sustained viral response (SVR) is obtained. In summary, the two key approaches to prevent development of HCV-related HCC are primary prevention of HCV infection (adequate programs to screen donor blood, universal precautions to stop medical transmission of blood-borne viruses, curbing transmission by IDU) and potent antiviral therapy of chronic HCV infection.

Introduction

Death from the complications of chronic hepatitis C virus (HCV) infection is a major health threat globally. Although the absolute number of HCV-infected people (~175 million) is less than half that of those infected with hepatitis B virus (HBV), HCV-related liver disease is a leading indication for liver transplantation in Western countries.[1–4] Moreover, the incidence of hepatocellular carcinoma (HCC) due to HCV infection is increasing in several Western countries, being responsible for approximately one-third of HCC cases in the USA,[1,3,4] and also important in Australia.[5] In most countries, chronic hepatitis C is thought to be increasing.[6–8] Conversely, the incidence of HCV-related HCC has been decreasing during the last decade in Japan,[9] following an epidemic first detected in the mid-1980s.[10] Because HCC is the most common cause of death from HCV-related cirrhosis in Japan, and rivals liver failure as a cause of death in other countries,[11,12] it is desirable to establish adequate strategies to prevent HCV infection, to arrest progression of HCV-related liver disease towards cirrhosis, and to devise a screening model for early detection of HCC resulting from HCV infection. This review will focus on the first two aspects; the third will be covered in a review on surveillance for HCC to be published in the Journal as the fifth article in this series.

Natural history of HCV infection

Until the late 1980s, the presence of HCV was inferred by cases of chronic hepatitis not accounted for by hepatitis A (HAV) or HBV infections, but could not be proven by traditional serological laboratory methods. Based on molecular technology, such as polymerase chain reaction (PCR) and cloning methods, molecular evidence of HCV infection was eventually demonstrated by Choo *et al.* in 1988.[13] Thereafter, the majority of so-called non-A and non-B hepatitis cases were proven to result from chronic HCV infection, and the natural history of resultant liver disease, chronic hepatitis C (CHC) was rapidly reported worldwide.[14–17] The Japanese experience is unique in that infected people tended to be older, and that iatrogenic infection such as from contaminated blood transfusion or receiving HCV-contaminated clotting factors was a common source of transmission.[17–19] The involvement of contaminated medical apparatus, such as syringes or needles, is

also thought to be possible, although the actual risk of possible HCV infection remains uncertain.

Due to the long course of CHC, the true natural history of liver disease resulting from chronic HCV infection is difficult to determine. In general, it is believed that 20–30 years are required to develop cirrhosis, although several reports indicate that this differs according to the age at infection, sex, chronic excessive alcohol intake, cigarette smoking, and obesity and insulin resistance.[18,20–29] After developing significant hepatic fibrosis (stage 3 fibrosis or cirrhosis), the incidence of HCC in CHC increases dramatically. In several Japanese series, patients with compensated cirrhosis have a 3–7% annual incidence of HCC, whereas those with only chronic hepatitis have an annual risk of 1%.[30] In Europe, Australia and the USA, the risk of HCC with CHC-cirrhosis appears to be in the range of 1–3%.[1,5,6,31] The cause of death with HCV-associated HCC is often end-stage liver disease (decompensated cirrhosis) rather than from other effects of hepatic malignancy, such as metastases or local complications. Overall, survival after the onset of decompensated cirrhosis is short unless liver transplantation is successfully performed. Accurate estimates of survival after diagnosis of HCC can be difficult to project because preservation of hepatic function significantly affects the selection of treatment options for such patients.

Risk factors

There are several risk factors for developing HCC in HCV-infected individuals (Table 1). These include advanced hepatic fibrosis (including cirrhosis), heavy alcohol use, diabetes mellitus, obesity, low platelet count, elevated alpha-fetoprotein (AFP) level, male sex, older age and increased hepatic iron stores.[8,27,30,32–34] Surveillance for HCC should be considered for HCV-infected individuals who have any of these risk factors, particularly those with advanced fibrotic disease who have not responded to antiviral therapy or are not suitable for such therapy. A recent Japanese study revealed that in people aged 70 years or older, sex is no a longer significant risk factor for the development of HCC.[35] HCC surveillance modalities and organization of a screening protocol are influenced by the social health-care system; details will be described in the review written by Amarapurkar and other participants of this working party to be published soon in the Journal.

Table 1 Possible risk factors for developing HCC in HCV infected individuals

Risk factor
Male sex
Presence of cirrhosis or advanced fibrosis
Elderly populations
Concomitant HBV or HIV infection
Presence of obesity
Excess of alcohol consumption

HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus.

Approaches for prevention of HCC caused by HCV infection (Table 2)

Prevention of HCV infection

Hepatitis C virus is transmitted through contaminated blood or blood products, either in the health-care setting or by injection drug users (IDU). Prevention in the health-care setting can be achieved by screening donor blood and application of universal precautions to prevent nosocomial blood-borne infections. Introduction of screening blood donors for anti-HCV antibodies has significantly reduced the incidence of transfusion-related HCV infection. Besides this, the wide-spread adoption of disposable medical devices, together with avoidance of multi-use vials for injectables ("universal precautions") appears to have contributed to reducing the incidence of medically transmitted HCV infection.[36] However, de novo infections still occur (estimated ~10 000/year in Australia alone) among IDU,[5] while tattooing and piercing by non-sterile practices (typically unlicensed premises) are possible routes of HCV infection.[1,5,37–40] Although such possible sources of transmission have been reported, adequate sterilization and proper handling of apparatus could further reduce the risk of HCV infection. In the USA, Australia and New Zealand, IDU are responsible for more than 90% of new HCV infections, and IDU have become an important mode of spread in many parts of Asia.[1,8,36,38,41–47] Avoiding sharing of needles and blood-contaminated syringes and use of communal articles to prepare injectables are important to prevent HCV transmission by IDU.

Table 2 Levels of prevention against HCV related hepatocellular carcinoma

Classification	Description	Example
Primary prevention	A. Prevention of HCV infection	Screen donor blood for HCV. Universal precautions to prevent blood contamination in health-care settings Educate IDU for possible transmission of HCV
	B. Measures to slow progression to cirrhosis, and alter susceptibility to HCC with liver diseases	Anti-viral treatment for HCV
Secondary prevention	Measure to prevent tumor recurrence after curative treatment	Anti-viral therapy Chemo-prevention (retinoids?) Iodine-131-labeled lipiodol trans-arterial chemoembolization
Tertiary prevention	Early detection to improve treatment outcomes	HCC screening (hepatic ultrasonography, alpha-fetoprotein and other serological markers)

HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IDU, injection drug users.

Table 3 Results of randomized controlled trials of interferon treatment on incidence of HCC among cirrhotic patients with HCV infection (adapted from Farrell and Fan[86])

Author (year)	*n*	Mean age (year)	Mean follow up (year)	*n* (%) with HCC			Other clinical outcomes
				Treatment group	Control group	*P*-value	
Nishiguchi (1995, 2001)[64,65]	90	56	4.5	2/45 (2%)	17/45 (38%)	<0.05	Improved survival
Mazzella (1996)[71]	284	53	2.7	5/193 (2.6%)	9/92 (9.8%)	<0.05	Slowed disease progression
Valla (1999)	99	57	3.1	5/47 (11%)	9/52 (17%)	>0.05	No improvement
Bernandinello (1999)	61	No data	5	2/38 (5.3%)	1/23 (4.3%)	>0.05	No improvement
Azzaroli (2004)[69]	60	56	5	0/30 (0)	9/30 (30%)	<0.05	Improved survival
Soga (2005)[70]	133	No data	5	5/103 (4.9%)	7/30 (23.3%)	<0.05	Not documented

HCC, hepatocellular carcinoma; HCV, hepatitis C virus.

Concomitant HIV infection is also frequent among HCV-infected IDU, ranging from less than 2% in Australia to more than 50% in South China.[41] The lower frequency in Australia is attributable to the lower prevalence of HIV in Australia in the early 1980s, when the incidence of new HCV infections was rising appreciably, and the introduction of public health measures that included "safe sex" messages and disposable needle and syringe programs.[41,42] Prevention of HCV infection by IDU is partly thwarted by sociopolitical factors that prevent more widespread introduction of "safe using" or other "harm reduction" strategies for those who use illicit drugs.[1,41–44,46,48] Attempts to prevent IDU by educational measures and public awareness campaigns have not been conspicuously successful.[49] On the other hand, community-based needle and syringe exchange programs in countries like Australia, while demonstrably preventing HIV, HBV and hepatitis D virus (HDV) infections, have had little impact on the incidence of new HCV infections.[50] The main reason for this is thought to be the high prevalence rate in the target subpopulation (those who use drugs) at the time of introduction of such programs, and other breeches of "safe injecting techniques" with the chaotic nature of IDU, particularly at inception. Moreover, it should be noted that the reported natural history as well as the risk for developing HCC is higher in HIV co-infection compared to HCV mono-infection.[51–53] Thus, the prevention of infection among IDU is important.

Eradication of viral infection by antiviral therapy

The optimal outcome of treatment against infectious diseases is permanent eradication of the infectious agent from the host body. Such eradication of HCV from human liver is possible, which differs from chronic HBV infection. In HBV infection, even after complete clinical recovery from the acute infection or with loss of hepatitis B surface antigen (HBsAg) during chronic infection, viral genome (covalently-closed circular HBV DNA) is frequently detected in the host liver. It has been proposed that such residual "occult HBV infection" comprises one of the reasons for HCC in non-B and non-C cases.[54,55] Spontaneous (natural) eradication of HCV in chronic infection is believed to be a rare phenomenon. However, different from HBV cases, permanent viral eradication is possible in HCV infection. It can be observed after acute HCV infection, with spontaneous recovery in 15–50% of cases (depending on age, genotype and vigor of initial hepatitis), or after successful interferon (IFN)-based antiviral treatment of acute or chronic HCV infection.

Sustained antiviral response (SVR) with hepatitis C treatment is equivalent to viral eradication in more than 99% of cases.[56] SVR is particularly likely with an IFN in combination with ribavirin.[56,57] Such treatment-induced eradication of HCV infection is associated with resolution of hepatitis and liver fibrosis, and prevention of hepatic decompensation.[58] Following successful antiviral therapy of chronic HCV infection, a lower incidence of developing HCC has been reported, irrespective of whether the disease stage is at the non-cirrhotic stages of chronic hepatitis, or with cirrhosis (Table 3).[59–71] Until the recent introduction of pegylated (PEG)-IFN and ribavirin combination therapy, a problem has been that the rate of SVR has been substantially lower in those with cirrhosis, among whom the incidence of developing HCC is substantially higher.[8,72–76] Accordingly, treatment of chronic HCV infection in its earlier stage is likely to produce the most favorable results for the prevention of HCC.

Recent treatment guidelines recommend the introduction of anti-viral therapy in HCV infection genotype 2 and 3, even with normal serum transaminase levels.[45,77–80] Because adverse effects from IFN and ribavirin are more severe in elderly people, treatment with either PEG-IFN/ribavirin or IFN/ribavirin (where cost of PEG-IFN is prohibitive) tends to be employed in patients aged 60 years or less.

Viral suppression

Even with the present standard-of-care, PEG-IFN plus ribavirin, therapy fails to eradicate the virus from the host liver in at least half of cases with genotype 1b HCV infection. In these cases, an improvement (normalization) of serum alanine aminotransferase (ALT) levels, reflecting decreased hepatitis activity, has been associated with lower incidence of HCC (Table 4).[64,66,67] Although any prolonged effects are yet to be established, these data have given rise to the suggestion to use IFN as maintenance therapy for suppressing the incidence of HCC in cases of chronic HCV infection in which there is failure to achieve SVR. However, several large studies using IFN (or PEG-IFN) as a possible agent to prevent development of HCC are currently underway or recently completed,[74,81,82] and all have failed to prove any preventive effect. It is noted that authors have observed that the period of observation

Table 4 Effect of viral response to IFN based treatment on incidence of HCC among patients with chronic hepatitis C (adapted from Farrell and Fan[86])

Author (year)	*n*	Mean follow up (year)	*n* (%) with HCC			
			Sustained viral response	Relapser	Non-responder	Non-treated
Imai (1998)[63]	419	4	1/151 (0.7%)	1/120 (5.8%)	20/148 (14%)	19/144 (12%)
Kasahara (1998)[75]	1022	7	5/313 (1.6%)	9/304 (3%)	32/405 (7.9%)	Not documented
Okanoue (1999)[66]	1148	2.7	3/316 (1%)	8/264 (3%)	41/568 (7%)	Not documented
Yu (2005)[76]	214	6	1/87 (1.1%)	Not documented	12/113 (11%)	Not documented
Hung (2004)[62]	132	3	5/73 (6.8%)	Not documented	12/113 (11)	Not documented
Yu (2006)[68]	1057	5	12/715 (1.7%)	Not documented	39/342 (11%)	54/562 (9.6%)

HCC, hepatocellular carcinoma; IFN, interferon.

in these studies may have been insufficient to find statistically significant differences, but no enticing trends have been observed either.

A pivotal concept in viral-induced hepatocarcinogenesis is that inflammation and fibrosis are both related to progression of chronic hepatitis to HCC. If this is the case, suppression of inflammation could reduce the incidence of HCC. Only limited data are available for evaluating the efficacy of maintenance therapies, designed to suppress activities of hepatitis for prevention of HCC. The recent study by Di Bisceglie *et al.* failed to demonstrate the favorable effects of maintenance therapy for preventing the development of HCC.[83] A design problem with these studies is that the long natural history of hepatitis C and the availability of potential curative therapy means that performance of a controlled study over a sufficiently long observation period would be extremely difficult. Use of ursodeoxycholic acid (UDCA) or glycyrrhizin (extract of a saponin component contained in the roots of the licorice plant) has been associated with an improvement of the serum ALT.[84] Phlebotomy, in an attempt to abrogate oxidative stress by returning hepatic iron stores to normal, has also been reported to improve biochemical tests in chronic hepatitis C, and was associated with improvement of liver histology.[85] Although the conceptual background for this approach is based on the fact that experimental HCC can be induced by excessive hepatic oxidative stress, any effect of phlebotomy on prevention of HCC among patients with chronic HCV infection is not yet established.

Concluding remarks

There is strong evidence that the incidence of HCC in patients with chronic HCV infection can be reduced in those who achieve SVR with anti-viral therapy, irrespective of the presence of cirrhosis. Among those without cirrhosis, SVR reduces the risk of developing cirrhosis and, ultimately, HCC. Although some expert opinion is in favor of maintenance IFN-based therapy for chronic hepatitis C, definitive evidence is lacking. Moreover, recent study did not support this hypothesis. Thus, we do not therefore recommend maintenance antiviral therapy until efficacy of this expensive and demanding form of therapy has been shown in global, large scale studies. Meanwhile, strong trends in reduction of new HCV infections from medical transmission are encouraging. It is now time to acknowledge rather than deny the importance of social factors, specifically IDU, in the "new wave" of HCV infections in Asia, and to work towards ways to counter this.

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HCC PREVENTION MINISERIES

Application of surveillance programs for hepatocellular carcinoma in the Asia–Pacific Region

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Abstract

Hepatocellular carcinoma (HCC) is a potential target for cancer surveillance (or screening) as it occurs in well-defined, at-risk populations and curative therapy is possible only for small tumors. Surveillance has been recommended by regional liver societies and is practiced widely, but its benefits are not clearly established. Hepatic ultrasonography with or without alpha fetoprotein (AFP) performed every 6 months is the preferred program. Surveillance of HCC has been well shown to detect small tumors for curative treatment, which may be translated to improved patient survival. However, most studies are limited by lead-time bias, length bias for early diagnosis of small HCC, different tumor growth rates and poor compliance with surveillance. Cost-effectiveness of surveillance programs depends on the rate of small HCC detected 'accidentally' (routine imaging) in a comparator group, annual incidence of HCC with various etiologies, patient age and the availability of liver transplantation. The incremental cost-effectiveness for 6-monthly AFP and ultrasound has been estimated from approximately $US26 000–74 000/quality adjusted life years (QALY). All cirrhotic patients are therefore recommended for HCC surveillance unless the disease is too advanced for any curative treatment. As chronic hepatitis B can develop into HCC without going through liver cirrhosis, high-risk non-cirrhotic chronic hepatitis B patients are also recommended for HCC surveillance. In conclusion, HCC surveillance could be effective at reducing disease-specific mortality with acceptable cost-effectiveness among selected patient groups, provided it is a well-organized program.

Introduction

In spite of significant improvements in diagnostic and therapeutic modalities, the prognosis of most gastrointestinal cancers remains poor, particularly because most patients present at very advanced stages of the disease. Several population-based mass screening and surveillance programs aimed at early detection and treatment (Barrett's esophagus, atrophic gastritis, gastric remnant, ulcerative colitis) are considered to be cost-ineffective, even though survival benefit attributable to the screening program has been documented.[1] Whether surveillance of hepatocellular carcinoma (HCC) is effective in improving survival and cost-effective as a public health measure has been a topic of debate for decades.

Hepatocellular carcinoma is a common cancer worldwide and a major public health problem in the Asia–Pacific region.[2,3] HCC ranks as the fifth most common cancer worldwide, and the third highest cause of cancer mortality. The highest mortality rates from HCC are reported from Southeast Asia and sub-Saharan Africa. The incidence of HCC is increasing in the USA and Australia, although the high rates of HCC in most Asia–Pacific countries appear to have reached a plateau.[3] The majority of such HCC develop in patients with cirrhosis, which is most often attributable to chronic hepatitis B virus (HBV) infection followed by chronic hepatitis C virus (HCV) infection in the Asia–Pacific region.[4] HBV-related cirrhosis is the main cause of HCC in the Asia–Pacific region except Japan where HCC is predominantly due to HCV infection.[2–7] HBV-related HCC can develop without the presence of underlying liver cirrhosis (~20% cases) whereas HCV-related HCC almost always occurs with cirrhosis. In older Japanese subjects, it remains a male-predominant disease with 2 : 1 male to female ratio for chronic HCV infection.[5] The annual incidence of HCC varies from 2–6% in HBV-related cirrhosis and 1.5–5% in HCV-related cirrhosis.

Natural history of HCC

The main objective of surveillance protocol is early detection of presymptomatic disease. Natural history of any cancer is a sequential multistep process with well-defined biological stages. The first stage is biological onset when the diseases are present but not detectable. In the second stage, the disease is giving rise to functional and structural changes but the patient is still asymptomatic. Availability of a proper test may be able diagnose disease in the early stage. In the third stage, the patient becomes symptomatic. The critical point for surveillance during disease progression is when treatment is either more effective or easier to apply than afterwards. The ideal critical point lies between the earliest possible time of diagnosis and usual time of clinical diagnosis. New molecular techniques may be able to identify biological onset of disease but surveillance strategies may not be feasible at this point.[1] Interpretation of surveillance protocol should be evaluated carefully for lead time bias and length time bias. Lead time bias is the apparent improved survival that comes from the diagnosis being made earlier in the course of a disease than diagnosed because of the development of symptoms. Unless lead time bias is properly controlled, studies of surveillance will show enhanced survival simply because the cancer is a diagnosed at an earlier stage. What we know about the natural history of HCC has been derived from clinically symptomatic patients and this may be just the tip of the iceberg. Cancer cannot be considered as an event but a process that extends over decades. Length bias is the apparent improvement in survival that occurs because surveillance preferentially detects slow growing cancers. More rapidly growing cancers may grow too large to be treated between visits. Survival benefits can therefore be attributable to the protocol itself even when therapy is worthless. It is well known that tumors with a long preclinical phase tend to have a long clinical phase as well. HCC is a unique cancer as the majority of the tumors develop on the background of liver cirrhosis. Management of HCC has to be considered in the context of liver cirrhosis and liver function. Surgical and loco-regional therapy may be adequate for patients with good liver reserve. For patients with advanced liver cirrhosis, liver transplantation is the best option but it is expensive and may not be available to the majority of patients.[2,8]

Application of surveillance programs for HCC

Hepatocellular carcinoma is a potentially viable target for surveillance as it occurs in well-defined risk populations (cirrhosis is a primary risk factor). HCC has a protracted subclinical phase. More than 20% of patients with cirrhosis may develop HCC over a period of 10 years. During the subclinical phase, there are no symptoms and the prognosis is improved if HCC can be diagnosed early. Prognosis of large HCC is dismal. Hence, HCC picked up by surveillance programs can be treated early and cure is possible.[6,9–13] Surveillance of HCC has been recommended by various organizations like the American Association For Study of Liver Disease (AASLD) and the Asia–Pacific Association For Study of Liver (APASL).[2,14] The recent consensus statement of the National Institute of Health on the management of hepatitis B has concluded that the balance of benefits and harms associated with screening for HCC is unknown and it is an area of future research.[15]

Surveillance has been practiced widely by gastroenterologists and hepatologists all over the world and it has become a standard practice even though evidence on its benefits has not been clearly established.[8,16] One study attempted to conduct a randomized controlled trial of surveillance for HCC, but more than 80% of the informed patients declined to participate because they preferred regular ultrasound examination than to be randomized to surveillance versus no surveillance.[17] Usefulness of a surveillance program for early diagnosis of HCC in clinical practice has been shown in several studies.[18–21] Two large population-based studies have demonstrated survival benefit of a surveillance program in chronic hepatitis B patients.[22,23] In the Alaskan study, 6-monthly determinations of alpha fetoprotein (AFP) in HBV-infected patients has led to the identification of curable HCC in 40% of patients.[22] A large-scale randomized control trial from Shanghai using abdominal ultrasound and serum AFP every 6 months in 18 816 patients aged 35–59 years with chronic hepatitis B and other risk factors for HCC showed a reduction in mortality by 37%.[23] One limitation of these studies is the unknown percentage of patients with liver cirrhosis. In the Shanghai study, liver transplantation was not available as a treatment option and compliance to surveillance was suboptimal.[23] In clinic-based surveillance studies, liver-specific mortality rates were reduced in cirrhotic patients with HCC detected during surveillance.[24,25] This was probably due to both early HCC detection and improvement in

treatment. Lead time bias and length time bias cannot be excluded in these studies. In five retrospective studies including more than 100 patients comparing HCC detected on surveillance versus those detected without surveillance, small and potentially curable HCC were detected in the surveillance groups leading to improved patient survival.[26–29] But surveillance program did not improve prognosis in patients with advanced cirrhosis. In the surveillance groups, HCC detected are usually small and relatively uniform. HCC in the non-surveillance groups are large and varied. HCC in the non-surveillance groups are usually detected in two different manners: (i) asymptomatic but discovered outside the regular surveillance program; and (ii) symptomatic. HCC detected without symptoms in the non-surveillance group are also smaller as compared to the symptomatic group. The efficacy of the surveillance program reported will be decided on number of asymptomatic patients detected incidentally in the non-surveillance group.[30] The benefits of HCC surveillance are summarized in Table 1.

Surveillance strategies for HCC

Screening tests should have an acceptable rate of accuracy and should be affordable. The screening strategy recommended for surveillance by the majority of associations is ultrasonography with or without AFP measurement. The sensitivity and specificity of AFP for HCC is in the range of 41–65% and 80%–95%, respectively, when an AFP cut-off of 20 ng/mL is used.[19] Up to 50% of patients with HCC have AFP lower than 20 ng/mL.[20] Therefore, AFP cannot be used as the sole tool for HCC surveillance. Furthermore, it is associated with significant false-positive results related to hepatitic activities. In other words, unnecessary additional diagnostic investigations and patient anxiety may be caused by a mildly elevated AFP. AFP values greater than 400 ng/mL are more diagnostic of HCC, but only very few HCC are associated with such high AFP values at screening. Very high AFP levels are usually found in patients with massive and advanced HCC.[20]

Ultrasound is the most popular imaging method for HCC surveillance because it is simple, inexpensive, and non-invasive and allows a real-time observation. In a systematic review including 14 studies of various designs from different patient groups, the pool estimates (95% confidence interval) of sensitivity, specificity, positive likelihood ratio and negative likelihood ratio of ultrasonography (USG) to detect HCC are 60.5% (44–76%), 96.9% (95–98%), 17.7 (8.5–36.9) and 0.5 (0.4–0.6), respectively.[31] The performance of ultrasound depends on the expertise of the operator, the ultrasound equipment available and the echo-texture of the liver. The evaluation of the actual sensitivity of ultrasound is however difficult due to the lack of a definite good standard for HCC.

Combined AFP and ultrasound can increase the HCC detection rates, but it also increases the costs and the false-positive rate from 2.9% (ultrasound alone) or 5.0% (AFP alone) to 7.5% (combined).[21] Owing to the limitations of AFP and ultrasound, it is a common practice to combine these two methods for HCC surveillance. Several studies using a combined AFP and ultrasound surveillance have proven survival benefit to patients by detecting smaller and curable HCC.[22,23,30,32,33] In general, 6-monthly AFP and ultrasound surveillance is sufficient. In a longitudinal study including 1018 chronic hepatitis B patients followed up for over 4 years, there was no additional survival benefit for an intensive surveillance program including lipiodal computed tomography over a

Table 1 Survival benefit of surveillance programs for hepatocellular carcinoma (HCC) in retrospective studies (HCC)

Author reference	Location	Patient population	HCC in surveillance group (*n*)	HCC in no surveillance group (*n*)	Survival in surveillance group	Survival in no surveillance group
Chie *et al.*[25]	Taiwan	Mixed	634	156	5-year survival 35%	5-year survival 29%
Yuen *et al.*[26]	Hong Kong	Mixed	142	164	Median survival 22 months	Median survival 5 months
Trevisani *et al.*[27]	Italy	Mixed	215 (6-month surveillance) 155 (12-month surveillance)	451	Median survival 36 months (6-month surveillance) and 34 months (12-month surveillance)	Median survival 14 months
Trevisani *et al.*[28]	Italy	Mixed	158	138 (incidental) 67 (symptomatic)	Median survival 30 months	Median survival 21 months (incidental) and 7 months (symptomatic)
Wong *et al.*[29]	Hong Kong	Mixed	79	393	Median survival 22 months; 2-year survival 49%	Median survival 6.5 months; 2-year survival 21%

HBV, hepatitis B virus; HCV, hepatitis C virus; NA, not available.

Table 2 Cost-effectiveness models for hepatocellular carcinoma (HCC) surveillance

Author reference	Target population	Treatment of HCC	ICER $/QALY vs no screening (screening strategy)
Sarasin *et al.*[39]	55-year-old Child's grade A cirrhosis	Resection	26 000–284 000 (6-monthly US + AFP) 24 000–240 000 (6-monthly US)
Arguedas *et al.*[40]	> 50-year-old hepatitis C cirrhosis	Resection, loco-ablative therapy, transplant	26 689 (6-monthly US + AFP) 16 605 (6-monthly CT + AFP) 118 000 (6-monthly MRI + AFP)
Saab *et al.*[41]	Patients awaiting transplant	Loco-ablative therapy, transplant	60 300 (6-monthly US) 74 000 (6-monthly US + AFP) 110 000 (6-monthly CT)
Lin *et al.*[42]	> 40-year-old hepatitis C related Child's grade A cirrhosis	Resection, loco-ablative therapy	23 043 (12-monthly US + AFP) 33 083 (12-monthly US, 6-monthly AFP) 73 789 (6-monthly US + AFP)
Patel *et al.*[43]	45–70-year-old hepatitis C cirrhosis	Resection, transplant	26 100–58 400 (6-monthly US + AFP)
Nouso *et al.*[44]	> 45-year-old Child's A cirrhosis	Resection, loco-ablative therapy, transplant	29 900–68 800 (6-monthly US)

AFP, alpha-fetoprotein; CT, computed tomography; ICER, incremental cost-effective ratio; MRI, magnetic resonance imaging; QALY, quality adjusted life years; US, Ultrasound.

regular 6-monthly combined AFP and ultrasound surveillance program even among patients with elevated AFP (> 20 ng/mL) or abnormal ultrasound findings.[34] There is no evidence to support the use of computed tomography (CT) for routine HCC surveillance as positive and negative predictive values are unknown. There is a significant cost and radiation exposure associated with four phase-contrast CT while there may be high false-positive rates in the absence of contrast CT. The other serological tests like AFP L3 and Des-γ carboxyprothrombin (DCP) have not shown to be useful for surveillance protocol.[35]

Cost-effectiveness of surveillance strategies

The current definition of worthwhile surveillance is based on the economic situation in 1992, and surveillance is defined cost-effective if it achieves a 3-month improvement in survival at a cost of less than $US50 000/life year saved.[36,37] Efficacy of surveillance program can be determined by randomized control trials but cost-effectiveness is generally determined by modeling study. Modeling studies are based on several assumptions and may not hold true in different geographical areas, economic status and health-care delivery systems in different countries. The cost-effectiveness of a surveillance program depends critically on the rate of small HCC detected accidentally in the non-surveillance group, annual incidence of HCC in various etiologies of HCC and the availability of liver transplantation as a treatment option. The aim of HCC surveillance is to detect small tumors that allow curative treatment. In an early cost-effective analysis, HCC surveillance by yearly USG and AFP could detect an early tumor at a cost of $US11 800.[38] There are six subsequent cost-effective analyses of the surveillance program based on the computerized decision analytical models using various training tools like ultrasound, CT scan, AFP at 6-monthly or yearly intervals, treatments offered like resection or liver transplantation with reference to life saved or quality adjusted life years (QALY) (Table 2).[39–44] The incremental cost-effectiveness ratio for 6-monthly AFP and ultrasound varied between approximately $US26 000 to $US74 000/QALY. Surveillance can be effective in reducing disease-specific mortality with acceptable cost-effectiveness among selected groups of patients. The cost-effectiveness depends on the: (i) rate of incidentally detected small HCC; (ii) annual incidence of HCC; (iii) adoption of transplant as a treatment strategy; and (iv) younger age of screen population. The majority of the earlier studies were conducted before liver transplantation was considered as an effective treatment for early HCC. Long-term survival improves in patients undergoing liver transplantation for HCC as liver transplantation is not only taking care of the HCC but also the underlying cirrhosis. However, whether it is a cost-effective strategy to treat HCC with liver transplantation warrants further investigation due to the high cost of the surgery.

Surveillance strategies according to the etiology and risk factors

According to randomized controlled trials, the risk of HCC in chronic hepatitis B is 0.27%/year and HCC surveillance is cost-effective.[22] In non-HBV-related cirrhosis, the risk of HCC is more than 1.4%/year and HCC surveillance is also cost-effective.[40]

HBV

Increasing evidence suggests that persistent HBV replication as indicated by high serum HBV DNA is a predictor of HCC. A large-scaled cohort study in Taiwan (REVEAL-HBV study) has recently shown that there was a biological gradient of risk for development of HCC in subsequent years based on the viral load at the first visit.[45] This study involved more than 3700 subjects aged 30–64 years and followed over more than 10 years. Subjects who had an initial viral load below 2000 IU/mL had the lowest risk of HCC and subjects who had HBV DNA exceeding 20 000 IU/mL started to have an increased risk of HCC. A subsequent study overcame the limitation of the previous one that the risk of HCC had been reflected by one-time measurement of serum HBV DNA at the start. In a cohort of 112 patients with HBV-

Table 3 Recommended at risk population for hepatocellular carcinoma (HCC) surveillance

Chronic hepatitis B
All patients with liver cirrhosis For non-cirrhotic patients
Male above age of 40 years and female above age of 50 years
Family history of HCC
High serum hepatitis B virus DNA (> 10 000 copies/mL)
Factors under investigation
Hepatitis B virus genotype C
Basal core promoter mutations
Chronic hepatitis C
All patients with liver cirrhosis, especially aged above 40 years
Special caution to patients with other risk factors such as alcoholism and co-infection with hepatitis B virus or human immunodeficiency virus
Other liver diseases
All patients with liver cirrhosis (evidence not certain)

related HCC and 1031 non-HCC subjects, pre-diagnostic multiple sera were collected for HBV DNA measurement over periods of up to 16 years. Multivariate analysis showed that persistently high viral load (detected HBV DNA at ≥ 50% of the visits) was associated with higher rate of HCC development.[46] Despite some controversies in cross-sectional and case-controlled studies, HBV genotype C, particularly subgenotype Ce, HBV has been found to be an independent risk factor for HCC development in several longitudinal studies.[47–49] This observation may be related to the more aggressive disease, delayed hepatitis e antigen (HBeAg) seroconversion and high prevalence of basal core promoter mutations associated with genotype C HBV than genotype B HBV.[50,51]

Older age is another independent predictive factor for HCC development. Regarding sex effect on HCC, male sex has been consistently reported to be more susceptible than female.[31,48–52] A meta-analysis of 17 case-control and three cohort studies reported a direct trend in risk with increasing alcohol consumption.[52] In a Korean 9-year prospective study, liver cirrhosis, chronic hepatitis, HCV infection, HBV infection and age exceeding 40 years were all independently associated with the risk of HCC development.[52,53]

Therefore, in HBV-infected patients, appropriate cases for surveillance are Asian men more than 40 years and women more than 50 years (for Africans, > 20 years). Patients with cirrhosis and patients with family history of HCC mainly among Asians and Africans are appropriate candidates for surveillance. As non-cirrhotic patients with chronic hepatitis B remain at risk for HCC,[54–57] AASLD recommends surveillance for HCC among not only HBV-related cirrhotic patients but also those at an older age, with family history of HCC, or with high serum HBV DNA levels (Table 3).[2] Whether HBV genotypes and basal core promoter mutation should be considered in the HCC surveillance program remains to be studied.

HCV and other liver diseases

The HCC derived from chronic HCV infection are responsible for the majority of HCC cases in the USA, Europe and Japan.[58–61] The HCC in chronic HCV-infected patients usually have pre-existing advanced fibrosis in the liver. The reported risk factors in chronic hepatitis C-related HCC include male sex, elderly population, alcohol intake and excessive oxidative stress.[62–70] However, in Japan where the estimated life expectancy exceeds 80 years, the incidence of HCC in female patients catches up with that of male patients in the population older than 70 years old.[71] Thus, strong caution should be taken toward HCV-infected people in this generation regardless of sex. This is important because Japanese patients with HCV infections seems to be 10–15 years older than the rest of the world, thus the Japanese experience will be reproduced again 10–15 years later. The sophisticated molecular technique demonstrated when and how the HCV infections have been spread throughout the world.[72–75]

The evaluation of significant hepatic fibrosis is not uniformly agreed by hepatologists. Although liver biopsy is the gold standard to evaluate hepatic fibrosis, its invasive nature abstains hepatologists to perform it as a routine procedure for all patients with HCV infection. Instead of liver biopsy, other assessment tools for evaluating hepatic fibrosis are developed and applied to HCV infections, such as simple platelet counts, Fibrotest, aspartate transaminases to platelet ratio index (APRI), or most recently elastography (Fibroscan). The accuracy of these methods are not systematically compared, thus large-scale studies are warranted to establish the value of these methods in estimating the risk of developing HCC in chronic hepatitis C.

In all, the rational for conducting a surveillance program towards chronic HCV infected patients is obvious, because these people are at high risk for developing HCC (especially at elderly age with advanced fibrosis) and easy to identify (e.g anti-HCV antibody positive) (Table 3).[76] In patients with hepatitis C-related cirrhosis, surveillance is effective. But in other forms of cirrhosis like alcoholic cirrhosis, non-alcoholic steatohepatitis-related cirrhosis, autoimmune hepatitis or cryptogenic cirrhosis, the efficacy of surveillance remains unproven.[76]

Conclusions

In the Asia–Pacific region, HCC is a major cause of morbidity and mortality. HCC surveillance can detect early tumors that are potentially amendable to curative treatment. Six-monthly USG examination with and without AFP seems to be the most optimal and cost-effective measure for HCC surveillance. All patients at risk of developing HCC with potential curative treatment available are recommended for regular HCC surveillance. Patients with early liver cirrhosis (or non-cirrhotic HBV-infected patients) are the best candidates for HCC surveillance as hepatic resection or loco-regional therapy can be applied once early HCC is detected. Among patients with advanced liver cirrhosis, HCC surveillance should still be carried out as far as liver transplantation remains a treatment option. On the other hand, HCC surveillance should not be conducted among patients with advanced liver cirrhosis but not being a liver transplant candidate for whatever reasons. For a HCC surveillance program to be effective, an increased awareness of HBV and HCV infection is mandatory as they are the most important etiologies of HCC in the Asia–Pacific region.

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