PEG IFN-α-2b, while 29 patients completed treatment with reduced dosages of RBV. Nine patients withdrew from the trial.

Time at which HCV-RNA became undetectable

Figure 2 shows the numbers of patients who achieved RVR, EVR, LVR and NR. Patients with RVR, EVR and LVR accounted for 9.8%, 50.0% and 18.3%, respectively. Patients with NR accounted for 22.0%. Patients with EVR accounted for half of all patients.

Response to therapy

Overall SVR rate for the total patient group was 57.3%. SVR rates for RVR, EVR and LVR patients were 100% (8/8), 80.5% (33/41) and 40.0% (6/15), respectively. Numbers of patients with SVR according to the time at which HCV-RNA became undetectable are shown in Figure 3.

Prediction of SVR and NR at baseline

Univariate analysis identified four parameters adversely influencing SVR: platelet count; HCV-RNA, mutant-type amino acid substitution at ISDR; and age. Multivariate analysis using the above variables identified two parameters independently influencing SVR: platelet count and mutant-type amino acid substitution at ISDR (Table 2).

Univariate analysis identified one parameter influencing NR: amino acid substitution at aa71 and aa90 in the core region. Multivariate analysis using this variable identified the same parameter as independently influencing NR (Table 3).

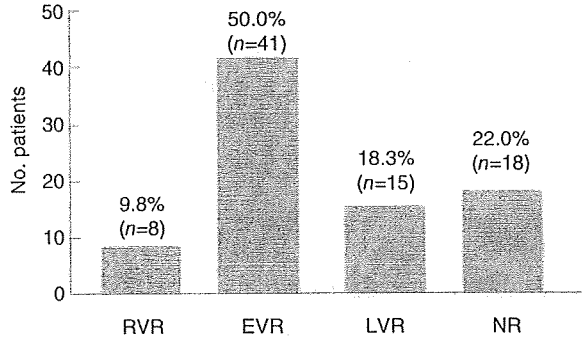

Figure 2 Time at which hepatitis C virus ribonucleic acid became undetectable. EVR, early viral response; LVR, late viral response; RVR, rapid viral response; NR, non-viral response.

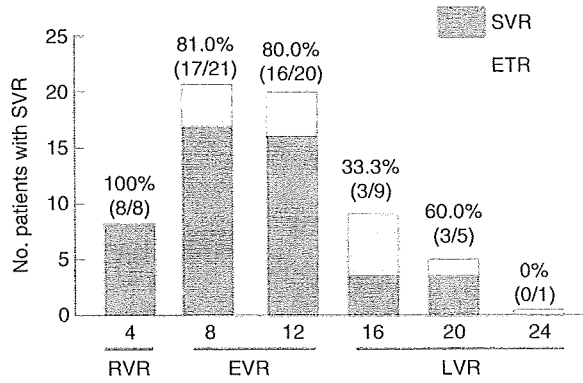

Figure 3 Number of patients with sustained viral response (SVR) and end-of-treatment reponse (ETR) according to the time when hepatitis C virus ribonucleic acid (HCV-RNA) became undetectable. EVR, early viral response; LVR, late viral response; RVR, rapid viral response; NR, non-viral response.

Prediction of SVR for patients with undetectable HCV-RNA by 24 weeks after commencement of therapy

In 64 patients, HCV-RNA was undetectable by 24 weeks after initiation of therapy. Of these, 17 patients experienced relapse (26.6%). Univariate analysis identified three parameters influencing SVR: platelet count, age and HCV-RNA. Multivariate analysis using these variables identified two parameters independently influencing SVR: age and HCV-RNA (Table 4).

Evaluation of predictive factors for SVR according to each treatment duration

We evaluated predictive factors for SVR according to each of the treatment durations by uni- and multivariate analysis. No parameters were significant in all treatment duration.

DISCUSSION

TREATMENT DURATION WAS based on the time at which HCV-RNA became undetectable under PEG IFN plus RBV therapy for genotype 1b and high viral load CHC patients. SVR rate of our response guided therapy was also investigated.

It is presently accepted that treatment duration of PEG IFN plus RBV therapy for LVR patients can be extended from 48 weeks to 72 weeks.[6] In RVR patients, standard 48-week therapy reached nearly 100% SVR rate.[13] EVR patients also achieved a high SVR rate by 48-week

Table 2 Predicting factors of sustained viral response by multivariate analysis

Factor	Category	Odds ratio (95% CI)	*P*
Platelets (10^4/mm^3)	0: < 13	1	0.018
	1: > 13	9.84 (2.34–41.3)	
Amino acid substitutions in the ISDR of NS5A	0: non mutant type	1	0.029
	1: mutant type	7.69 (1.23–48.0)	

CI, confidence intervals; ISDR, interferon sensitivity-determining region.

Table 3 Predicting factors of non-viral response by multivariate analysis

Factor	Category	Odds ratio (95% CI)	*P*
Amino acid substitutions in aa 70 and/or aa91 of the core region	0: Non double-wild	1	0.049
	1: Double-wild	0.315 (0.1–0.994)	

CI, confidence intervals.

therapy.[14] Interestingly, it has been observed that the SVR rate decreases in a stepwise manner among EVR patients whose HCV-RNA became undetectable at different times. Therefore, it was hypothesized in the present study that it would be effective to add stepwise short-time treatment duration, and we set treatment duration to 52 weeks for EVR patients with HCV-RNA undetected at week 8 and 56–60 weeks for EVR patients with HCV-RNA undetected at week 12.

Mangia *et al.* reported that the SVR rates of standard 48-week therapy for RVR patients, EVR patients whose HCV-RNA became undetectable at week 8, and EVR patients whose HCV-RNA became undetectable at week 12 were 87%, 70.3% and 38.1%, respectively.[14]

In contrast, the results of the present study showed that the SVR rate of our protocol in RVR patients, EVR patients whose HCV-RNA became undetectable at week 8 and EVR patients with HCV-RNA undetected at week 12 were 100%, 81.0% and 80.0%, respectively. The SVR rate of EVR patients with HCV-RNA undetected at week 12 was even higher than the SVR rate of the 72-week therapy that Mangia *et al.* reported. Only two cases with HCV-RNA undetected at 12 weeks had the treatment duration of 56 weeks. Since these two cases reached SVR, we concluded that even if we had a standardized 60-week therapy for patients with HCV-RNA undetected at 12 weeks, it would not have made any difference to our result. In contrast, the availability of the 56-week therapy for patients with HCV-RNA undetected at 12 weeks remains unclear. Thus, further investigation is clearly required to establish a more appropriately designed treatment protocol.

Table 4 Predicting factors of sustained viral response for patients with undetectable HCV-RNA by 24 weeks after start of therapy by multivariate analysis

Factor	Category	Odds ratio (95% CI)	*P*
Age (years)	0: < 55	1	0.0284
	1: > 55	0.079 (0.01–0.76)	
HCV-RNA (KIU/mL)	0: < 4000	1	0.029
	1: > 4000	0.123 (0.03–0.62)	

CI, confidence intervals.

This treatment extension protocol was well tolerated, with a total frequency of treatment discontinuation of only 11.0%. Dose reduction following adverse events was used for 25 patients with a reduction in RBV, one patient with a reduction in PEG IFN and four patients with reductions in both drugs. Reductions in PEG IFN or RBV have previously been suggested as a reason for lower SVR rates.[15] Conversely, in the present study, a factor contributing to higher SVR rates was that a large number of patients were treated with sufficient volumes of both drugs. Evaluation of the time at which HCV-RNA became undetectable showed EVR in 50.0% of patients. This EVR rate is similar to results from other reports in Japan, including clinical trials, showing EVR in about half of patients who receive PEG IFN plus RBV therapy. Conversely, SVR rates have been about 70% in EVR patients from other Japanese clinical trials.[8] If we achieve a 10% increase in the SVR rate for patients with EVR, this represents a 5% advantage in the overall SVR rate. Increasing SVR among EVR patients with short-term extended therapy thus offers an important contribution to increases in overall SVR.

Baseline factors that might be useful to predict SVR and NR were evaluated in the present study. One patient factor associated with SVR was a higher platelet count on pretreatment blood tests. Decreased platelet counts are known to be correlated with more advanced hepatic fibrosis. Decreased platelet count in conjunction with advanced hepatic fibrosis seems to represent a factor associated with treatment resistance. However, no histological factors were identified, but only a limited number of biopsies were carried out. A viral factor associated with SVR was ISDR amino acid mutations in the NS5A region. Enomoto *et al.* reported ISDR mutations as predictive of treatment response to IFN monotherapy[10] and IFN plus RBV therapy.[16] Based on the present findings, ISDR mutations are also useful in predicting response to PEG IFN plus RBV therapy. Zuezem *et al.* reported no correlation between ISDR mutations and treatment response, whereas another study reported a correlation that was characteristic of a Japanese-specific subtype of HCV genotype 1b.[17] This issue clearly warrants further investigation. Viral factors associated with NR were amino acid substitutions at aa70 and aa91. Akuta *et al.* have also reported ISDR region mutations as contributing factors to SVR and core region mutations as contributing factors to NR.[12] The present findings are in agreement with those results. In contrast, no patient factors associated with NR were identified.

Patients with end-of-treatment response (ETR) were also evaluated. ETR patients displayed a low relapse rate of 26.6%. Analysis of ETR identified age and viral load as factors contributing to SVR. In other words, treatment protocols (including further treatment extension) must be considered for older patients and high baseline HCV-RNA levels. In Japan, sex has also recently attracted interest as a treatment resistance factor. In the present study, 11 of 17 patients with relapse were female. Although sex was not identified as a significant factor in the present study, we suggest this is because of the small size of this study.

The present study could not identify any significant predictive factors for SVR according to each of the treatment durations. So, we conclude that a larger subject population might identify this as a significant factor in treatment resistance.

The TaqMan PCR assay, now widely used in clinical practice, is more sensitive for detecting serum HCV than the Amplicor Monitor assay. In the present study, the time at which HCV-RNA became undetectable was based on 50 IU/mL as the lower limit of detection using the Amplicor Monitor assay. However, the TaqMan PCR assay, with a lower limit of detection of 10 IU/mL, may allow a more accurate evaluation of viral response.[18,19] Thus, in some relapsing patients, TaqMan PCR assay might have allowed viral detection for a longer period of time. This might be more suitable to evaluate the effects of a short-term extension of treatment.

In conclusion, the present study showed a higher SVR rate than previously reported, although the number of patients was small. To more accurately evaluate the usefulness of short-term extensions to the treatment protocol, a large-scale, multicenter, standard-controlled study is desirable. New drugs for the treatment of refractory CHC are being developed at a rapid pace, but it will be several years before such drugs can be applied in clinical practice. In the meantime, the CHC patient population in Japan is aging, disease severity is progressing, and the pharmacotherapeutic options remain limited. Modification of treatment protocols is one possible option to reduce relapse rates. The results of the present study indicate that short-treatment extension of PEG IFN plus RBV treatment protocols in EVR patients can improve SVR rates.

REFERENCES

1 Manns MP, McHutchison JG, Gordon SC *et al.* 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: 958–65.
2 Fried MW, Shiffman ML, Reddy KR *et al.* Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; 347: 975–82.
3 Sarrazin C, Rouzier R, Wagner F *et al.* SCH 503034, a novel hepatitis C virus protease inhibitor, plus pegylated interferon alpha-2b for genotype 1 nonresponders. *Gastroenterology* 2007; 132: 1270–8.
4 Kieffer TL, Sarrazin C, Miller JS *et al.* Telaprevir and pegylated interferon-alpha-2a inhibit wild-type and resistant genotype 1 hepatitis C virus replication in patients. *Hepatology* 2007; 46: 631–9.
5 Davis GL, Wong JB, McHutchison JG *et al.* Early virologic response to treatment with peginterferon alfa-2b plus ribavirin in patients with chronic hepatitis C. *Hepatology* 2003; 38: 645–52.
6 Pearlman BL, Ehleben C, Saifee S. Treatment extension to 72 weeks of peginterferon and ribavirin in hepatitis c genotype 1-infected slow responders. *Hepatology* 2007; 46: 1688–94.
7 Sánchez-Tapias JM, Diago M, Escartín P *et al.* Peginterferon-alfa2a plus ribavirin for 48 versus 72 weeks in patients with detectable hepatitis C virus RNA at week 4 of treatment. *Gastroenterology* 2006; 131: 451–60.
8 Iino S, Okita K, Omata M' *et al.* Clinical efficacy of PEG-Interferon alpha 2b and ribavirin combination therapy for

48 weeks in chronic hepatitis C patients with genotype 1 and high viral load - retrospective comparison with Interferon alpha 2b and ribavirin combination therapy for 24 weeks. *Kan Tan Sui* 2004; 49: 1099–121.
9 Kuboki M, Iino S, Okuno T *et al.* Peginterferon alpha 2a (40KD) plus ribavirin for the treatment of chronic hepatitis C in Japanese patients. *J Gastroenterol Hepatol* 2007; 22: 645–52.
10 Enomoto N, Sakuma I, Asahina Y *et al.* Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. *N Engl J Med* 1996; 334: 77–81.
11 Akuta N, Suzuki F, Kawamura Y *et al.* Predictive factors of early and sustained responses to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b: amino acid substitutions in the core region and low-density lipoprotein cholesterol levels. *J Hepatol* 2007; 46: 403–10.
12 Akuta N, Suzuki F, Sezaki H *et al.* Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 1b high viral load and non-virological response to interferon-ribavirin combination therapy. *Intervirology* 2005; 48: 372–80.
13 Martinez-Bauer E, Crespo J, Romero-Gomez M *et al.* Development and validation of two medels for early prediction of response to therapy in genotype 1 chronic hepatitis C. *Hepatology* 2006; 43: 72–80.
14 Mangia A, Minerva N, Bacca D *et al.* Individualized treatment duration for hepatitis C genotype 1 patients: a randomized controlled trial. *Hepatology* 2008; 47: 43–50.
15 McHutchison JG, Manns M, Patel K *et al.* Adherence to combination therapy enhances sustained response in genotype-1-infected patients with chronic hepatitis C. *Gastroenterology* 2002; 123: 1061–9.
16 Ueda H, Enomoto N, Sakamoto N *et al.* Change of HCV quasispecies during combination therapy and ribavirin. *Hepatol Res* 2004; 29: 89–96.
17 Zeuzem S, Lee JH, Roth WK. Mutations in the nonstructural 5A gene of European hepatitis C virus isolates and response to interferon alfa. *Hepatology* 1997; 25: 740–4.
18 Pittaluga F, Allice T, Abate M *et al.* Clinical evaluation of the COBAS Ampliprep/COBAS TaqMan for HCV RNA quantitation in comparison with the branched-DNA assay. *J Med Virol* 2008; 80: 254–60.
19 Carlsson T, Quist A, Weiland O. Rapid viral response and treatment outcome in genotype 2 and 3 chronic hepatitis C: comparison between two HCV RNA quantitation methods. *J Med Virol* 2008; 80: 803–7.

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Enhanced intracellular retention of a hepatitis B virus strain associated with fulminant hepatitis

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ABSTRACT

A plasmid carrying 1.3-fold HBV genome was constructed from a HBV strain that caused five consecutive cases of fulminant hepatitis (pBFH2), and HepG2 cells were transfected with pBFH2 or its variants. The pBFH2 construct with A1762T/G1764A, G1862T, and G1896A showed the largest amount of core particle-associated intracellular HBV DNA, but no significant increase of extracellular HBV DNA in comparison with the wild construct, suggesting that these mutations might work together for retention of the replicative intermediates in the cells. The retention might relate to the localization of hepatitis B core antigen (HBcAg) in the nucleus of HepG2, which was observed by confocal fluorescence microscopy. HBcAg immunohistochemical examination of liver tissue samples obtained from the consecutive fulminant hepatitis patients showed stronger staining in the nucleus than acute hepatitis patients. In conclusion, the fulminant HBV strain caused retention of the core particles and the core particle-associated HBV DNA in the cells.

Introduction

Hepatitis B virus (HBV) causes a spectrum of liver diseases such as acute self-limiting or fulminant hepatitis, chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. Fulminant hepatitis, which is lethal during a short period in many cases, occurs in approximately 1% of patients with acute HBV infection (Lee, 1993). The pathogenesis that leads to fulminant hepatitis B is considered to result from enhanced replication of the virus (Baumert et al., 1996; Hasegawa et al., 1994) and the exuberant immune response of the host (Rivero et al., 2002), but it is not fully understood.

HBV contains a small (3.2 kb), circular, partially double-stranded DNA genome. This genome includes four, partly overlapping open reading frames: the precore/core gene encoding for the hepatitis B e antigen (HBeAg) and the core antigen (HBcAg), the polymerase gene encoding for the polymerase protein, the preS/S gene encoding for the hepatitis B surface antigen (HBsAg), and the X gene encoding for the X protein. In the course of replication, four kinds of viral RNAs, 3.5, 2.4, 2.1, and 0.8 kb in size, are transcribed (Ganem and Varmus, 1987). There are two types of 3.5 kb RNAs whose 5′ termini and functions are different: the pregenomic RNA (pgRNA) and the precore mRNA. The pgRNA, which is encapsidated as the template for reverse transcription to generate viral DNA, serves as the mRNA for HBcAg and the polymerase protein (Summers and Mason, 1982). The precore mRNA is translated into the precore/core fusion protein that is post-translationally modified to HBeAg (Roossinck et al., 1986; Standring et al., 1988). The transcription of the pgRNA and the precore mRNA are regulated by the core promoter corresponding to nucleotide (nt) 1613–1849 (Kramvis and Kew, 1999). The double core promoter mutations of A1762T/G1764A, which were frequently observed in HBeAg-negative chronic hepatitis patients (Okamoto et al., 1994) and fulminant hepatitis patients (Sato et al., 1995), were reported to reduce the production of HBeAg and enhance the replication of HBV in an in vitro study (Buckwold et al., 1996; Moriyama et al., 1996). It was also documented that the precore mutation of G1896A, which makes a stop codon and abrogates HBeAg (Carman et al., 1989), was associated with fulminant hepatitis (Liang et al., 1991; Omata et al., 1991), and the enhanced replication of HBV with G1896A in vitro has been described (Ozasa et al., 2006; Scaglioni et al., 1997).

Recently, we reported five consecutive cases of fulminant hepatitis B that were caused by the same strain of HBV (Nagasaki et al., 2008). The full-length sequences of HBV obtained from them

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were 99.8–100% identical to each other. This strain belonged to genotype B2 (Ba), which was reported to cause fulminant hepatitis less frequently than genotypes A, C, D, and other subgenotypes of genotype B (Ozasa et al., 2006). The fulminant strain was found to have several mutations in the core promoter and precore regions. The aim of this study is to investigate the significance of these mutations in the pathogenesis of fulminant hepatitis, using an in vitro culture system.

Results

Mutations in the fulminant strain of HBV

In the fulminant HBV strain FH-2, many mutations throughout the genome were observed. The full-length sequence (accession number: AB302943) (Nagasaki et al., 2008) was compared with a genotype B2 consensus sequence that was deduced from 52 full-length sequences deposited on GenBank/EMBL/DDBJ. A total of 45 nucleotide mutations, including the double core promoter mutations of A1762T/G1764A and the precore stop mutation of G1896A, were found. In the precore region, a distinctive mutation of G1862T located within the bulge of the ε signal as an RNA structure (Fig. 1C) was also found. Other nucleotide mutations were not found in the core promoter and precore regions except for G1632C. Because nt 1632 varies in the known genotype B2 strains, the nucleotide was considered not to have an important role in the pathogenesis of fulminant hepatitis.

As for amino acid (aa), 17 mutations in the polymerase gene, 7 mutations in the preS/S gene, 6 mutations in the precore/core gene, and 6 mutations in the X gene were observed (Table 1). Focusing on the core promoter and precore regions, an amino acid mutation of residue 17 of Val to Phe (V17F) in the precore region was found besides the precore stop mutation and the double core promoter mutations (corresponding to aa 130 and 131 in the X gene). V17F in the precore region corresponds to the G1862T mutation. We suspected that the nonsynonymous mutations of G1862T might have some effects on the development of fulminant hepatitis, besides A1762T/G1764A and G1896A.

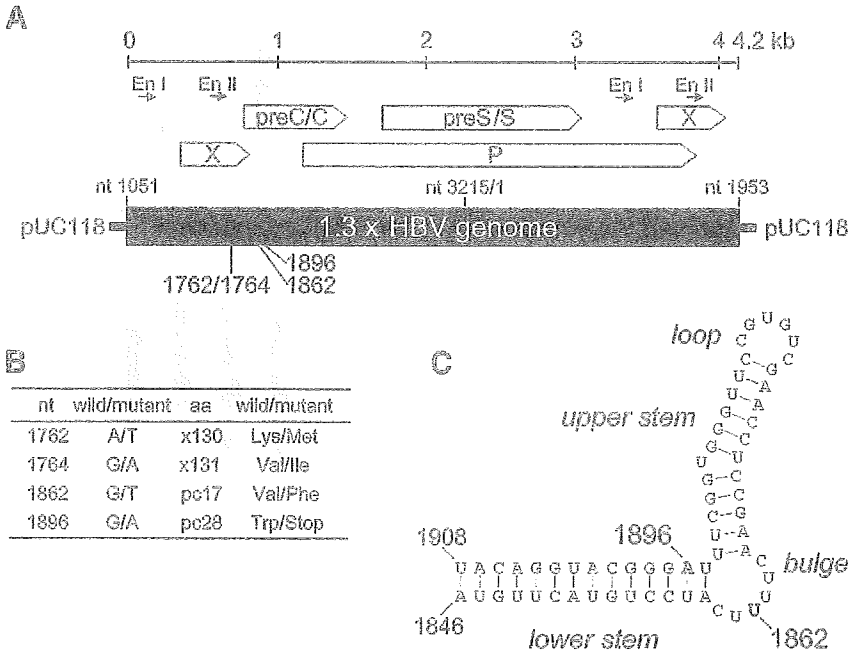
Validation of the replication capacity and the transfection efficiency

A plasmid containing 1.3-fold genome of the FH-2 strain, named pBFH2, was transfected to HepG2 or Huh7 cells, and the replication capacity of the strain was compared in these cell lines. Because the amount of HBsAg and HBV DNA in the culture medium was significantly larger in HepG2 than Huh7 (Fig. 2A), HepG2 was used in the following experiments.

To investigate the significance of the mutations of A1762T/G1764A, G1862T, and G1896A in the FH-2 strain, pBFH2 and its variants were transfected to HepG2 cells and compared. First, the efficiency of transfection was validated using the SEAP reporter system. It was shown that the differences in the activity of SEAP between pBFH2 and its variants coincided with that of expressed HBsAg (Fig. 2B). Therefore, the assay of HBsAg was thought to be suitable for the validation of the transfection efficiency in this system.

Intracellular replicative intermediates of HBV

Southern blotting analysis was performed using HepG2 collected 3 days after transfection. Intracellular core particle-associated HBV DNA was shown as replicative intermediates such as relaxed circular DNA, double-stranded linear DNA, and single-stranded DNA (Fig. 3A). The amount of intracellular HBV DNA was measured by densitometry (Fig. 3B). When compared with the all-wild construct (A1762/G1762, G1862, and G1896), the intracellular HBV DNA was decreased significantly in the construct with the single mutation of G1862T. This finding was in agreement with a previous report (Guarnieri et al., 2006). However, the suppression of HBV replication was overcome by the coexisting mutation of A1762T/G1764A or G1896A. Moreover, only the all-mutant original construct, pBFH2, showed significantly larger amounts of intracellular HBV DNA than the all-wild construct. The double mutations of



nt	wild/mutant	aa	wild/mutant
1762	A/T	x130	Lys/Met
1764	G/A	x131	Val/Ile
1862	G/T	pc17	Val/Phe
1896	G/A	pc28	Trp/Stop

Fig. 1. Construction of a plasmid (pBFH2) and mutations in the strain. (A) Schema of the plasmid containing 1.3-fold HBV genome and the positions of nucleotide mutations found in the core promoter and the precore region. (B) Nucleotide mutations and corresponding amino acid mutations in the core promoter and the precore region in pBFH2. (C) The structure of the ε signal as an RNA secondary structure. It consists of two base-paired segments (lower and upper stems), a bulge, and a loop. nt 1862 is within the bulge and nt 1896 is within the lower stem. En, enhancer; preC/C, precore/core; P, polymerase.

Table 1
Differences of amino acids between the B2 consensus sequence and the FH-2 strain.

Gene	aa position	B2 consensus[a]	FH-2
Polymerase	50	Ile	Thr
	93	Lys	Glu
	104	Asn	Thr
	118	Asn	Lys
	150	Thr	Ile
	212	Pro	Ser
	261	Gly	Asp
	366	His	Asn
	301	Ala	Thr
	464	Asn	Asp
	470	Asn	His
	474	Thr	Asn
	480	Asn	Asp
	566	Leu	Ile
	678	Ser	Arg
	679	Lys	Asn
	809	Thr	Ser (Phe)[b]
preS/S	81	Ala	Thr
	120	Met	Ile
	132	Gln	His
	214	Asn	Ser
	250	Cys	Tyr
	294	Pro	Thr
	358	Val	Ala
Precore/core	17	Val	Phe
	28	Trp	Stop
	106	Glu	Gln
	108	Pro	Gln
	113	Leu	Ala
	210	Ser	Pro
X	37	Leu	Val
	44	Val	Ala
	48	Asp	Ile
	87	Gly	Arg
	130	Lys	Met
	131	Val	Ile

[a] Deduced from 52 full-length sequences of genotype B2 HBV registered on GenBank/EMBL/DDBJ.
[b] Found in the other isolates obtained from consecutive cases of fulminant hepatitis.

A1762T/G1764A, or the triple mutations of A1762T/G1764A and G1896A, had a tendency to increase the replicative intermediates, but not significantly. The single G1896A mutation did not seem to increase them.

Released HBV virions and HBeAg

The amount of the yielded HBV virions in the culture supernatant of pBFH2-transfected HepG2 was assayed by real-time PCR and compared with pBFH2-variant constructs (Fig. 4). The single mutation of G1862T reduced it significantly whereas the double mutation of A1762T/G1764A increased it. Notably, the amount was not increased significantly for pBFH2, in contrast to the result of intracellular HBV DNA. These data suggested that pBFH2 might cause accumulation of the replicative intermediates in the cells due to the mutations in the core promoter and precore region.

The secreted HBeAg was reduced in the pBFH2-variant constructs with A1762T/G1764A and/or G1896A expectedly (Fig. 4), but the effect of A1762T/G1764A was limited in this study. Consistent with a previous report (Guarnieri et al., 2006), G1862T did not seem to affect the expression of HBeAg.

Distribution of HBcAg in HepG2 cells

To investigate the mechanism of the retention of viral replicative intermediates in the cells, the relationship between the distribution of HBcAg in the cells and the mutations of HBV was analyzed using confocal microscopy (Fig. 5). The all-wild construct showed weak HBcAg staining mainly in the nucleus. The construct with only A1762T/G1764A showed the predominant distribution of HBcAg in the cytoplasm, which was concordant with a previous report (Kawai et al., 2003; Liu et al., 2009). The predominant distribution of HBcAg in the nucleus was observed in the construct with the single mutation of G1896A. Although the construct with only G1862T showed HBcAg staining mainly in the cytoplasm, strong staining of HBcAg was observed in the nucleus of the transfected cells of the all-mutant construct, pBFH2. The relationship between the predominance of the HBcAg distribution and the amount of intracellular/extracellular HBV DNA is shown in Table 2. The constructs demonstrating predominant HBcAg distribution in the cytoplasm, such as the construct having only A1762T/G1764A, had a tendency to yield a large amount of extracellular HBV DNA in comparison with intracellular HBV DNA. In contrast, pBFH2, which produced the largest amount of intracellular HBV DNA, showed HBcAg distribution in the nucleus. Whereas the HBcAg distribution in the cytoplasm might lead to the efficient release of the HBV particles, its distribution in the nucleus might be related to the retention of replicative intermediates in the cells. The same results were obtained also using a mouse monoclonal anti-HBcAg antibody (Hyb-3120; Institute of Immunology, Tokyo, Japan), which recognizes a capsid conformation-specific epitope and not HBeAg (Conway et al., 2003) (data not shown). Taken together, the presence of all of the mutations of A1762T/G1764A, G1896A, and G1862T was considered to work together to accumulate HBV DNA in the cells via retention of HBV core particles in the nucleus.

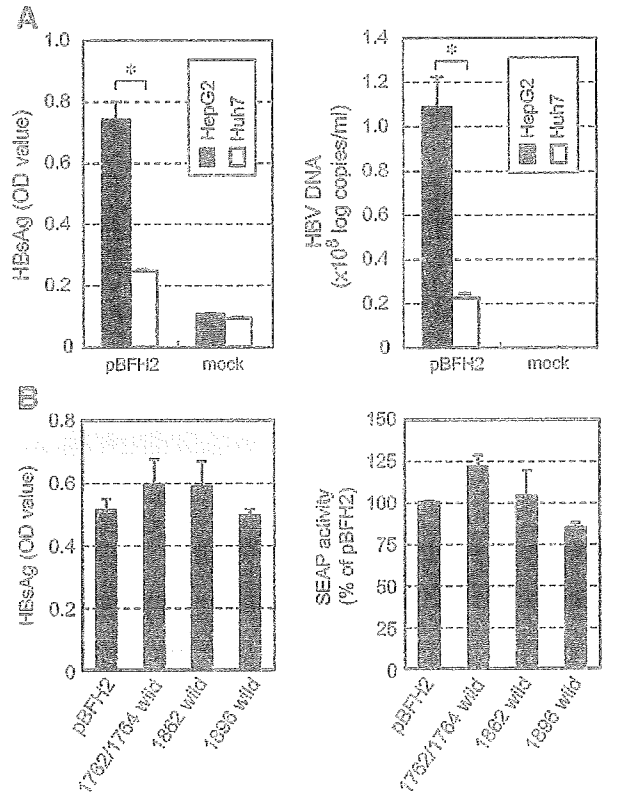


Fig. 2. (A) Comparison of replicative capacity of the FH-2 strain in HepG2 and Huh7. HBsAg and HBV DNA in the culture medium were assayed by ELISA and real-time PCR, respectively. *, $P<0.05$. (B) Validation of transfection efficiency using the SEAP reporter system. The SEAP activity in the culture supernatant was compared with HBsAg, and it was evaluated to determine whether the HBsAg assay was appropriate for the validation of transfection efficiency. Values represent means of triplicate experiments ± standard deviation.

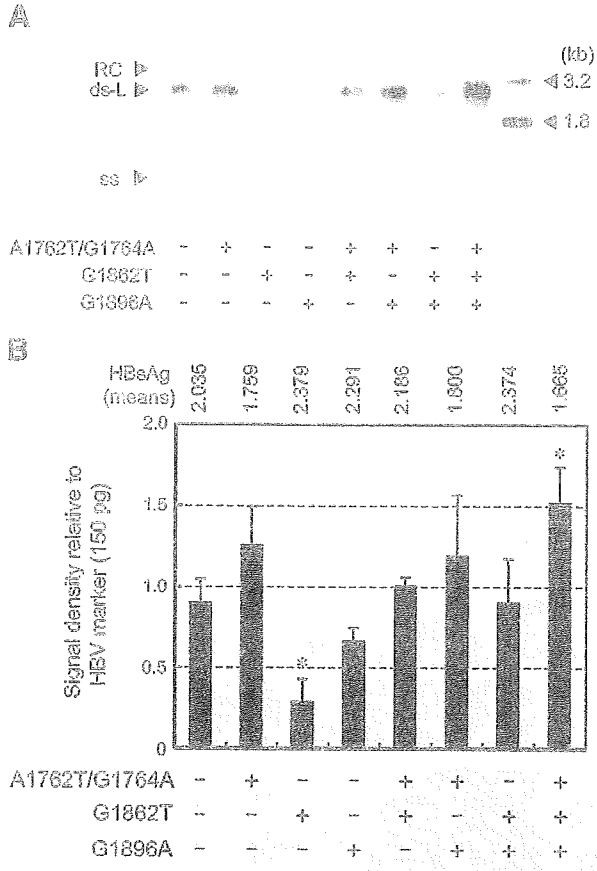


Fig. 3. Detection of replicative intermediates of HBV by Southern blotting analysis. (A) A result of a representative experiment of Southern blotting analysis. + indicates the mutant nucleotides in each nucleotide and — indicates the wild-type nucleotides. RC, relaxed circular HBV DNA; ds-L, double-stranded linear HBV DNA; ss, single-stranded HBV DNA. (B) Amount of replicative intermediates measured by densitometry, in comparison with the density of 150 pg HBV marker. The bars indicate the mean value of triplicate experiments ± standard deviation. Means of HBsAg assayed by ELISA were shown as markers for transfection efficiency. *, $P<0.05$ in comparison with the all-wild construct.

Distribution of HBcAg in tissue samples

To evaluate the results of in vitro study, immunohistochemical examination of liver specimens was performed using the same primary antibody to HBcAg (Fig. 6). In the samples obtained from three of five consecutive fulminant hepatitis B patients, both the nucleus and the cytoplasm of hepatocytes were stained (Figs. 6A–C). In contrast, nuclear staining was rarely observed in acute hepatitis B patients (Figs. 6D–F). In a chronic hepatitis B patient for positive control, nuclear staining was observed (Fig. 6G). Although the cytoplasmic HBcAg was observed more strongly in the tissue samples of fulminant hepatitis than the pBFH2-transfected cells in vitro, it was shown that HBcAg was retained in the nucleus of hepatocytes of these fulminant hepatitis patients. It was considered that the findings from the tissue samples supported the results from pBFH2-transfected cells, in which the core particle-associated replicative intermediates of HBV were retained.

Discussion

A large body of evidence has demonstrated that HBV-related liver disease is immune-mediated (Chisari, 1997; Kondo et al., 1997; Perrillo, 2001). Whereas strong and multispecific T cell responses are observed during acute HBV infection, patients with chronic hepatitis B tend to have weak and narrowly focused immune responses (Chisari, 1997). However, immunocompromised hosts such as patients undergoing hemodialysis can develop fulminant hepatitis B (Igaki et al., 2003), indicating that both viral factors as well as the host immune response are involved in the pathogenesis of fulminant hepatitis. In our study, all of the five consecutive patients with fulminant hepatitis B were over 60 years old and might have had relatively weaker immune responses to HBV. Therefore, the HBV strain obtained from them is considered to be appropriate for the study of the viral factors that lead to fulminant hepatitis.

The fulminant strain of genotype B2 in this study had several mutations including A1762T/G1764A and G1896A, and additionally, G1862T was found in the precore region. It was reported that G1862T was found frequently in the HBV of fulminant hepatitis patients without A1762T/G1764A and G1896A in China, where genotype B2 HBV is prevalent (Hou et al., 2002). In that report, G1862T caused a reduction of HBeAg, which might be associated with the development of fulminant hepatitis. However, another report using a genotype D strain described that the mutation did not change the expression of HBeAg but reduced the expression of the core protein and impaired HBV replication (Guarnieri et al., 2006). These reports are controversial and further analysis of this mutation is needed.

This study demonstrated that the precore G1862T mutation caused the accumulation of the core particle-associated HBV DNA in the cells, in conjunction with the core promoter A1762T/G1764A and precore G1896A mutations. nt 1862 could have two possible functions in the replication of HBV based on the position in the precore

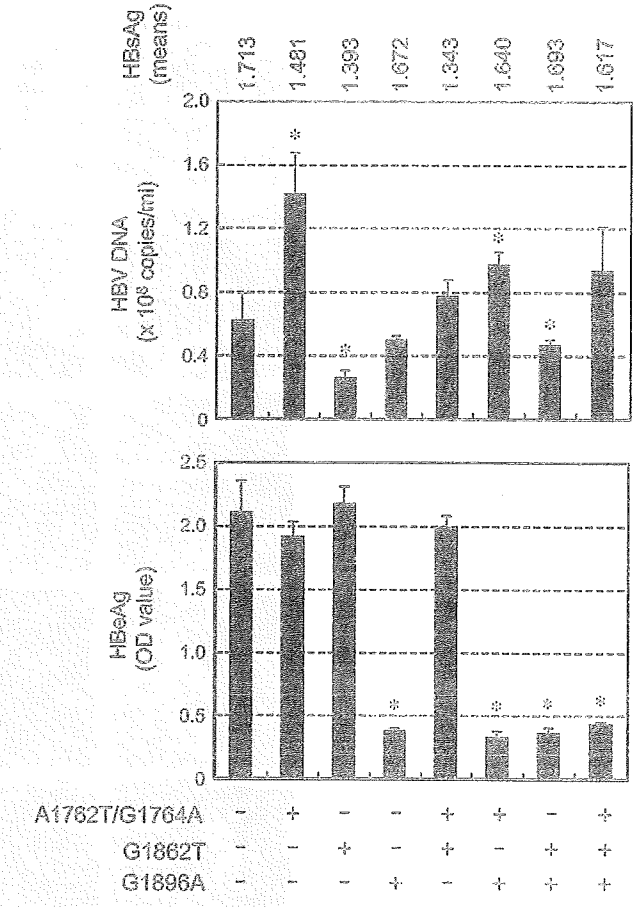


Fig. 4. Released HBV particle and HBeAg in the culture supernatant. The amount of HBV particle was measured by real-time PCR and HBeAg was assayed by ELISA. In the top, the means of HBsAg in the culture supernatant are shown. Values represent means of triplicate experiments ± standard deviation. *, $P<0.05$ in comparison with the all-wild construct.

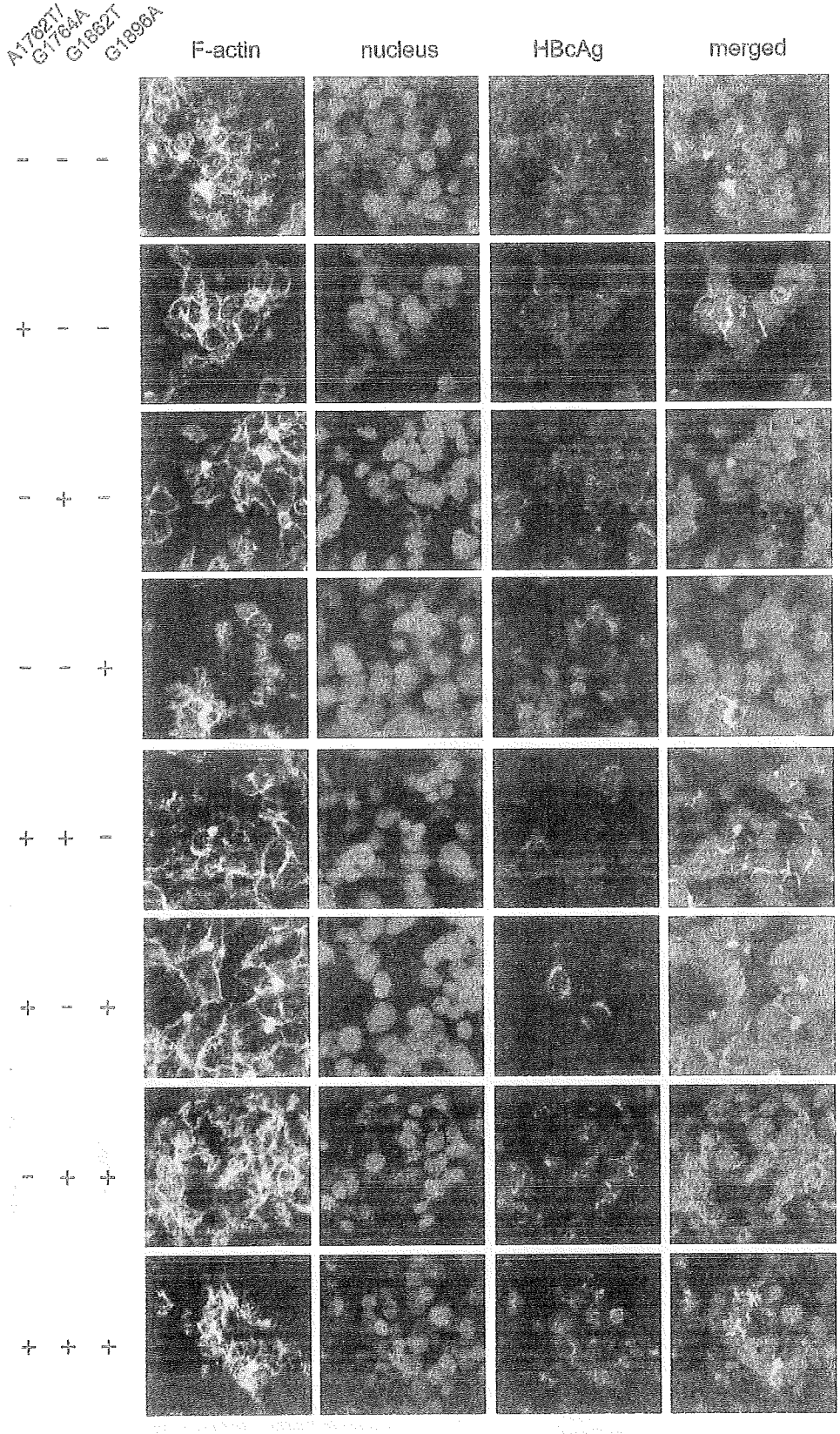


Fig. 5. Comparison of the distribution of HBcAg in the transfected HepG2 cells observed by confocal microscopy according to the HBV mutations. HBcAg was stained using a rabbit polyclonal anti-HBcAg antibody (Dako), and F-actin and nucleus were stained simultaneously.

Table 2
The summarized results of the intracellular/extracellular HBV DNA and distribution of HBcAg in the nucleus according to HBV mutations.

HBV mutations			Intracellular	Extracellular	HBcAg distribution[b]
A1762T/G1764A	G1862T	G1896A	HBV DNA[a]	HBV DNA[a]	
−	−	−	+	+	N>C
+	−	−	+	++	C>N
−	+	−	±	±	C>N
−	−	+	+	+	N>C
+	+	−	+	+	C>N
+	−	+	+	++	C>N
−	+	+	+	±	N>C
+	+	+	++	+	N>C

[a] ++ indicates that the amount of HBV DNA was significantly larger than the all-wild (A1762/G1764, G1862, and G1896) construct, and ± indicates that the amount was significantly lower than the all-wild construct. + indicates no significant change relative to the all-wild construct.

[b] N>C indicates that the cells having HBcAg in the nucleus were observed more predominantly than those having HBcAg in the cytoplasm, and C>N indicates the opposite frequency.

sequence: (i) its mutation converts aa 17 of valine to phenylalanine in the precore peptide and can affect processing of the precore/core protein into HBeAg (Chen et al., 2008; Hou et al., 2002) and (ii) it is within the bulge of the ε signal, which works as the template for the primers to synthesize the negative-strand DNA (Nassal and Rieger, 1996), and is recognized by HBV polymerase in the encapsidation of the pgRNA into the core particle (Rieger and Nassal, 1995). As for the former, aa 17 in the precore peptide is located at the −3 position of the signal peptidase cleavage site, and phenylalanine in this position is considered a "forbidden" amino acid, which may abrogate the cleavage of the precore/core protein by the signal peptidase. Although the effect of G1862T on the production of HBeAg is controversial (Chen et al., 2008; Guarnieri et al., 2006; Hou et al., 2002), it could be supposed that the single mutation of G1862T has a suppressive effect on HBV replication, at least in in vitro studies using genotype D strains (Chen et al., 2008; Guarnieri et al., 2006). A protein of 22 kDa (p22) including precore peptide, which is a product from the precore/core protein other than HBeAg, was reported to inhibit encapsidation (Kimura et al., 2005; Scaglioni et al., 1997). Hence, it was suspected that the possibly increased p22, which might not be cleaved due to G1862T, might lower the capacity of HBV encapsidation and replication. The finding in this study that the replication capacity was overcome by the precore stop mutation G1896A, which abrogates the precore/core protein, supports this speculation. In view of the function of the bulge of the ε signal, G1862T could make the extended templates for the initiation of the negative-strand DNA replication. Whereas the primer of TGAA is made using the 3′-part of the bulge as the template in wild-type HBV, the extended primer of TGAAA or TGAAAA which can anneal to the direct repeat 1 (DR1) and the continued 5′ nucleotides (5′-*CTTTTTCACCTCTGCCT*-3′, italic-typed nucleotides are the DR1 sequence and underlined nucleotides are complement to the extended primer) can be made in the presence of G1862T. Although it is unknown whether the extended primer can actually initiate the extension of the negative-strand DNA, the primer may have some positive effects on reverse transcription. Some of the possible effects of G1862T on the viral replication can be affected by other mutations and the phenotype may be changed.

The core promoter mutation of A1762T/G1764A, which is known to enhance the transcription of pgRNA and reduce the precore mRNA (Moriyama et al., 1996), was described as being associated with the cytoplasmic distribution of HBcAg (Kawai et al., 2003; Liu et al., 2009). In this study, the localization of HBcAg was changed to the nucleus when both G1862T and G1896A were present. Whereas HBcAg is a 21 kD protein (p21) and can be transported across the nuclear membrane by diffusion, the core particle, which consists of approximately 180 subunits of a core protein, cannot diffuse across it (Forbes, 1992; Kawai et al., 2003). It has been speculated that high density HBcAg resulting from the increased pgRNA in the presence of A1762T/G1764A might favor the formation of the core particle in the cytoplasm, and the release of the particle as an infectious virus particle after envelopment. A part of the incompletely processed precore/core protein, p22, can also assemble into the core particle, but the particle cannot encapsidate the pgRNA (Scaglioni et al., 1997). The empty core particle could be detected also in this study using the polyclonal anti-HBcAg antibody. As G1862T reduces HBcAg expression (Guarnieri et al., 2006) and G1896A abrogates the precore/core protein, the presence of these mutations may lower the density of p21 and p22 leading to inefficient assembly of the core particle in the cytoplasm. After that, unassembled p21 is transported across the nuclear membrane by the function of the putative nucleolar localization signal of HBcAg (Ning and Shih, 2004) and be assembled in the nucleus, followed by formation of the core particle. As the particles cannot be transported across the nuclear membrane, they may accumulate in the nucleus.

The core promoter mutations of A1762T/G1764A change overlapping X protein, and there is a possibility that the change of X protein may have effect on HBV replication or localization. A recent

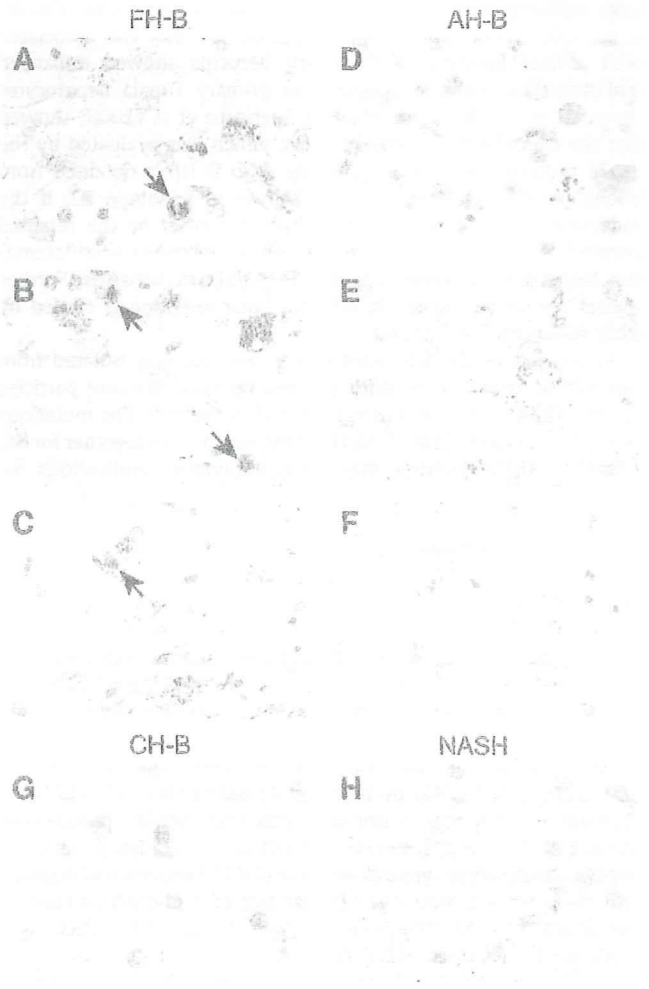


Fig. 6. HBcAg immunohistochemical examination of the liver tissue samples obtained from fulminant hepatitis B (FH-B) patients and acute hepatitis B (AH-B) patients using a rabbit polyclonal anti-HBcAg antibody (Dako). (A–C) Tissue samples of three of five consecutive fulminant hepatitis B patients from whom the FH-2 strain was isolated. The arrows indicate the hepatocytes with nuclear HBcAg staining. (D–F) Tissue samples obtained from three acute hepatitis B patients. (G) A sample obtained from a chronic hepatitis B (CH-B) patient as positive control. (H) A sample of a nonalcoholic steatohepatitis (NASH) patient as negative control.

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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References

Abdelhamed, A.M., Kelley, C.M., Miller, T.G., Furman, P.A., Isom, H.C., 2002. Rebound of hepatitis B virus replication in HepG2 cells after cessation of antiviral treatment. J. Virol. 76 (16), 8148–8160.

Baumert, T.F., Rogers, S.A., Hasegawa, K., Liang, T.J., 1996. Two core promotor mutations identified in a hepatitis B virus strain associated with fulminant hepatitis result in enhanced viral replication. J. Clin. Invest. 98 (10), 2268–2276.

Baumert, T.F., Yang, C., Schurmann, P., Kock, J., Ziegler, C., Grullich, C., Nassal, M., Liang, T.J., Blum, H.E., von Weizsacker, F., 2005. Hepatitis B virus mutations associated with fulminant hepatitis induce apoptosis in primary Tupaia hepatocytes. Hepatology 41 (2), 247–256.

Buckwold, V.E., Xu, Z., Chen, M., Yen, T.S., Ou, J.H., 1996. Effects of a naturally occurring mutation in the hepatitis B virus basal core promoter on precore gene expression and viral replication. J. Virol. 70 (9), 5845–5851.

Carman, W.F., Jacyna, M.R., Hadziyannis, S., Karayiannis, P., McGarvey, M.J., Makris, A., Thomas, H.C., 1989. Mutation preventing formation of hepatitis B e antigen in patients with chronic hepatitis B infection. Lancet 2 (8663), 588–591.

Chen, C.Y., Crowther, C., Kew, M.C., Kramvis, A., 2008. A valine to phenylalanine mutation in the precore region of hepatitis B virus causes intracellular retention and impaired secretion of HBe-antigen. Hepatol. Res. 38 (6), 580–592.

Chirillo, P., Pagano, S., Natoli, G., Puri, P.L., Burgio, V.L., Balsano, C., Levrero, M., 1997. The hepatitis B virus X gene induces p53-mediated programmed cell death. Proc. Natl. Acad. Sci. U. S. A. 94 (15), 8162–8167.

Chisari, F.V., 1997. Cytotoxic T cells and viral hepatitis. J. Clin. Invest. 99 (7), 1472–1477.

Conway, J.F., Watts, N.R., Belnap, D.M., Cheng, N., Stahl, S.J., Wingfield, P.T., Steven, A.C., 2003. Characterization of a conformational epitope on hepatitis B virus core antigen and quasiequivalent variations in antibody binding. J. Virol. 77 (11), 6466–6473.

Foo, N.C., Ahn, B.Y., Ma, X., Hyun, W., Yen, T.S., 2002. Cellular vacuolization and apoptosis induced by hepatitis B virus large surface protein. Hepatology 36 (6), 1400–1407.

Forbes, D.J., 1992. Structure and function of the nuclear pore complex. Annu. Rev. Cell Biol. 8, 495–527.

Ganem, D., Varmus, H.E., 1987. The molecular biology of the hepatitis B viruses. Annu. Rev. Biochem. 56, 651–693.

Guarnieri, M., Kim, K.H., Bang, G., Li, J., Zhou, Y., Tang, X., Wands, J., Tong, S., 2006. Point mutations upstream of hepatitis B virus core gene affect DNA replication at the step of core protein expression. J. Virol. 80 (2), 587–595.

Hasegawa, K., Huang, J., Rogers, S.A., Blum, H.E., Liang, T.J., 1994. Enhanced replication of a hepatitis B virus mutant associated with an epidemic of fulminant hepatitis. J. Virol. 68 (3), 1651–1659.

Hou, J., Lin, Y., Waters, J., Wang, Z., Min, J., Liao, H., Jiang, J., Chen, J., Luo, K., Karayiannis, P., 2002. Detection and significance of a G1862T variant of hepatitis B virus in Chinese patients with fulminant hepatitis. J. Gen. Virol. 83 (Pt. 9), 2291–2298.

Hussain, Z., Jung, H.S., Ryu, D.K., Ryu, W.S., 2009. Genetic dissection of naturally occurring basal core promoter mutations of hepatitis B virus reveals the silent phenotype in the overlapping X gene. J. Gen. Virol. 90 (pt. 9), 2272–2281.

Igaki, N., Nakaji, M., Moriguchi, R., Akiyama, H., Tamada, F., Oimomi, M., Goto, T., 2003. An outbreak of fulminant hepatitis B in immunocompromised hemodialysis patients. J. Gastroenterol. 38 (10), 968–976.

Jardi, R., Rodriguez, F., Buti, M., Costa, X., Cotrina, M., Valdes, A., Galimany, R., Esteban, R., Guardia, J., 2001. Quantitative detection of hepatitis B virus DNA in serum by a new rapid real-time fluorescence PCR assay. J. Viral Hepat. 8 (6), 465–471.

Kawai, K., Horiike, N., Michitaka, K., Onji, M., 2003. The effects of hepatitis B virus core promoter mutations on hepatitis B core antigen distribution in hepatocytes as detected by laser-assisted microdissection. J. Hepatol. 38 (5), 635–641.

Kimura, T., Ohno, N., Terada, N., Rokuhara, A., Matsumoto, A., Yagi, S., Tanaka, E., Kiyosawa, K., Ohno, S., Maki, N., 2005. Hepatitis B virus DNA-negative dane particles lack core protein but contain a 22-kDa precore protein without C-terminal arginine-rich domain. J. Biol. Chem. 280 (23), 21713–21719.

Kondo, T., Suda, T., Fukuyama, H., Adachi, M., Nagata, S., 1997. Essential roles of the Fas ligand in the development of hepatitis. Nat. Med. 3 (4), 409–413.

Kramvis, A., Kew, M.C., 1999. The core promoter of hepatitis B virus. J. Viral Hepat. 6 (6), 415–427.

Lee, W.M., 1993. Acute liver failure. N. Engl. J. Med. 329 (25), 1862–1872.

Liang, T.J., Hasegawa, K., Rimon, N., Wands, J.R., Ben-Porath, E., 1991. A hepatitis B virus mutant associated with an epidemic of fulminant hepatitis. N. Engl. J. Med. 324 (24), 1705–1709.

Liu, C.J., Jeng, Y.M., Chen, C.L., Cheng, H.R., Chen, P.J., Chen, T.C., Liu, C.H., Lai, M.Y., Chen, D.S., Kao, J.H., 2009. Hepatitis B virus basal core promoter mutation and DNA load correlate with expression of hepatitis B core antigen in patients with chronic hepatitis B. J. Infect. Dis. 199 (5), 742–749.

Moriyama, K., Okamoto, H., Tsuda, F., Mayumi, M., 1996. Reduced precore transcription and enhanced core-pregenome transcription of hepatitis B virus DNA after replacement of the precore-core promoter with sequences associated with e antigen-seronegative persistent infections. Virology 226 (2), 269–280.

Nagasaki, F., Ueno, Y., Niitsuma, H., Inoue, J., Kogure, T., Fukushima, K., Kobayashi, K., Shimosegawa, T., 2008. Analysis of the entire nucleotide sequence of hepatitis B causing consecutive cases of fatal fulminant hepatitis in Miyagi Prefecture Japan. J. Med. Virol. 80 (6), 967–973.

Nassal, M., Rieger, A., 1996. A bulged region of the hepatitis B virus RNA encapsidation signal contains the replication origin for discontinuous first-strand DNA synthesis. J. Virol. 70 (5), 2764–2773.

Ning, B., Shih, C., 2004. Nucleolar localization of human hepatitis B virus capsid protein. J. Virol. 78 (24), 13653–13668.

Okamoto, H., Tsuda, F., Akahane, Y., Sugai, Y., Yoshiba, M., Moriyama, K., Tanaka, T., Miyakawa, Y., Mayumi, M., 1994. Hepatitis B virus with mutations in the core promoter for an e antigen-negative phenotype in carriers with antibody to e antigen. J. Virol. 68 (12), 8102–8110.

Omata, M., Ehata, T., Yokosuka, O., Hosoda, K., Ohto, M., 1991. Mutations in the precore region of hepatitis B virus DNA in patients with fulminant and severe hepatitis. N. Engl. J. Med. 324 (24), 1699–1704.

Ozasa, A., Tanaka, Y., Orito, E., Sugiyama, M., Kang, J.H., Hige, S., Kuramitsu, T., Suzuki, K., Tanaka, E., Okada, S., Tokita, H., Asahina, Y., Inoue, K., Kakumu, S., Okanoue, T., Murawaki, Y., Hino, K., Onji, M., Yatsuhashi, H., Sakugawa, H., Miyakawa, Y., Ueda, R., Mizokami, M., 2006. Influence of genotypes and precore mutations on fulminant or chronic outcome of acute hepatitis B virus infection. Hepatology 44 (2), 326–334.

Perrillo, R.P., 2001. Acute flares in chronic hepatitis B: the natural and unnatural history of an immunologically mediated liver disease. Gastroenterology 120 (4), 1009–1022.

Rieger, A., Nassal, M., 1995. Distinct requirements for primary sequence in the 5′- and 3′-part of a bulge in the hepatitis B virus RNA encapsidation signal revealed by a combined in vivo selection/in vitro amplification system. Nucleic Acids Res. 23 (19), 3909–3915.

Rivero, M., Crespo, J., Fabrega, E., Casafont, F., Mayorga, M., Gomez-Fleitas, M., Pons-Romero, F., 2002. Apoptosis mediated by the Fas system in the fulminant hepatitis by hepatitis B virus. J. Viral Hepat. 9 (2), 107–113.

Roossinck, M.J., Jameel, S., Loukin, S.H., Siddiqui, A., 1986. Expression of hepatitis B viral core region in mammalian cells. Mol. Cell. Biol. 6 (5), 1393–1400.

Sato, S., Suzuki, K., Akahane, Y., Akamatsu, K., Akiyama, K., Yunomura, K., Tsuda, F., Tanaka, T., Okamoto, H., Miyakawa, Y., Mayumi, M., 1995. Hepatitis B virus strains with mutations in the core promoter in patients with fulminant hepatitis. Ann. Intern. Med. 122 (4), 241–248.

Scaglioni, P.P., Melegari, M., Wands, J.R., 1997. Posttranscriptional regulation of hepatitis B virus replication by the precore protein. J. Virol. 71 (1), 345–353.

Standring, D.N., Ou, J.H., Masiarz, F.R., Rutter, W.J., 1988. A signal peptide encoded within the precore region of hepatitis B virus directs the secretion of a heterogeneous population of e antigens in *Xenopus* oocytes. Proc. Natl. Acad. Sci. U. S. A. 85 (22), 8405–8409.

Sugiyama, M., Tanaka, Y., Kato, T., Orito, E., Ito, K., Acharya, S.K., Gish, R.G., Kramvis, A., Shimada, T., Izumi, N., Kaito, M., Miyakawa, Y., Mizokami, M., 2006. Influence of hepatitis B virus genotypes on the intra- and extracellular expression of viral DNA and antigens. Hepatology 44 (4), 915–924.

Summers, J., Mason, W.S., 1982. Replication of the genome of a hepatitis B-like virus by reverse transcription of an RNA intermediate. Cell 29 (2), 403–415.

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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.

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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)[59]	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.

References

1 Armstrong GL, Wasley A, Simard EP, McQuillan GM, Kuhnert WL, Alter MJ. The prevalence of hepatitis c virus infection in the United States, 1999 through 2002. *Ann. Intern. Med.* 2006; **144**: 705–14.

2 Davila JA, Morgan RO, Shaib Y, McGlynn KA, El-Serag HB. Hepatitis C infection and the increasing incidence of hepatocellular carcinoma: a population-based study. *Gastroenterology* 2004; **127**: 1372–80.

3 El-Serag HB, Davila JA, Petersen NJ, McGlynn KA. The continuing increase in the incidence of hepatocellular carcinoma in the United States: an update. *Ann. Intern. Med.* 2003; **139**: 817–23.

4 Kim WR, Gores GJ, Benson JT, Therneau TM, Melton LJ 3rd. Mortality and hospital utilization for hepatocellular carcinoma in the United States. *Gastroenterology* 2005; **129**: 486–93.

5 Razali K, Amin J, Dore GJ, Law MG. Modelling and calibration of the hepatitis C epidemic in Australia. *Stat. Methods Med. Res.* 2008.

6 Deuffic-Burban S, Mohamed MK, Larouze B, Carrat F, Valleron AJ. Expected increase in hepatitis C-related mortality in Egypt due to pre-2000 infections. *J. Hepatol.* 2006; **44**: 455–61.

7 El-Serag HB. Epidemiology of hepatocellular carcinoma in USA. *Hepatol. Res.* 2007; **37** (Suppl. 2): S88–94.

8 Farrell GC. New hepatitis C guidelines for the Asia–Pacific region: APASL consensus statements on the diagnosis, management and treatment of hepatitis C virus infection. *J. Gastroenterol. Hepatol.* 2007; **22**: 607–10.

9 Tanaka Y, Kurbanov F, Mano S *et al.* Molecular tracing of the global hepatitis C virus epidemic predicts regional patterns of hepatocellular carcinoma mortality. *Gastroenterology* 2006; **130**: 703–14.

10 Tanaka Y, Hanada K, Mizokami M *et al.* Inaugural article: a comparison of the molecular clock of hepatitis C virus in the United States and Japan predicts that hepatocellular carcinoma incidence in the United States will increase over the next two decades. *Proc. Natl Acad. Sci. USA* 2002; **99**: 15584–9.

11 Kiyosawa K, Tanaka E. Characteristics of hepatocellular carcinoma in Japan. *Oncology* 2002; **62** (Suppl. 1): 5–7.

12 Umemura T, Kiyosawa K. Epidemiology of hepatocellular carcinoma in Japan. *Hepatol. Res.* 2007; **37** (Suppl. 2): S95–100.

13 Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* 1989; **244**: 359–62.

14 Bruix J, Barrera JM, Calvet X *et al.* Prevalence of antibodies to hepatitis C virus in Spanish patients with hepatocellular carcinoma and hepatic cirrhosis. *Lancet* 1989; **2**: 1004–6.

15 Colombo M, Kuo G, Choo QL *et al.* Prevalence of antibodies to hepatitis C virus in Italian patients with hepatocellular carcinoma. *Lancet* 1989; 2: 1006–8.
16 Farci P, Alter HJ, Wong D *et al.* A long-term study of hepatitis C virus replication in non-A, non-B hepatitis. *N. Engl. J. Med.* 1991; 325: 98–104.
17 Takahashi M, Yamada G, Miyamoto R, Doi T, Endo H, Tsuji T. Natural course of chronic hepatitis C. *Am. J. Gastroenterol.* 1993; 88: 240–3.
18 Tsukuma H, Hiyama T, Tanaka S *et al.* Risk factors for hepatocellular carcinoma among patients with chronic liver disease. *N. Engl. J. Med.* 1993; 328: 1797–801.
19 Yano M, Kumada H, Kage M *et al.* The long-term pathological evolution of chronic hepatitis C. *Hepatology* 1996; 23: 1334–40.
20 Budhu A, Wang XW. The role of cytokines in hepatocellular carcinoma. *J. Leukoc. Biol.* 2006; 80: 1197–213.
21 Furutani T, Hino K, Okuda M *et al.* Hepatic iron overload induces hepatocellular carcinoma in transgenic mice expressing the hepatitis C virus polyprotein. *Gastroenterology* 2006; 130: 2087–98.
22 Hassan MM, Hwang LY, Hatten CJ *et al.* Risk factors for hepatocellular carcinoma: synergism of alcohol with viral hepatitis and diabetes mellitus. *Hepatology* 2002; 36: 1206–13.
23 Ikeda K, Marusawa H, Osaki Y *et al.* Antibody to hepatitis B core antigen and risk for hepatitis C-related hepatocellular carcinoma: a prospective study. *Ann. Intern. Med.* 2007; 146: 649–56.
24 Kamegaya Y, Hiasa Y, Zukerberg L *et al.* Hepatitis C virus acts as a tumor accelerator by blocking apoptosis in a mouse model of hepatocarcinogenesis. *Hepatology* 2005; 41: 660–7.
25 Kenny-Walsh E. Clinical outcomes after hepatitis C infection from contaminated anti-D immune globulin. Irish Hepatology Research Group. *N. Engl. J. Med.* 1999; 340: 1228–33.
26 Moriya K, Fujie H, Shintani Y *et al.* The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. *Nat. Med.* 1998; 4: 1065–7.
27 Ohki T, Tateishi R, Sato T *et al.* Obesity is an independent risk factor for hepatocellular carcinoma development in chronic hepatitis C patients. *Clin. Gastroenterol. Hepatol.* 2008; 6: 459–64.
28 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: 825–32.
29 Vogt M, Lang T, Frosner G *et al.* Prevalence and clinical outcome of hepatitis C infection in children who underwent cardiac surgery before the implementation of blood-donor screening. *N. Engl. J. Med.* 1999; 341: 866–70.
30 Makuuchi M, Kokudo N, Arii S *et al.* Development of evidence-based clinical guidelines for the diagnosis and treatment of hepatocellular carcinoma in Japan. *Hepatol. Res.* 2008; 38: 37–51.
31 Perz JF, Alter MJ. The coming wave of HCV-related liver disease: dilemmas and challenges. *J. Hepatol.* 2006; 44: 441–3.
32 Bruix J, Sherman M. Management of hepatocellular carcinoma. *Hepatology* 2005; 42: 1208–36.
33 Maki A, Kono H, Gupta M *et al.* Predictive power of biomarkers of oxidative stress and inflammation in patients with hepatitis C virus-associated hepatocellular carcinoma. *Ann. Surg. Oncol.* 2007; 14: 1182–90.
34 Sherman M. Screening for hepatocellular carcinoma. *Hepatol. Res.* 2007; 37 (Suppl. 2): S152–65.
35 Miki D, Aikata H, Uka K *et al.* Clinicopathological features of elderly patients with hepatitis C virus-related hepatocellular carcinoma. *J. Gastroenterol.* 2008; 43: 550–7.
36 Alter MJ. Healthcare should not be a vehicle for transmission of hepatitis C virus. *J. Hepatol.* 2008; 48: 2–4.
37 Armstrong GL, Alter MJ, McQuillan GM, Margolis HS. The past incidence of hepatitis C virus infection: implications for the future burden of chronic liver disease in the United States. *Hepatology* 2000; 31: 777–82.
38 Micallef JM, Macdonald V, Jauncey M *et al.* High incidence of hepatitis C virus reinfection within a cohort of injecting drug users. *J. Viral. Hepat.* 2007; 14: 413–18.
39 Wiese M, Grungreiff K, Guthoff W, Lafrenz M, Oesen U, Porst H. Outcome in a hepatitis C (genotype 1b) single source outbreak in Germany – a 25-year multicenter study. *J. Hepatol.* 2005; 43: 590–8.
40 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: 1562–9.
41 Alter MJ. Epidemiology of viral hepatitis and HIV co-infection. *J. Hepatol.* 2006; 44 (Suppl. 1): S6–9.
42 Buffington J, Mast E. Viral hepatitis. In: Wallace R, Kohatsu N, eds. *Public Health and Preventive Medicine*, 15th edn. New York: McGraw-Hill Companies, 2008; 211–28.
43 Esteban JI, Sauleda S, Quer J. The changing epidemiology of hepatitis C virus infection in Europe. *J. Hepatol.* 2008; 48: 148–62.
44 Khan MH, Farrell GC, Byth K *et al.* Which patients with hepatitis C develop liver complications? *Hepatology* 2000; 31: 513–20.
45 McCaughan GW, Omata M, Amarapurkar D *et al.* Asian Pacific Association for the Study of the Liver consensus statements on the diagnosis, management and treatment of hepatitis C virus infection. *J. Gastroenterol. Hepatol.* 2007; 22: 615–33.
46 Pawlotsky JM, Dusheiko G, Hatzakis A *et al.* Virologic monitoring of hepatitis B virus therapy in clinical trials and practice: recommendations for a standardized approach. *Gastroenterology* 2008; 134: 405–15.
47 Treloar C, Laybutt B, Jauncey M *et al.* Broadening discussions of "safe" in hepatitis C prevention: a close-up of swabbing in an analysis of video recordings of injecting practice. *Int. J. Drug Policy* 2008; 19: 59–65.
48 Page-Shafer K, Hahn JA, Lum PJ. Preventing hepatitis C virus infection in injection drug users: risk reduction is not enough. *AIDS* 2007; 21: 1967–9.
49 Madden A, Cavalieri W. Hepatitis C prevention and true harm reduction. *Int. J. Drug Policy* 2007; 18: 335–7.
50 Beek I, Dwyer R, Dore GJ, Luo K, Kaldor JM. Infection with HIV and hepatitis C virus among injecting drug users in a prevention setting: retrospective cohort study. *BMJ* 1998; 317: 433–7.
51 Brau N, Fox RK, Xiao P *et al.* Presentation and outcome of hepatocellular carcinoma in HIV-infected patients: a U.S.-Canadian multicenter study. *J. Hepatol.* 2007; 47: 527–37.
52 Qurishi N, Kreuzberg C, Luchters G *et al.* Effect of antiretroviral therapy on liver-related mortality in patients with HIV and hepatitis C virus coinfection. *Lancet* 2003; 362: 1708–13.
53 Bonacini M, Louie S, Bzowej N, Wohl AR. Survival in patients with HIV infection and viral hepatitis B or C: a cohort study. *AIDS* 2004; 18: 2039–45.
54 Yotsuyanagi H, Shintani Y, Moriya K *et al.* Virologic analysis of non-B, non-C hepatocellular carcinoma in Japan: frequent involvement of hepatitis B virus. *J. Infect. Dis.* 2000; 181: 1920–8.
55 Brechot C. Pathogenesis of hepatitis B virus-related hepatocellular carcinoma: old and new paradigms. *Gastroenterology* 2004; 127 (Suppl. 1): S56–61.
56 McHutchison JG, Poynard T, Esteban-Mur R *et al.* Hepatic HCV RNA before and after treatment with interferon alone or combined with ribavirin. *Hepatology* 2002; 35: 688–93.
57 McHutchison JG, Patel K. Future therapy of hepatitis C. *Hepatology* 2002; 36 (Suppl. 1): S245–52.

58 Shiratori Y, Imazeki F, Moriyama M *et al.* Histologic improvement of fibrosis in patients with hepatitis C who have sustained response to interferon therapy. *Ann. Intern. Med.* 2000; **132**: 517–24.
59 Azzaroli F, Accogli E, Nigro G *et al.* Interferon plus ribavirin and interferon alone in preventing hepatocellular carcinoma: a prospective study on patients with HCV related cirrhosis. *World J. Gastroenterol.* 2004; **10**: 3099–102.
60 Camma C, Giunta M, Andreone P, Craxi A. Interferon and prevention of hepatocellular carcinoma in viral cirrhosis: an evidence-based approach. *J. Hepatol.* 2001; **34**: 593–602.
61 Fartoux L, Degos F, Trepo C *et al.* Effect of prolonged interferon therapy on the outcome of hepatitis C virus-related cirrhosis: a randomized trial. *Clin. Gastroenterol. Hepatol.* 2007; **5**: 502–7.
62 Hung CH, Lee CM, Lu SN *et al.* Long-term effect of interferon alpha-2b plus ribavirin therapy on incidence of hepatocellular carcinoma in patients with hepatitis C virus-related cirrhosis. *J. Viral. Hepat.* 2006; **13**: 409–14.
63 Imai Y, Kawata S, Tamura S *et al.* Relation of interferon therapy and hepatocellular carcinoma in patients with chronic hepatitis C. Osaka Hepatocellular Carcinoma Prevention Study Group. *Ann. Intern. Med.* 1998; **129**: 94–9.
64 Nishiguchi S, Kuroki T, Nakatani S *et al.* Randomised trial of effects of interferon-alpha on incidence of hepatocellular carcinoma in chronic active hepatitis C with cirrhosis. *Lancet* 1995; **346**: 1051–5.
65 Nishiguchi S, Shiomi S, Nakatani S *et al.* Prevention of hepatocellular carcinoma in patients with chronic active hepatitis C and cirrhosis. *Lancet* 2001; **357**: 196–7.
66 Okanoue T, Itoh Y, Minami M *et al.* Interferon therapy lowers the rate of progression to hepatocellular carcinoma in chronic hepatitis C but not significantly in an advanced stage: a retrospective study in 1148 patients. Viral Hepatitis Therapy Study Group. *J. Hepatol.* 1999; **30**: 653–9.
67 Yoshida H, Shiratori Y, Moriyama M *et al.* Interferon therapy reduces the risk for hepatocellular carcinoma: national surveillance program of cirrhotic and noncirrhotic patients with chronic hepatitis C in Japan. IHIT Study Group. Inhibition of Hepatocarcinogenesis by Interferon Therapy. *Ann. Intern. Med.* 1999; **131**: 174–81.
68 Yu ML, Lin SM, Chuang WL *et al.* A sustained virological response to interferon or interferon/ribavirin reduces hepatocellular carcinoma and improves survival in chronic hepatitis C: a nationwide, multicentre study in Taiwan. *Antivir. Ther.* 2006; **11**: 985–94.
69 Bernardinello E, Cavalletto L, Chemello L *et al.* Long-term clinical outcome after beta-interferon therapy in cirrhotic patients with chronic hepatitis C. TVVH Study Group. *Hepatogastroenterology* 1999; **46**: 3216–22.
70 Soga K, Shibasaki K, Aoyagi Y. Effect of interferon on incidence of hepatocellular carcinoma in patients with chronic hepatitis C. *Hepatogastroenterology* 2005; **52**: 1154–8.
71 Mazzella G, Accogli E, Sottili S *et al.* Alpha interferon treatment may prevent hepatocellular carcinoma in HCV-related liver cirrhosis. *J. Hepatol.* 1996; **24**: 141–7.
72 Jacobson IM, Brown RS Jr, Freilich B *et al.* Peginterferon alfa-2b and weight-based or flat-dose ribavirin in chronic hepatitis C patients: a randomized trial. *Hepatology* 2007; **46**: 971–81.
73 Manns MP, McHutchison JG, Gordon SC *et al.* 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**: 958–65.
74 Shiffman ML, Di Bisceglie AM, Lindsay KL *et al.* Peginterferon alfa-2a and ribavirin in patients with chronic hepatitis C who have failed prior treatment. *Gastroenterology* 2004; **126**: 1015–23; discussion 947.
75 Kasahara A, Hayashi N, Mochizuki K *et al.* Risk factors for hepatocellular carcinoma and its incidence after interferon treatment in patients with chronic hepatitis C. Osaka Liver Disease Study Group. *Hepatology* 1998; **27**: 1394–402.
76 Yu ML, Dai CY, Chen SC *et al.* High versus standard doses interferon-alpha in the treatment of naive chronic hepatitis C patients in Taiwan: a 10-year cohort study. *BMC Infect. Dis.* 2005; **5**: 27.
77 Berenguer M. Systematic review of the treatment of established recurrent hepatitis C with pegylated interferon in combination with ribavirin. *J. Hepatol.* 2008; **49**: 274–87.
78 Dienstag JL, McHutchison JG. American Gastroenterological Association medical position statement on the management of hepatitis C. *Gastroenterology* 2006; **130**: 225–30.
79 Okanoue T, Itoh Y, Minami M *et al.* Guidelines for the antiviral therapy of hepatitis C virus carriers with normal serum aminotransferase based on platelet counts. *Hepatol. Res.* 2008; **38**: 27–36.
80 Strader DB, Wright T, Thomas DL, Seeff LB. Diagnosis, management, and treatment of hepatitis C. *Hepatology* 2004; **39**: 1147–71.
81 Everson GT, Hoefs JC, Seeff LB *et al.* Impact of disease severity on outcome of antiviral therapy for chronic hepatitis C: lessons from the HALT-C trial. *Hepatology* 2006; **44**: 1675–84.
82 Lok AS, Ghany MG, Goodman ZD *et al.* Predicting cirrhosis in patients with hepatitis C based on standard laboratory tests: results of the HALT-C cohort. *Hepatology* 2005; **42**: 282–92.
83 Di Bisceglie AM, Shiffman ML, Everson GT *et al.* Prolonged therapy of advanced chronic hepatitis C with low-dose peginterferon. *N. Engl. J. Med.* 2008; **359**: 2429–41.
84 Arase Y, Ikeda K, Murashima N *et al.* The long term efficacy of glycyrrhizin in chronic hepatitis C patients. *Cancer* 1997; **79**: 1494–500.
85 Wakusawa S, Ikeda R, Takikawa T, Hayashi H, Yano M, Yoshioka K. Combined phlebotomy and ursodeoxycholic acid treatment in the patients with chronic hepatitis C. *Hepatol. Res.* 2000; **18**: 54–62.
86 Farrell GC, Fan J. Prevention of hepatocellular carcinoma. In: Al Knawy B, Reddy K, Bolondi L, eds. *Hepatocellular Carcinoma: A Practical Approach*. Bensheim: Reichert, 2009 (in press).

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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

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