Association Between the Various Mutations in Viral Core Promoter Region to Different Stages of Hepatitis B, Ranging of Asymptomatic Carrier State to Hepatocellular Carcinoma

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OBJECTIVES: **The objective of this study was to determine the association of 19 mutations with frequencies ≥10% in the core promoter region of hepatitis B virus (HBV) with chronic hepatitis B (CHB), liver cirrhosis, and hepatocellular carcinoma (HCC).**

METHODS: **Eight hundred forty-six asymptomatic hepatitis B surface antigen carriers (ASCs), 235 CHB patients, 188 cirrhosis patients, and 190 HCC patients with intact data of HBV genotyping, DNA sequencing, and serological parameters were studied. Nucleotides with the highest frequencies in HBV genotypes B and C from all ASCs were treated as wild-type nucleotides.**

RESULTS: **Mutations at nt.1674, nt.1719, nt.1762, nt.1764, nt.1846, nt.1896, and nt.1913 in genotype C were significantly associated with CHB, cirrhosis, and HCC, as compared with ASCs. C1673T, A1726C, A1727T, C1730G, C1766T, T1768A, C1773T, and C1799G in genotype C were significantly associated with cirrhosis compared with the CHB patients, whereas these mutations were inversely associated with HCC compared with the cirrhosis patients. Multivariate regression analyses showed that age, male, abnormal alanine aminotransferase (ALT), T1768A, A1762T/G1764A, and A1846T were independently associated with cirrhosis compared with ASCs and the patients with CHB. Age, abnormal ALT, HBV DNA (≥10[4] copies/ml), genotype C, C1653T, T1674C/G, T1753V, and A1762T/G1764A were independently associated with HCC compared with those without HCC. Haplotypic carriages with two or more HBV mutations were significantly associated with HCC. T1674C/G, C1653T, and T1753V were specific for HCC. A1762T/G1764A had a moderate sensitivity and specificity for HCC.**

CONCLUSIONS: **C1673T, A1726C, A1727T, C1730G, C1766T, T1768A, C1773T, and C1799G in genotype C are specific for cirrhosis. A1846T and T1674C/G are novel factors independently associated with cirrhosis and HCC, respectively.**

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INTRODUCTION

Hepatitis B virus (HBV) infection remains a major public health problem globally. More than two billion people have been infected worldwide, and of these, >350 million suffer from chronic HBV infection (1). Chronic HBV infection results in approximately one third of all cases of liver cirrhosis (LC) and more than three quarters of all cases of hepatocellular carcinoma (HCC) worldwide (2). Chronic hepatitis B (CHB), LC, and HCC are progressive stages of chronic HBV infection.

HBV belongs to the hepadnaviridae, a family of enveloped viruses with an incomplete double-stranded DNA genome of 3.2 kb. HBV genome contains four overlapping open reading frames that encode the surface, core, X, and polymerase proteins. HBV core promoter region including the basal core promoter (nucleotides 1751–1769) and the enhancer II (nucleotides 1636–1744) overlaps with the X open reading frame (nucleotides 1374–1835). The basal core promoter, which is regulated by the enhancer II and to some extent by the enhancer I, controls the transcription of precore mRNA and pregenomic RNA (3). The precore region encodes the precore protein, which is processed in the endoplasmic reticulum to produce secreted hepatitis B e antigen (HBeAg). HBeAg expression indicates active viral replication. HBeAg expression and high viral load are associated with an increased risk of HCC (4–6). According to a sequence divergence >8% in the entire HBV genome, 10 genotypes (A–J) have been identified so far (7–10). HBV genotypes have distinct geographical distributions worldwide, and have been shown to differ with regard to clinical diseases, prognosis, and response to interferon treatment (10–19). Genotypes B and C are endemic in Asia. Infection with genotype C is often associated with the risks of LC and HCC at older age compared with infection with genotype B (10–16). HBV genotypes have distinct patterns of the core promoter mutations that have been shown to be associated with the risk of HCC (20–31). Our recent meta-analysis has shown that C1653T, T1753V, and A1762T/G1764A are each associated with an increased risk of HCC, and these mutations are increasingly more prevalent as chronic HBV infection progress from asymptomatic hepatitis B surface antigen (HBsAg) carrier (ASC) state to LC or HCC (32). A1762T/G1764A has been shown to be a valuable biomarker for identifying a subset of HBV-infected subjects who are at extremely high risk of HCC in prospective studies (26,29,30).

However, the association of the HBV mutations in the core promoter region with HCC remains controversial because of conflicting data (33,34). Most of the studies only had a limited number of participants, and often examined only specific viral mutations and rarely analyzed other mutations that might have important roles. To elucidate the association of HBV mutations with the progressive liver diseases, it is essential to clarify "wild-type" patterns of given HBV genotypes from the subjects without clinical liver diseases. However, no epidemiological study with sufficient participants has shown the "wild-type" pattern of a given HBV genotype and the genotype-specific mutation pattern in the core promoter region of HBV as well. Although prospective study is more reliable than cross-sectional study in elucidating causal relationship between HBV mutations and HCC, the number of persons who developed HCC during follow-up was very limited, and some confounding factors like unavoidably receiving antiviral treatment and exposure to other risk factors might be introduced during the long-term follow-up. Thus, cross-sectional case–control study, which compares the frequencies of HBV mutations in ASCs with those in the patients with CHB, in the patients with LC, and in those with HCC, should be reliable for analyzing the association of HBV mutation with HCC. In this study, we initially identified the wild-type nucleotides in the core promoter region of HBV genotypes B and C from 846 ASCs, and then evaluated the association of all mutations with the frequencies >10% in this region with CHB, LC, or HCC.

METHODS

Study subjects

ASCs were screened in our community-based epidemiological bases in Shanghai and four surrounding cities Ningbo, Soochow, Huzhou, and Zhangjiagang from January to March 2008 and revisited from May to July 2009. The HCC patients and the LC patients were newly diagnosed at the three affiliated hospitals of this university and the first people hospital of Zhangjiagang from October 2008 to March 2009. The CHB patients were newly diagnosed at the first people hospital of Zhangjiagang from October 2008 to July 2009. All participants were positive for HBsAg. The included ASCs fulfilled the following criteria: (i) seropositive for HBsAg for at least 6 months, (ii) no evidence of LC or HCC based on the clinical criteria and ultrasound examination, and (iii) normal alanine aminotransferase (ALT) (upper limit, 45 U/l). The patients with HCC were diagnosed on the basis of cytologic or pathologic findings or an elevated α-fetoprotein (≥400 ng/ml) combined with the positive image on computerized tomography, regardless of cirrhosis or inflammation state. The patients with LC were diagnosed by histologic analysis of liver biopsy specimens or by findings of repeated ultrasonography that were suggestive of cirrhosis and supplemented with clinical criteria indicating portal hypertension (i.e., the presence of ascites, thrombocytopenia, and esophageal varices). The patients with clear signs of cirrhosis and free of HCC were diagnosed as having LC, regardless of inflammation state. The diagnosis of CHB was basically according to the previous report (35): (i) seropositive for HBsAg for at least 6 months, (ii) serum HBV DNA $>1\times10^5$ copies/ml for HBeAg-positive and $>1\times10^4$ copies/ml for HBeAg-negative patients, (iii) persistent or intermittent elevation in ALT level, (iv) liver biopsy showing chronic hepatitis with moderate or severe necroinflammation. The patients with apparent inflammatory activity and free of ultrasonographic or histologic cirrhosis and HCC were diagnosed as having CHB. We excluded the subjects whose medical records showed a history of having received antiviral (such as interferon, lamivudine, or adefovir) or immunosuppressive treatments and the patients with acute hepatitis B who were diagnosed as previously described (36). Patients who had other possible causes of hepatitis or LC, including autoimmune hepatitis, coinfection with hepatitis C virus or D virus, primary biliary cirrhosis, Wilson's disease, fatty liver, hemochromatosis,

and other concurrent illness, were also excluded. Nine hundred sixty-seven ASCs, 327 patients with CHB, 209 patients with LC, and 275 patients with HCC who had intact clinical and serological data were subjected for HBV genotyping and DNA sequencing. The study protocol conformed to the 1975 Declaration of Helsinki and was approved by the ethics committee of this university. All patients gave informed consents.

Examination of serological HBV markers, liver function, and HBV DNA concentration and HBV genotyping

Before clinical treatment, 5 ml fasting blood of each subject was collected with a vacuum blood collection tube without anticoagulant. The serum was separated by centrifugation at 4°C and stored in a sterile tube at −80°C within 4 h of sample collection. Serological HBV markers, liver function, and HBV DNA concentration were examined as previously described (17, 37). HBV genotypes and subgenotypes were determined by using a multiplex PCR assay (17). HBV genotypes of samples with low level of HBV DNA were identified by nested multiplex PCR. The primer set for the first round of the nested PCR was 5′-TTTGCGGGTCACCATA TTCTTGG-3′ and 5′-CGAACCACTGAACAAATGGCACTAG-3′, amplifying a fragment of 1,106 bp (from nt.2815 to nt.705). PCR was performed in 25 μl mixture containing 3 μl diluted HBV DNA, 1×PCR buffer, 1.5 U Taq polymerase (TaKaRa Biotechnology, Dalian, China), 0.2 μM each primer (synthesized by Sangon Biotechnology, Shanghai, China), and 0.2 mM dNTP (TaKaRa). The amplification was performed with annealing temperature of 58°C for 60 s for 35 cycles by using an Autorisierter thermocycler (Eppendorf AG, Hamburg, Germany). The product (2 μl) of the first round PCR served as a template for routine multiplex PCR in total volume of 50 μl. The amplicons were examined as previously described (37).

HBV DNA sequencing and mutation analysis

The core promoter region of HBV was amplified by nested PCR. The primer set for the first round of the nested PCR was 5′-TGCACTTCGCTTCACCTCTG-3′ and 5′-TAAGCGGGAGG AGTGCGAAT-3′, amplifying a fragment of 717 bp (from nt.1594 to nt.2310). The primer set for the second round of the nested PCR was 5′-TCGCATGGAGACCACCGTGA-3′ and 5′-ATAGCTTGCCTGAGTGC-3′, amplifying a fragment of 473 bp (from nt.1604 to nt.2076). PCR was performed in 25 μl mixture containing 3 μl HBV DNA, 1×PCR buffer, 1.5 U Taq polymerase (Promega, Madison, WI), 0.2 μM each primer (synthesized by Sangon Biotechnology), and 0.2 mM dNTP (Promega). The thermocycler was programmed to initially denature the samples for 3 min at 95°C, followed by 30 cycles consisting of 94°C for 60 s, 53°C for 55 s, 72°C for 60 s for the first round reaction, and followed by a final elongation step at 72°C for 10 min. For the second round reaction, the annealing temperature was set as 55°C, and the other conditions were the same as the first round reaction. Direct DNA sequencing was carried out by using ABI PRISM BigDye sequencing kits and an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA). All sequences were analyzed in both forward and backward directions. For viral mutation analysis, we sequenced

the core promoter region from 1,459 subjects who were selected from those infected with unique HBV genotype, either genotype B (B2, 100.0%) or genotype C (C2, 98.5%). These sequences were blunted (from nt.1626 to nt.2004) and deposited in GenBank with accession numbers (GQ858378–GQ859134, GQ277657–GQ278209, GQ278220–GQ278282, GQ278284–GQ278335, and GQ278353–GQ278386). Phylogenetic analysis was performed to confirm HBV genotyping results selectively. Sequence alignment and phylogenetic analysis were performed by using MEGA 4.0 software (38).

Definition of wild-type and mutated nucleotides

After the alignment, a nucleotide with the highest frequency at each site in the core promoter region of HBV genotype B or genotype C from ASCs was termed as a wild-type nucleotide. Nucleotide substitutions with other three nucleotides and deletion at each site were termed as mutations. A site with the frequencies of mutations in combination >10%, either in genotype B or genotype C from all participants, was termed as a hotspot.

Statistical analysis

χ^2 test was used to determine the differences in categorical variables, such as HBeAg positivities. Continuous variables, such as serum viral loads with skewed distribution, were adjusted to normal distribution by transformation into logarithmic function, and then tested by Student's t-test or analysis of variance. We also evaluated linear trend in the frequencies of each HBV mutation with increasing age. Forward stepwise multivariate regression analysis ($P_{entry} = 0.05$, $P_{removal} = 0.10$) was performed to obtain the adjusted odds ratios of factors, which were significantly associated with the disease in univariate analysis for the risk of LC or HCC and their 95% confidence intervals. All statistical tests were two sided, and performed by using the Statistical Program for Social Sciences (SPSS15.0 for Windows, SPSS, Chicago, IL). A P value of <0.05 was considered as statistically significant. For multiple comparisons, P values were corrected by the Bonferroni correction. The estimated haplotype frequencies of HBV mutations in combination were determined as previously described (37). The sensitivity and the specificity of single mutation and combined mutations for HCC were calculated as previously described (32).

RESULTS

Clinical and virological characteristics of study subjects

Eight hundred forty-six ASCs, 235 CHB patients, 188 LC patients, and 190 HCC patients who had intact data of HBV genotyping and HBV DNA sequencing were included in the analysis. Clinical and virological characteristics of the study subjects are listed in **Table 1**. Of the HCC patients, 139 (73.2%) had LC. The patients with HCC were significantly older, and had a significantly higher proportion of men than ASCs and the patients with CHB. There were no significant differences in ALT levels and the compositions of age and sex between the patients with LC and those with HCC. There was no difference in viral load between the patients with HCC and those with CHB. The frequency of HBeAg expression in

Table 1. Clinical and virological characteristics of the study subjects

Characteristic	ASCs	CHB patients	LC patients	HCC patients	*P* value
No.	846	235	188	190	
Age (years)[a]	29.2±16.6	40.3±14.4	50.9±11.4	50.2±11.0	0.000
Male (%)	497 (58.7)	154 (65.5)	147 (78.2)	151 (79.5)	0.000
HBV genotype (%)					
B	284 (33.6)	54 (23.0)	47 (25.0)	21 (11.1)	
C	562 (66.4)	181 (77.0)	141 (75.0)	169 (88.9)	0.000
ALT (U/l)[a]	21.9±9.8	134.9±245.6	106.4±188.3	89.4±120.5	0.000
HBeAg (%)					
Negative	337 (39.9)	82 (38.1)	107 (73.3)	105 (55.3)	
Positive	507 (60.1)	133 (61.9)	39 (26.7)	85 (44.7)	0.000
HBV DNA levels (log$_{10}$ copies/ml)[a]	4.19±2.19	4.88±2.02	4.27±1.57	5.17±1.54	0.000

ALT, alanine aminotransferase; ASCs, asymptomatic hepatitis B surface antigen carriers; CHB, chronic hepatitis B; HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; HCC, hepatocellular carcinoma; LC, liver cirrhosis.
[a]Data are mean±s.d.

the patients with LC was significantly lower than that in those with CHB and those with HCC after the adjustment for age and sex (adjusted odds ratio=0.35, 95% confidence interval=0.21–0.57; adjusted odds ratio=0.44, 95% confidence interval=0.27–0.71, respectively).

Mutations in hotspots of HBV genotypes B and C and their association with HBeAg expression

The hotspots were observed at nt.1652, nt.1653, nt.1673, nt.1674, nt.1703, nt.1719, nt.1726, nt.1727, nt.1730, nt.1753, nt.1762, nt.1764, nt.1766, nt.1768, nt.1773, nt.1799, nt.1846, nt.1896, and nt.1913. The frequencies of mutations at nt.1653, nt.1674, nt.1703, nt.1719, nt.1727, nt.1753, nt.1762, nt.1764, nt.1766, and nt.1768 were significantly higher in genotype C than genotype B, and the opposite was true for nt.1652, nt.1726, nt.1896, and nt.1913 in all study subjects. Wild-type nucleotides determined by using the sequences from all ASCs were the same as those from ASCs seropositive for HBeAg. Among these hotspots, wild-type nucleotides at nt.1652, nt.1673, nt.1726, nt.1727, nt.1730, and nt.1799 were different between genotype B and genotype C in all ASCs. The frequencies of 78.9% (15/19) mutations in genotype B and 63.2% (12/19) mutations in genotype C were significantly higher in the HBeAg-negative than in the HBeAg-positive ASCs infected with the corresponding genotype (see **Supplementary Table 1** online).

Association of mutations in the core promoter region of HBV genotypes C and B with progressive liver diseases

To investigate the associations of mutations in the core promoter region of HBV genotypes B and C with CHB, LC, and HCC, we initially treated ASCs as controls. Mutations at nt.1674, nt.1719, nt.1762, nt.1764, nt.1846, nt.1896, and nt.1913 in genotype C were significantly associated with CHB, LC, and HCC, after the adjustment for age and sex, respectively (see **Supplementary Table 2**). As compared with the patients with CHB, mutations at nt.1673, nt.1727, nt.1730, nt.1764, nt.1766, nt.1768, nt.1773, nt.1799, and nt.1896 in both genotypes were significantly associated with LC after the adjustment (see **Supplementary Table 3**). Interestingly, mutation at nt.1719 was significantly associated with LC in genotype B, whereas it was inversely associated with LC in genotype C. As compared with the patients with LC, mutations at nt.1653, nt.1674, nt.1719, nt.1753, and nt.1762 in genotype C were significantly associated with HCC, whereas mutations at nt.1673, nt.1703, nt.1726, nt.1727, nt.1730, nt.1766, nt.1768, nt.1773, and nt.1799 in genotype C were inversely associated with HCC after the adjustment (see **Supplementary Table 4**). The associations of mutations at nt.1653, nt.1674, nt.1719, nt.1753, and nt.1762 in genotype C with HCC were only found in the HBeAg-negative patients, as compared with the patients with LC. Mutation at nt.1846 was significantly associated with HCC in the HBeAg-negative patients, whereas it was inversely associated with HCC in the HBeAg-positive patients after the adjustment (see **Supplementary Table 5**). **Figure 1** presents mutations significantly associated with CHB, LC, or HCC after the adjustment.

C1653T, T1674C, G1719T, A1762T, and A1846T were significantly associated with HCC, whereas A1652G, C1673T, A1726C, A1727T, C1730G, T1768A, C1773T, and C1799G were inversely associated with HCC, as compared with the patients with LC in the genotype C HBV-infected patients seronegative for HBeAg. C1673T, A1726C, A1727T, C1730G, C1766T, T1768A, C1773T, and C1799G were significantly associated with LC, as compared with the patients with CHB, whereas these mutations were inversely associated with HCC, as compared with the patients with LC (**Table 2**). As compared with ASCs and the patients with CHB, C1673T, T1674C, A1703G, A1726C, C1730G, C1799G, A1846T, and C1913A in genotype C and C1653T, G1719T, A1727G, T1753A/C, A1762T, G1764A, C1766T, T1768A, C1773T, and

Table 2. Associations of the nucleotide substitutions in HBV genotype C with LC and HCC, as compared with the patients with CHB and those with LC, respectively

Substitution	CHB (n=181)	LC (n=141)	HCC (n=169)	OR (95% CI)
C1673T				
—	0	2	0	
A	1	0	1	
C	164	92	143	1
T	16	47	25	5.24 (2.81–9.76), $P<0.001$[a,c]; 0.34 (0.20–0.59), $P<0.001$[b,c]
A1726C				
—	0	2	1	
A	169	89	151	1
C	10	50	15	9.49 (4.59–19.62), $P<0.001$[a,c]; 0.18 (0.09–0.33), $P<0.001$[b,c]
G	2	0	2	
A1727T				
—	0	1	1	
A	53	23	53	1
C	0	1	2	
G	113	63	97	
T	15	53	16	8.14 (3.83–17.30), $P<0.001$[a,c]; 0.13 (0.06–0.28), $P<0.001$[b,c]
C1730G				
—	0	1	1	
A	1	3	1	
C	162	83	149	1
G	17	53	18	6.09 (3.32–11.17), $P<0.001$[a,c]; 0.19 (0.10–0.34), $P<0.001$[b,c]
T	1	1	0	
C1766T				
—	8	3	3	
C	154	98	140	1
G	2	0	1	
T	17	40	25	3.70 (1.99–6.88), $P<0.001$[a,c]; 0.44 (0.25–0.77), $P=0.012$[b,c]
T1768A				
—	9	3	3	
A	4	34	17	13.65 (4.71–39.58), $P<0.001$[a,c]; 0.35 (0.19–0.67), $P=0.004$[b,c]
G	1	0	2	
T	167	104	147	1
C1773T				
—	1	0	1	
A	5	2	6	
C	165	103	154	1
G	1	2	3	
T	9	34	5	6.05 (2.79–13.13), $P<0.001$[a,c]; 0.10 (0.04–0.26), $P<0.001$[b,c]
C1799G				
C	164	91	146	1
G	17	50	21	5.30 (2.89–9.73), $P<0.001$[a,c]; 0.26 (0.15–0.46), $P<0.001$[b,c]
T	0	0	2	

CI, confidence interval; CHB, chronic hepatitis B; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; LC, liver cirrhosis; OR, odds ratio.
[a]The risk of LC compared with the patients with CHB.
[b]The risk of HCC compared with the patients with LC.
[c]Corrected using the Bonferroni correction.

G1896A in both genotypes were significantly associated with LC (see **Supplementary Table 6**). As compared with those without HCC, C1653T, T1674C/G, G1719T, A1727T, T1753V, C1913A in genotype C and T1753G, A1762T, G1764A, A1846T, and G1896A in both genotypes were significantly associated with HCC (see **Supplementary Table 7**).

Factors independently associated with LC as compared with ASCs and the CHB patients

The multiple factors, containing age (years), sex (male vs. female), HBV DNA level (≥10^4 vs. <10^4 copies/ml), HBV genotype (C vs. B), ALT (abnormal vs. normal), HBeAg status (positive vs. negative), and the presence of C1673T, A1703G, A1726C, C1727G, A1762T/G1764A, C1766T, T1768A, C1773T, A1846T, and G1896A were introduced into the multivariate regression model. Old age, male, abnormal ALT, T1768A, A1762T/G1764A, and

A1846T were found to be independently associated with LC, as compared with ASCs and the patients with CHB (**Table 3**).

Factors independently associated with HCC as compared with those without HCC

The multiple factors, containing age (years), sex (male vs. female), HBV DNA level (≥10^4 vs. <10^4 copies/ml), HBV genotype (C vs. B), ALT (abnormal vs. normal), HBeAg status (positive vs. negative), and the presence of C1653T, T1674C/G, G1719T, A1762T/G1764A, T1753V, A1846T, and C1913A were introduced into the multivariate regression model. Old age, abnormal ALT, HBV DNA (≥10^4 copies/ml), genotype C, C1653T, T1674C/G, T1753V, and A1762T/G1764A were independently associated with HCC, as compared with the subjects without HCC (**Table 4**).

Table 3. Factors independently associated with the risk of LC compared with ASCs and the patients with CHB by stepwise multivariate regression analysis

Factor	AOR (95% CI)	*P* value
Age (year)	1.05 (1.03 – 1.07)	0.000
Sex (male)	1.94 (1.02 – 3.70)	0.044
ALT (≥45 U/l)	2.91 (1.66 – 5.12)	0.000
T1768A	3.05 (1.32 – 7.02)	0.009
A1846T	1.94 (1.07 – 3.52)	0.029
A1762T/G1764A	2.85 (1.51 – 5.37)	0.001

ALT, alanine aminotransferase; AOR, adjusted odds ratio; ASC, asymptomatic hepatitis B virus surface antigen carrier; CI, confidence interval; CHB, chronic hepatitis B; LC, liver cirrhosis.

Table 4. Factors independently associated with the risk of HCC compared with those without HCC by stepwise multivariate regression analysis

Factor	AOR (95% CI)	*P* value
Age (year)	1.04 (1.03 – 1.06)	0.000
ALT (≥45 U/l)	1.94 (1.28 – 2.93)	0.002
HBV DNA ($\log_{10}$ copies/ml≥4)	4.53 (2.96 – 6.92)	0.000
Genotype C	1.93 (1.08 – 3.45)	0.026
C1653T	1.69 (1.03 – 2.76)	0.037
T1674C/G	1.65 (1.05 – 2.59)	0.031
T1753V	1.89 (1.17 – 3.05)	0.009
A1762T/G1764A	2.59 (1.60 – 4.20)	0.000

ALT, alanine aminotransferase; AOR, adjusted odds ratio; CI, confidence interval; HBV, hepatitis B virus; HCC, hepatocellular carcinoma.

Table 5. Estimated haplotype frequencies of the HBV polymorphisms in the patients with HCC and the HBV-infected subjects without HCC

Haplotype[a]	Non-HCC (*n*=908)	HCC (*n*=170)	OR (95% CI)	*P* value
1653C-1674T-1753T-1762A/1764G	0.566	0.112	0.10 (0.06 – 0.16)	0.000
1653C-1674T-1753T-1762T/1764A	0.173	0.194	1.15 (0.76 – 1.75)	0.505
1653C-1674T-1753V-**1762A/1764G**	0.011	0.006	0.53 (0.07 – 4.18)	0.845
1653T-**1674T-1753T-1762A/1764G**	0.013	0.006	0.44 (0.06 – 3.42)	0.674
1653C-1674G/C-**1753T-1762A/1764G**	0.039	0.018	0.45 (0.14 – 1.47)	0.175
1653C-1674G/C-**1753T**-1762T/1764A	0.030	0.082	2.93 (1.50 – 5.71)	0.001
1653C-1674T-1753V-1762T/1764A	0.065	0.170	2.96 (1.83 – 4.78)	0.000
1653T-**1674T-1753T**-1762T/1764A	0.045	0.159	3.99 (2.38 – 6.70)	0.000
1653C-1674G/C-1753V-1762T/1764A	0.023	0.129	6.28 (3.37 – 11.70)	0.000
1653T-**1674T**-1753V-1762T/1764A	0.009	0.035	4.12 (1.41 – 12.02)	0.015
1653T-1674G/C-**1753T**-1762T/1764A	0.014	0.071	5.23 (2.34 – 11.67)	0.000
1653T-1674G/C-1753V-1762T/1764A	0.003	0.018	7.27 (1.61 – 32.78)	0.013

CI, confidence interval; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; OR, odds ratio.
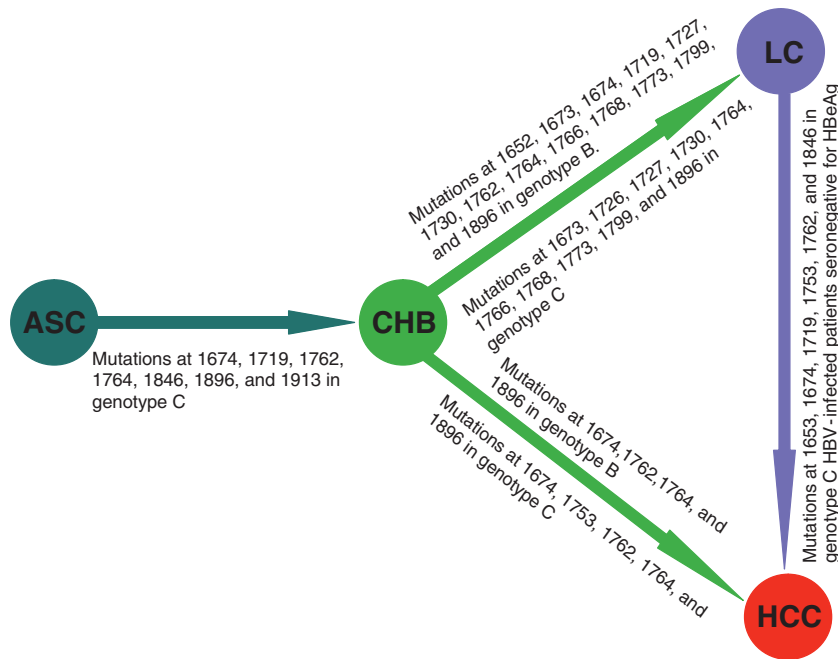[a]Wild-type nucleotides are marked in bold.

Figure 1. The associations of mutations in the core promoter region of hepatitis B virus (HBV) genotypes B and C with chronic hepatitis B (CHB), liver cirrhosis (LC), or hepatocellular carcinoma (HCC) after the adjustment for age and sex.

Changes in the frequencies of mutations with increasing age of the subjects at different disease states

All participants were divided into five age groups. The proportion of ASCs consecutively decreased with increasing age ($P_{trend} < 0.001$), the proportions of the LC patients and the HCC patients consecutively increased with increasing age ($P_{trend} < 0.001$ for each) (see **Supplementary Figure 1**). **Figure 2** presents changes in the frequencies of the six mutations independently associated with LC or HCC with increasing age of the subjects at different disease states. In the HBV-infected subjects without the end-stage liver diseases, the frequencies of C1653T, T1753V, T1768A, A1846T, and A1762T/G1764A, rather than T1674C/G, consecutively increased with increasing age ($P_{trend} < 0.05$ for each). The frequencies of the six mutations did not increase with increasing age in the patients with HCC. The frequencies of C1653T, T1674C/G, T1768A, A1846T, and A1762T/G1764A, rather than T1753V, did not increase with increasing age in the patients with LC. Average frequencies of C1653T, T1674C/G, T1753V, and A1762T/G1764A in the HCC patients were 30.3%, 34.6%, 33.0%, and 79.3% and significantly higher than those (8.8%, 11.8%, 9.3%, and 27.0%) in the HBV-infected subjects without HCC, respectively ($P < 0.001$ for each). Average frequencies of T1768A, A1846T, and A1762T/G1764A in the LC patients were 24.3%, 29.0%, and 61.9% and significantly higher than those (5.3%, 9.8%, and 21.0%) in ASCs and the CHB patients, respectively ($P < 0.001$ for each).

Association of estimated haplotype frequencies of HBV mutations in combination with HCC

Table 5 shows the estimated haplotype frequencies of C1653T, T1674C/G, T1753V, and A1762T/G1764A in combination and their association with HCC. Haplotypic carriage with wild-type forms of the four polymorphisms was inversely associated with HCC. In the absence of other three mutations, haplotypic carriage with A1762T/G1764A alone was not significantly associated with HCC. The same was true for C1653T, T1674C/G, and T1753V. Of the four HBV mutations, haplotypic carriages with two or more HBV mutations were significantly associated with HCC.

Sensitivity and specificity of HBV mutations for HCC

We then evaluated the potential value of the presence of HBV mutations that were independently associated with HCC for the indication of HCC. As shown in **Table 6**, T1674C/G, C1653T, and T1753V were specific for HCC. A1762T/G1764A had a moderate sensitivity and specificity for HCC. A1762T/G1764A or T1753V, A1762T/G1764A or C1653T, and A1762T/G1764A or T1674C/G were sensitive for HCC.

DISCUSSION

In this study, we excluded the subjects who had received antiviral treatment. As the presence of A1762T/G1764A is associated with a high rate of antiviral response in HBeAg-positive patients (39), antiviral therapy could decrease the ability to detect mutations. Mutations examined in this study are of naturally occurring HBV core promoter mutations during chronic HBV infection. We successfully sequenced 87.5%, 71.9%, 90.0%, and 69.1% HBV samples from ASCs, the CHB patients, the LC patients, and the HCC patients, respectively. Failure to amplify the core promoter region due to low viral concentration in sera or HBV mutations in the primer binding sites is the major reason for unsuccessfully sequencing the samples.

LIVER

Table 6. Sensitivity and specificity of HBV mutations alone or in combination for HCC

Mutation status	No. of HCC patients	No. of subjects without HCC	Sensitivity (95% CI) (%)	Specificity (95% CI) (%)
T1674C/G				
+	65	130	34.6 (27.8–41.4)	88.2 (86.3–90.1)
–	123	969		
C1653T				
+	56	92	30.3 (23.6–36.9)	91.2 (89.4–92.9)
–	129	949		
T1753V				
+	64	122	33.7 (27.0–40.4)	90.4 (88.8–92.0)
–	126	1,147		
A1762T/G1764A				
+	152	360	80.0 (74.3–85.7)	71.6 (69.2–74.1)
–	38	909		
C1653T or T1753V				
+	109	203	58.9 (51.8–66.0)	80.7 (78.3–83.1)
–	76	850		
A1762T/G1764A or T1753V				
+	154	382	81.1 (75.5–86.6)	69.9 (67.4–72.4)
–	36	887		
A1762T/G1764A or C1653T				
+	158	387	84.0 (78.8–89.3)	63.7 (60.8–66.6)
–	30	680		
A1762T/G1764A or T1674C/G				
+	164	420	86.8 (81.9–91.6)	62.4 (59.6–65.2)
–	25	697		

CI, confidence interval; HBV, hepatitis B virus; HCC, hepatocellular carcinoma.

In this study, 19 hotspots in HBV core promoter region were evaluated. We first determined wild-type nucleotides in this region of genotypes B and C from 846 ASCs, and found that wild-type nucleotides were the same as those from ASCs seropositive for HBeAg. The frequencies of 78.9% mutations in genotype B and 63.2% mutations in genotype C were significantly higher in HBeAg-negative than in HBeAg-positive ASCs infected with the corresponding HBV genotype. In the HBeAg-positive period, serum viral load is high and immune selection is week. Immune selection may increase during HBeAg seroconversion. HBeAg negativity has been associated with the presence of mutations in the basal core promoter and/or in the precore regions (40). Six of the 19 hotspots had different "wild-type" nucleotides between genotype B and genotype C. These evidences support that our definition of HBV wild-type nucleotides in the core promoter region is reasonable and the wild-type nucleotide in each hotspot may serve as a reference to evaluate the association of HBV mutations with the progressive liver diseases.

Some of the HBV mutations consecutively increase with aging and the advanced liver diseases are more frequent in men than in women (10,26). Age and sex may be the major confounders. We adjusted age and sex in evaluating the association of HBV mutations with CHB, LC, or HCC. In the subjects with genotype C, mutations at nt.1674, nt.1719, nt.1762, nt.1764, nt.1846, nt.1896, and nt.1913 were each associated with CHB, LC, and HCC, as compared with ASCs; whereas mutations at nt.1674, nt.1753, and nt.1762 were each associated with HCC compared with the CHB patients or the LC patients. In the subjects with genotype B, mutations at nt.1674, nt.1762, nt.1764, and nt.1896 were associated with HCC compared with the CHB patients (**Figure 1**). These mutations may indicate different stages of hepatitis B.

The majority of HBV-associated HCC (73.2% in this study) has LC. LC is an important end-stage liver disease of chronic HBV infection, and is common in HBeAg-negative patients. Old age, reactivation of hepatitis B, high ALT level, and genotype C have been shown to be risk factors of LC from the subjects chronically infected with HBV (41–45). Serum viral load has been found to be significantly associated with LC in the HBeAg-negative HBV-infected subjects (44,45). In this study, we found that mutations at nt.1653, nt.1674, nt.1719, nt.1753, nt.1762, and nt.1846 in genotype C were associated with HCC compared with the LC
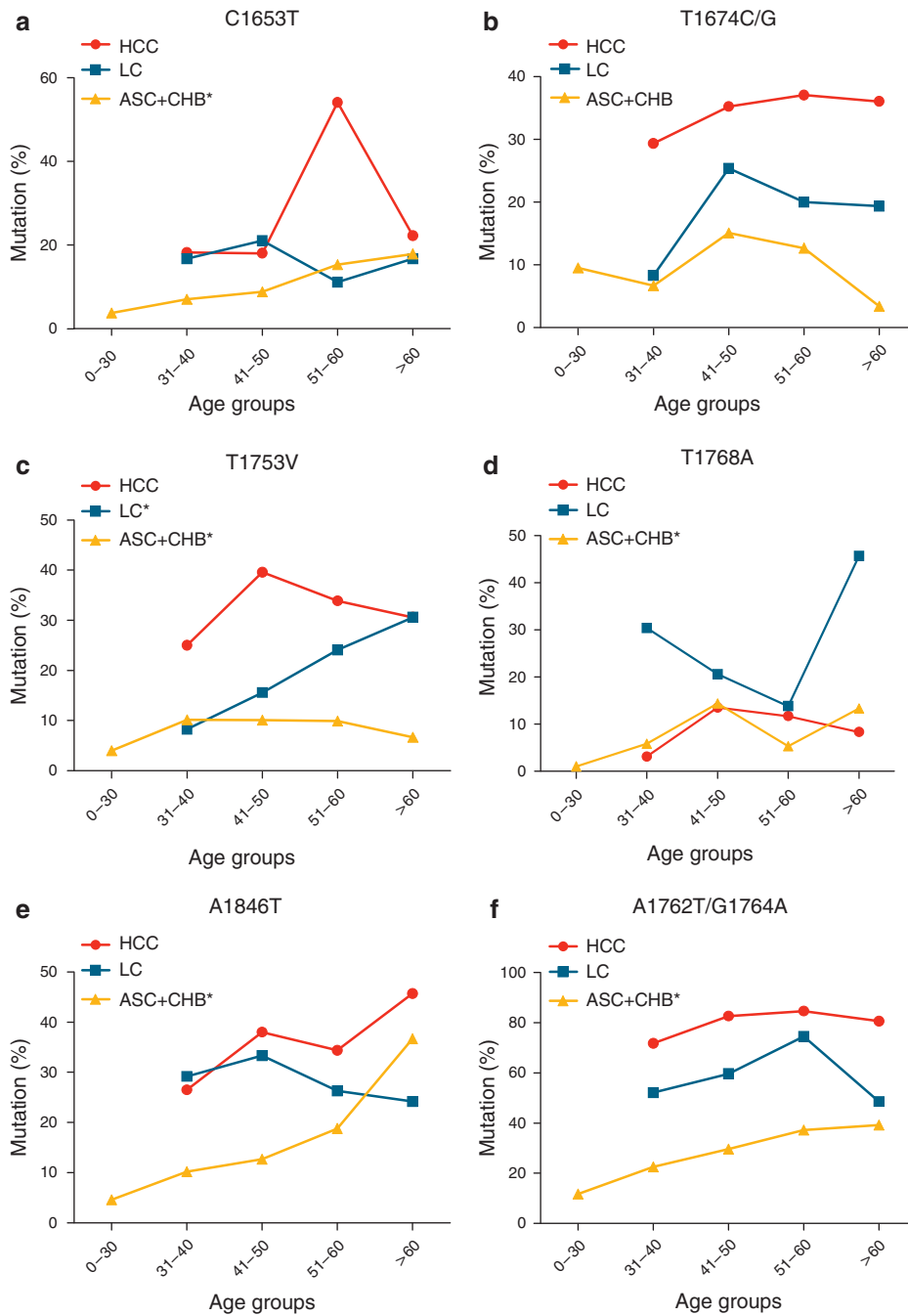
Figure 2. Changes in the frequencies of the mutations independently associated with liver cirrhosis (LC) or hepatocellular carcinoma (HCC) with increasing age of the study subjects at different disease states. (**a**, C1653T; **b**, T1674C/G; **c**, T1753V; **d**, T1768A; **e**, A1846T; **f**, A1762T/G1764A). *$P_{trend} < 0.05$.

patients, and these associations were only found in HBeAg-negative, rather than in HBeAg-positive subjects (see **Supplementary Table 5**). Interestingly, C1673T, A1726C, A1727T, C1730G, T1768A, C1773T, and C1799G were significantly associated with LC compared with the patients with CHB, whereas these mutations were inversely associated with HCC compared with the patients with LC. This result indicates that C1673T, A1726C, A1727T, C1730G, T1768A, C1773T, and C1799G are specific for LC in those infected with genotype C. These mutations might be associated with the natural wound healing proc-

ess to necroinflammation, rather than carcinogenesis. The multivariate analysis showed that old age, male sex, abnormal ALT, T1768A, A1762T/G1764A, and A1846T were independently associated with LC (**Table 3**). T1768A and A1762T/G1764A have been shown to be associated with LC (40,44). In this study, we added A1846T as a novel factor independently associated with LC. Thus, continuing liver damage in older, male subjects infected with HBV genotype C with mutations T1768A, A1762T/G1764A, and A1846T are apt to develop LC, especially for the HBV-infected subjects seronegative for HBeAg.

Multivariate regression analysis showed that C1653T, T1674C/G, A1762T/G1764A, and T1753V were independently associated with HCC compared with the subjects without HCC (**Table 4**). C1653T, T1753V, and A1762T/G1764A have been demonstrated to be significantly associated with HCC (32). In our study for analyzing another group of study subjects mostly recruited from May to October 2009, T1753V and A1762T/G1764A have been demonstrated to be independently associated with HCC, as compared with the age, sex-matched HBV-infected subjects without HCC (37). The results of this study are quite consistent with the previous findings. We demonstrated, for the first time, that T1674C/G was a novel factor independently associated with HCC. The HCC-associated HBV mutants in the core promoter region, for example, A1762T/G1764A mutants, may not transmit *via* mother-to-child vertical transmission because the children whose mothers carrying HBV mutants were mostly found to be infected with wild-type form of the same viruses (46,47). In this study, we found that although the frequencies of C1653T, T1753V, and A1762T/G1764A, rather than T1674C/G, consecutively increased with increasing age in ASCs and the patients with CHB, the frequencies of the six HCC-associated mutations went up to high levels in the patients with HCC even at relative young age (**Figure 2**). We therefore hypothesize that the HCC-associated HBV mutations are likely selected at one or several key steps of hepatocarcinogenesis.

Average frequencies of T1674C/G, C1653T, T1753V, and A1762T/G1764A were >30% in the patients with HCC, which make clinical examination feasible and clearly discriminate the HBV-infected patients with and without HCC. To elucidate the association of combined mutations in a given HBV genome with the risk of HCC, we innovatively investigated the estimated haplotype frequency of HBV mutations in combination and its association with HCC. Of the four HBV mutations independently associated with HCC, haplotypic carriages with two or more HBV mutations, rather than single one, were significantly associated with HCC. In the analysis of the sensitivity and specificity of these mutations, we found that T1674C/G, C1653T, and T1753V were specific for HCC, whereas A1762T/G1764A had moderate sensitivity and specificity for HCC (**Table 6**). These mutations, alone or in combination, may be reasonably arranged as molecular markers for HCC in the HBV-infected subjects.

HBV genotype-associated mutations in the core promoter region might be selected by HBV–host interaction during HBV chronic infection and in turn promote host hepatocarcinogenesis. It is also possible that the mutated X protein transactivates host oncogenes responsible for the development of HCC or that transactivators encoded by some oncogenes select the specific HBV mutations during hepatocarcinogenesis. HBV genotype C is independently associated with HCC, so are the core promoter mutations. We therefore hypothesized that the mutations in the core promoter region of genotype C might alter the binding sites of some nuclear factors related to hepatocarcinogenesis. To test this hypothesis, we determined "wild-type" HBV sequence from nt.1651 to nt.1920 by using the sequences of HBV genotype C from 562 ASCs, and then created a paired "mutated" sequence by replacing the "wild-type" nucleotides with the corresponding mutations that were significantly associated with the risk of HCC compared with ASCs after the adjustment (see **Supplementary Table 2**). With the use of TFSEARCH software online (http://www.cbrc.jp/research/db/TFSEARCH.html), we found different transcriptional factor binding sites between the "wild-type" sequence and the "mutated" sequence (see **Supplementary Figure 2**). Generation of HFH-2-, HNF-3b-, Skn-1-, Cdx-1-, cap-, and Oct-1-binding sites in the "mutated" sequence might represent novel virus–host interactions related to the development, viral replication, protooncogene activation, and carcinogenesis (48–50). The core promoter region encodes the C-terminal of HBV X protein. C1653T, T1674G/C, T1753V, A1762T, and G1764A mutations leaded to the amino acid (aa) transitions at aa94 (H-to-T), aa101 (S-to-A/P), aa127 (I-to-T/N/S), aa130 (K-to-M), and aa131 (V-to-I) sites. In addition, A1762T introduced a translation initiation site ATG at the C-terminal of HBx protein. HBV X mutations, especially the C-terminal deletions, have been frequently found in tumor tissues of HCC (51,52). Most of the C-terminally truncated HBx proteins lose their inhibitory effects on cell proliferation and transformation, retain their ability to bind to p53, and attenuate DNA repair and p53-mediated apoptosis, which may provide a selective clonal advantage for preneoplastic or neoplastic hepatocytes and contribute to hepatocarcinogenesis (53). However, the role of the amino acids substitutions at the C-terminal of HBx protein on hepatocarcinogenesis is rarely elucidated. It has been demonstrated that the core promoter mutations other than those at positions 1762 and 1764 have major impact on viral DNA replication and high viral load is the most important risk factor for HCC occurrence and recurrence after resection (5,6,11,12,54,55). High viral load due to HBV core promoter mutations may be one of the important causes of HBV-associated HCC.

In conclusion, this study systemically investigated the associations of HBV core promoter mutations with CHB, LC, or HCC. We found that C1673T, A1726C, A1727T, C1730G, T1768A, C1773T, and C1799G were specific for LC. A1846T and T1674C/G were novel factors independently associated with cirrhosis and HCC, respectively. Haplotypic carriages with two or more HBV mutations, rather than single one, were significantly associated with HCC. The frequencies of C1653T, T1753V, and A1762T/G1764A, rather than T1674C/G, consecutively increased with increasing age in ASCs and the patients with CHB, but not in the patients with HCC. Average frequencies of T1674C/G, C1653T, T1753V, and A1762T/G1764A were >30% in the patients with HCC. Sequential examination of these HBV mutations may be helpful in classifying the HBV-infected subjects who will develop HCC in future and need active antiviral treatments to postpone the occurrence of HCC. Although some mutations statistically associated with LC or HCC were found in this study, further study is needed to see if these mutations predict the development of LC or HCC in those who carry the mutation but do not yet have the associated disease.

CONFLICT OF INTEREST

Study Highlights

WHAT IS CURRENT KNOWLEDGE

✓ Age, male, abnormal alanine aminotransferase, high hepatitis B virus (HBV) load, and genotype C are associated with hepatocellular carcinoma (HCC).

✓ A1762T/G1764A, C1653T, and T1753V are associated with HCC.

✓ A1762T/G1764A and T1768A are associated with liver cirrhosis.

✓ The impact of HBV mutations on different stages of hepatitis B is poorly documented.

WHAT IS NEW HERE

✓ Hepatitis B virus (HBV) genotype-related viral mutations on chronic hepatitis B (CHB), liver cirrhosis (LC), or hepatocellular carcinoma (HCC) are well documented.

✓ C1673T, A1726C, A1727T, C1730G, T1768A, C1773T, and C1799G are specific for LC.

✓ A1846T is independently associated with LC.

✓ T1674C/G is independently associated with HCC and is specific for HCC.

✓ The frequency of T1674C/G does not increase with increasing age at any disease state.

✓ Haplotypic carriages with two or more HBV mutations, rather than single one, are significantly associated with HCC.

REFERENCES

1. Lavanchy D. Worldwide epidemiology of HBV infection, disease burden, and vaccine prevention. J Clin Virol 2005;34 (Suppl 1): S1–3.
2. Perz JF, Armstrong GL, Farrington LA et al. The contributions of hepatitis B virus and hepatitis C virus infections to cirrhosis and primary liver cancer worldwide. J Hepatol 2006;45:529–38.
3. Kay A, Zoulim F. Hepatitis B virus genetic variability and evolution. Virus Res 2007;127:164–76.
4. Yang HI, Lu SN, Liaw YF et al. Hepatitis B e antigen and the risk of hepatocellular carcinoma. N Engl J Med 2002;347:168–74.
5. Chen CJ, Yang HI, Su J et al. Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. JAMA 2006;295:65–73.
6. Wu CF, Yu MW, Lin CL et al. Long-term tracking of hepatitis B viral load and the relationship with risk for hepatocellular carcinoma in men. Carcinogenesis 2008;29:106–12.
7. Schaefer S. Hepatitis B virus taxonomy and hepatitis B virus genotypes. World J Gastroenterol 2007;13:14–21.
8. Olinger CM, Jutavijittum P, Hubschen JM et al. Possible new hepatitis B virus genotype, southeast Asia. Emerg Infect Dis 2008;14:1777–80.
9. Tatematsu K, Tanaka Y, Kurbanov F et al. A genetic variant of hepatitis B virus divergent from known human and ape genotypes isolated from a Japanese patient and provisionally assigned to new genotype J. J Virol 2009;83:10538–47.
10. Cao GW. Clinical relevance and public health significance of hepatitis B virus genomic variations. World J Gastroenterol 2009;15:5761–9.
11. Chan HL, Tse CH, Mo F et al. High viral load and hepatitis B virus subgenotype ce are associated with increased risk of hepatocellular carcinoma. J Clin Oncol 2008;26:177–82.
12. Yu MW, Yeh SH, Chen PJ et al. Hepatitis B virus genotype and DNA level and hepatocellular carcinoma: a prospective study in men. J Natl Cancer Inst 2005;97:265–72.
13. Chan HL, Hui AY, Wong ML et al. Genotype C hepatitis B virus infection is associated with an increased risk of hepatocellular carcinoma. Gut 2004;53:1494–8.
14. Kao JH, Chen PJ, Lai MY et al. Hepatitis B genotypes correlate with clinical outcomes in patients with chronic hepatitis B. Gastroenterology 2000;118:554–9.
15. Chan HL, Wong GL, Tse CH et al. Hepatitis B virus genotype C is associated with more severe liver fibrosis than genotype B. Clin Gastroenterol Hepatol 2009;7:1361–6.
16. Ni YH, Chang MH, Wang KJ et al. Clinical relevance of hepatitis B virus genotype in children with chronic infection and hepatocellular carcinoma. Gastroenterology 2004;127:1733–8.
17. Yin J, Zhang H, Li C et al. Role of hepatitis B virus genotype mixture, subgenotypes C2 and B2 on hepatocellular carcinoma: compared with chronic hepatitis B and asymptomatic carrier state in the same area. Carcinogenesis 2008;29:1685–91.
18. Erhardt A, Blondin D, Hauck K et al. Response to interferon alfa is hepatitis B virus genotype dependent: genotype A is more sensitive to interferon than genotype D. Gut 2005;54:1009–13.
19. Wai CT, Chu CJ, Hussain M et al. HBV genotype B is associated with better response to interferon therapy in HBeAg(+) chronic hepatitis than genotype C. Hepatology 2002;36:1425–30.
20. Kao JH, Chen PJ, Lai MY et al. Basal core promoter mutations of hepatitis B virus increase the risk of hepatocellular carcinoma in hepatitis B carriers. Gastroenterology 2003;124:327–34.
21. Yuen MF, Tanaka Y, Mizokami M et al. Role of hepatitis B virus genotypes Ba and C, core promoter and precore mutations on hepatocellular carcinoma: a case control study. Carcinogenesis 2004;25:1593–8.
22. Chou YC, Yu MW, Wu CF et al. Temporal relationship between hepatitis B virus enhancer II/basal core promoter sequence variation and risk of hepatocellular carcinoma. Gut 2008;57:91–7.
23. Yuen MF, Tanaka Y, Shinkai N et al. Risk for hepatocellular carcinoma with respect to hepatitis B virus genotypes B/C, specific mutations of enhancer II/core promoter/precore regions and HBV DNA levels. Gut 2008;57:98–102.
24. Sung JJ, Tsui SK, Tse CH et al. Genotype-specific genomic markers associated with primary hepatomas, based on complete genomic sequencing of hepatitis B virus. J Virol 2008;82:3604–11.
25. Guo X, Jin Y, Qian G et al. Sequential accumulation of the mutations in core promoter of hepatitis B virus is associated with the development of hepatocellular carcinoma in Qidong, China. J Hepatol 2008;49:718–25.
26. Yang HI, Yeh SH, Chen PJ et al. Associations between hepatitis B virus genotype and mutants and the risk of hepatocellular carcinoma. J Natl Cancer Inst 2008;100:1134–43.
27. Dong Q, Chan HL, Liu Z et al. A1762T/G1764A mutations of hepatitis B virus, associated with the increased risk of hepatocellular carcinoma, reduce basal core promoter activities. Biochem Biophys Res Commun 2008;374:773–6.
28. Kim JK, Chang HY, Lee JM et al. Specific mutations in the enhancer II/core promoter/precore regions of hepatitis B virus subgenotype C2 in Korean patients with hepatocellular carcinoma. J Med Virol 2009;81:1002–8.
29. Yuan JM, Ambinder A, Fan Y et al. Prospective evaluation of hepatitis B 1762(T)/1764(A) mutations on hepatocellular carcinoma development in Shanghai, China. Cancer Epidemiol Biomarkers Prev 2009;18:590–4.
30. Fang ZL, Sabin CA, Dong BQ et al. HBV A1762T, G1764A mutations are a valuable biomarker for identifying a subset of male HBsAg carriers at extremely high risk of hepatocellular carcinoma: a prospective study. Am J Gastroenterol 2008;103:2254–62.

LIVER

31. Liu CJ, Kao JH. Core promoter mutations of hepatitis B virus and hepatocellular carcinoma: story beyond A1762T/G1764A mutations. J Gastroenterol Hepatol 2008;23:347–50.

32. Liu S, Zhang H, Gu C *et al.* Associations between hepatitis B virus mutations and the risk of hepatocellular carcinoma: a meta-analysis. J Natl Cancer Inst 2009;101:1066–82.

33. Choi CS, Cho EY, Park R *et al.* X gene mutations in hepatitis B patients with cirrhosis, with and without hepatocellular carcinoma. J Med Virol 2009;81:1721–5.

34. Livingston SE, Simonetti JP, McMahon BJ *et al.* Hepatitis B virus genotypes in Alaska native people with hepatocellular carcinoma: preponderance of genotype F. J Infect Dis 2007;195:5–11.

35. Lok AS, McMahon BJ. Chronic hepatitis B. Hepatology 2007;45:507–39.

36. Zhang HW, Yin JH, Li YT *et al.* Risk factors for acute hepatitis B and its progression to chronic hepatitis in Shanghai, China. Gut 2008;57:1713–20.

37. Liu S, Xie J, Yin J *et al.* A matched case-control study of hepatitis B virus mutations in the preS and core promoter regions associated independently with hepatocellular carcinoma. J Med Virol 2010; doi:10.1002/jmv.00000 (in press).

38. Tamura K, Dudley J, Nei M *et al.* MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 2007;24:1596–9.

39. Tangkijvanich P, Komolmit P, Mahachai V *et al.* Low pretreatment serum HBsAg level and viral mutations as predictors of response to PEG-interferon alpha-2b therapy in chronic hepatitis B. J Clin Virol 2009;46:117–23.

40. Chen CH, Lee CM, Lu SN *et al.* Clinical significance of hepatitis B virus (HBV) genotypes and precore and core promoter mutations affecting HBV e antigen expression in Taiwan. J Clin Microbiol 2005;43:6000–6.

41. Fung J, Lai CL, But D *et al.* Prevalence of fibrosis and cirrhosis in chronic hepatitis B: implications for treatment and management. Am J Gastroenterol 2008;103:1421–6.

42. Chu CM, Liaw YF. Incidence and risk factors of progression to cirrhosis in inactive carriers of hepatitis B virus. Am J Gastroenterol 2009;104:1693–9.

43. Nguyen MH, Garcia RT, Trinh HN *et al.* Histological disease in Asian-Americans with chronic hepatitis B, high hepatitis B virus DNA, and normal alanine aminotransferase levels. Am J Gastroenterol 2009;104:2206–13.

44. Chen CH, Hung CH, Lee CM *et al.* Pre-S deletion and complex mutations of hepatitis B virus related to advanced liver disease in HBeAg-negative patients. Gastroenterology 2007;133:1466–74.

45. Wong GL, Wong VW, Choi PC *et al.* Evaluation of alanine transaminase and hepatitis B virus DNA to predict liver cirrhosis in hepatitis B e antigen-negative chronic hepatitis B using transient elastography. Am J Gastroenterol 2008;103:3071–81.

46. Cheng H, Su H, Wang S *et al.* Association between genomic heterogeneity of hepatitis B virus and intrauterine infection. Virology 2009;387:168–75.

47. Shen T, Yan XM, Zou YL *et al.* Virologic characteristics of hepatitis B virus in patients infected via maternal-fetal transmission. World J Gastroenterol 2008;14:5674–82.

48. Long Y, Chen E, Liu C *et al.* The correlation of hepatocyte nuclear factor 4 alpha and 3 beta with hepatitis B virus replication in the liver of chronic hepatitis B patients. J Viral Hepat 2009;16:537–46.

49. Jonckheere N, Vincent A, Perrais M *et al.* The human mucin MUC4 is transcriptionally regulated by caudal-related homeobox, hepatocyte nuclear factors, forkhead box A, and GATA endodermal transcription factors in epithelial cancer cells. J Biol Chem 2007;282:22638–50.

50. Yanagiya A, Svitkin YV, Shibata S *et al.* Requirement of RNA binding of mammalian eukaryotic translation initiation factor 4GI (eIF4GI) for efficient interaction of eIF4E with the mRNA cap. Mol Cell Biol 2009;29:1661–9.

51. Wang Y, Lau SH, Sham JS *et al.* Characterization of HBV integrants in 14 hepatocellular carcinomas: association of truncated X gene and hepatocellular carcinogenesis. Oncogene 2004;23:142–8.

52. Chen GG, Li MY, Ho RL *et al.* Identification of hepatitis B virus X gene mutation in Hong Kong patients with hepatocellular carcinoma. J Clin Virol 2005;34:7–12.

53. Hussain SP, Schwank J, Staib F *et al.* TP53 mutations and hepatocellular carcinoma: insights into the etiology and pathogenesis of liver cancer. Oncogene 2007;26:2166–76.

54. Parekh S, Zoulim F, Ahn S *et al.* Genome replication, virion secretion, and e antigen expression of naturally occurring hepatitis B virus core promoter mutants. J Virol 2003;77:6601–12.

55. Hung IF, Poon RT, Lai CL *et al.* Recurrence of hepatitis B-related hepatocellular carcinoma is associated with high viral load at the time of resection. Am J Gastroenterol 2008;103:1663–73.