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| Author(s) | Yuen, MF; Tanaka, Y; Shinkai, N; Poon, RT; But, DYK; Fong, DYT; Fung, J; Wong, DKH; Yuen, JCH; Mizokami, M; Lai, CL |
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# 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

M-F Yuen,[1] Y Tanaka,[2] N Shinkai,[2] R T Poon,[3] D Yiu-Kuen But,[1] D Y-T Fong,[4] J Fung,[1] D Ka-Ho Wong,[1] J Chi-Hang Yuen,[1] M Mizokami,[2] C-L Lai[1]

[1] Department of Medicine, The University of Hong Kong, Queen Mary Hospital, Hong Kong; [2] Department of Clinical Molecular Informative Medicine, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan; [3] Department of Surgery, The University of Hong Kong, Queen Mary Hospital, Hong Kong; [4] Department of Nursing Studies, The University of Hong Kong, Queen Mary Hospital, Hong Kong

Correspondence to:
Dr M F Yuen, Department of Medicine, The University of Hong Kong, Queen Mary Hospital, Pokfulam Road, Hong Kong; mfyuen@hkucc.hku.hk

## ABSTRACT

**Background/aim:** To examine the risks for hepatocellular carcinoma (HCC) with respect to hepatitis B virus (HBV) genotypes, specific viral mutations (MT), serum HBV DNA levels, and cirrhosis.

**Methods:** HBV genotypes, 1653/1753/core promoter (CP)/precore MT and HBV DNA levels were determined in 248 HBV patients with HCC and 248 HBV controls.

**Results:** Genotype C, CP-MT, T1653, HBV DNA levels $\geqslant 4$ $\log_{10}$ copies/ml and cirrhosis had a higher risk for HCC compared to patients with genotype B ($p = 0.001$, OR 1.9), CP wild-type (WT) ($p < 0.001$, OR 4.1), C1653 ($p = 0.028$, OR 2.4), HBV DNA $< 4$ $\log_{10}$ copies/ml ($p = 0.003$, OR 2.1) and without cirrhosis ($p < 0.001$, OR 4.0) respectively. Multivariate analysis showed that CP-MT, T1653, HBV DNA $\geqslant 4$ $\log_{10}$ copies/ml and cirrhosis were independent factors for HCC (all $p < 0.05$). A receiver operating characteristics curve showed no cut-off HBV DNA level associated with minimal chance of HCC. Patients with CP-MT and cirrhosis had a 22.2-fold increased risk of HCC compared to patients with CP-WT and without cirrhosis. Patients with CP-MT and HBV DNA levels $\geqslant 4$ $\log_{10}$ copies/ml had a 7.2-fold increased risk of HCC compared to patients with CP-WT and HBV DNA levels $< 4$ $\log_{10}$ copies/ml. Patients with CP-MT and T1653 had a 9.9-fold increased risk of HCC compared to patients with wild-type for both regions.

**Conclusions:** CP-MT, T1653, HBV DNA levels $\geqslant 4$ $\log_{10}$ copies/ml and cirrhosis are independent factors for development of HCC. The risks increased substantially in patients having these factors in combination.

Hepatocellular carcinoma (HCC) is a disease of global concern, occurring in over 20% of the 400 million people with chronic hepatitis B infection (CHB). While the exact mechanisms of hepatocarcinogenesis with CHB infection remain elusive, several virological factors have been identified to be possibly associated with a higher risk of development of HCC. These include hepatitis B virus load (HBV DNA) levels, HBV genotypes, core promoter and precore mutations. These factors are also associated with the development of cirrhosis and its complications.[1][2]

The majority of the published studies examining HBV genotypes compare genotypes B and C in relation to the disease profile of CHB because these are the two main genotypes prevailing in Asia, a region contributing around 75% of the world's population of CHB. However, while some studies suggest genotype C has a higher risk of development of HCC,[3–5] this observation is not substantiated by others.[6–8] One large study conducted in Taiwan shows that genotype B is more commonly found in patients with HCC developed at a young age.[9] In the Caucasian and Indian populations, genotype D is associated with a greater risk for HCC than genotype A.

Concerning the common naturally occurring mutations at the precore (G1896A) and core promoter (A1762T and G1764A) regions, some studies show that patients with precore mutants have more aggressive disease including reactivation of CHB and fulminating course of the disease,[10][11] These observations have not been substantiated in other studies partly because the predominant genotypes are different between Asia and Europe/USA.[12][13] For core promoter mutations, some studies report a higher risk of development of HCC in patients with core promoter mutations compared to those with wild-type.[3][6][7][14–16] Again, this has not been confirmed by other studies.[5][17] In addition to these two common mutations, two other mutations, C to T at 1653 in the enhancer II region and T to C/A/G (V) at 1753 in the core promoter region, have recently been found to be associated with the development of HCC.[18–20]

The uncertainty as to whether these virological factors are genuine risk factors for the development of HCC may be due to several reasons. Most of the studies only have a limited number of patients. These studies often examine only specific virological factors; for example, genotypes without considering the possible confounding effect of other parameters, such as viral mutations and HBV DNA levels. Indeed the associations between genotype B with precore mutations and genotype C with core promoter mutations have been shown to be confounding factors.[21] Whether there are any additive or synergistic effects on the risks of HCC development with different combinations of genotypes/precore/core promoter and mutations in the enhancer II region and HBV DNA levels have not been studied. Finally, the risks for development of HCC of these factors in the setting of cirrhosis have not been examined.

Therefore we sought to examine the risks of HBV DNA levels, HBV genotypes, core promoter/precore/T1653/V1753 mutations and cirrhosis individually and in combination for the development of HCC in a large population study.

## PATIENTS AND METHODS

### Patients

A total of consecutive 248 Chinese CHB patients with HCC were recruited from Department of Medicine and Department of Surgery, The University of Hong Kong, Queen Mary Hospital, Hong Kong from 2000 to 2004. All patients had the diagnosis of HCC for the first time, during regular follow-up in our centre (n = 198) or in other hospitals (n = 50). Patients with recurrent HCC were excluded from the present study. One hundred and twenty patients had histologically proven HCC. The remaining 128 patients had elevated α-fetoprotein (AFP) with typical imaging features in computerised tomography and/ or magnetic resonance imaging and/or hepatic angiogram.

During the same period of recruitment of patients with HCC, 4825 CHB Chinese patients without HCC were being followed up in the University Liver Clinic of Queen Mary Hospital, Hong Kong. A consecutive 248 CHB patients without HCC were recruited as controls. These control patients were matched individually with each patient with HCC for gender, age (less than 2 years difference) and hepatitis B e antigen (HBeAg)/ antibody to HBeAg (anti-HBe) status in a 1:1 ratio. The absence of HCC was assured by the absence of any space occupying lesion by ultrasonography performed on two separate occasions 1 year apart.

All patients were positive for hepatitis B surface antigen (HBsAg) checked by radioimmunoassay (AUSRIA II, Abbott Laboratories, North Chicago, IL) for at least 6 months. HBeAg/ anti-HBe was also determined by the same assay. Patients with other concomitant diseases including hepatitis C or D virus infection, autoimmune hepatitis, Wilson's disease, primary biliary cirrhosis, alcoholic liver disease and fatty liver (diagnosed by ultrasonography) were excluded.

Liver cirrhosis is defined by the score of >2 according to the aspartate aminotransferase (AST) to platelet ratio index (APRI) calculated from the following formula: ([AST/upper limit of normal]/platelet count [×10$^9$/litre]) × 100.[22]

### Methods

Stored serum at −70°C were thawed for the determination of the HBV DNA levels, HBV genotypes, core promoter and precore mutations and finally the mutations at the enhancer II region. The HBV DNA levels were measured by Cobas Amplicor HBV Monitor test (Roche Diagnostics, Branchburg, NJ) with a lower limit of detection of 300 copies/ml.

HBV genotypes were determined by the enzyme linked immunosorbent assay (ELISA). The detailed methodology of the assay was described in our previous study.[23] The sequence of core promoter and precore regions including A1762T/G1764A (core promoter mutations) and G1896A (precore mutation) were determined by direct sequencing. The methodology was described in our previous study.[20] The two recently identified HCC-related mutations at the enhancer II and core promoter regions namely, C to T at 1653 and T to C/A/G (V) at 1753, were also sequenced according to the methods described in our previous study[20] in 140 patients with HCC and 100 control patients with adequate sera available for sequencing. There were no differences in the median age (range), male to female and HBeAg: anti-HBe ratios between these two subgroups of 140 and 100 patients [56.6 years (29–83.7) vs. 59.8 years (24.8–81.6), p = 0.13 for age; 114:26 vs. 79: 21, p = 0.65 for male to female ratio; and 40:100 vs. 23:77, p = 0.28 for HBeAg: anti-HBe ratio).

### Statistical analysis

All statistical analyses were performed using the SPSS 14.0 for Windows, SPSS Inc., Chicago, IL). The Mann–Whitney U test was used to compare continuous variables between patients with HCC and control patients. The $\chi^2$ test with Yates correction factor or Fisher's exact test was used to compare categorical variables between two groups. A receiver operating characteristic (ROC) curve was used to determine whether there is a cut-off HBV DNA which was associated with no risk of HCC. Logistic regression was adopted to determine independent risk factors for HCC. The adjusted odds ratios (OR) for development of HCC of different combinations of variables were also calculated by the logistic regression analysis with a selected combination defined as the reference. All estimates were accompanied by a 95% confidence interval (CI), where appropriate and a p-value <0.05 was considered as statistical significance.

## RESULTS

### Demographics

The demographic data for 248 patients with HCC and 248 control patients are listed in table 1. Patients with HCC had a significantly poorer liver biochemical parameters and higher median AFP level compared to control patients. Patients with HCC also had a higher prevalence of liver cirrhosis compared to control patients. The OR for patients with cirrhosis was 4.0 [95% CI, 2.8 to 5.9].

### HBV genotypes

A total of 478 out of 496 (96.2%) samples had positive genotype results from EIA test, but this test gave indeterminate results for the remaining 18 samples (10 from patients with HCC, eight from control patients). Of the 238 patients with HCC with genotype results, 67 (28.2%) had genotypes B, 170 (71.4%) had genotypes C and one (0.4%) had genotype D. Of the 240 control patients with genotype results, 100 (41.7%) had genotypes B, 135 (56.3%) had genotype C, three (1.3%) had genotypes D and two (0.8%) had mixed genotypes.

Comparing patients with either genotypes B or C, patients with HCC had a higher prevalence of genotype C compared to control patients [170/237 (71.2%) vs. 135/235 (57.4%) respectively; p = 0.001; OR 1.9; 95% CI, 1.3 to 2.8].

### Core promoter and precore mutations

Of all the 496 samples, direct sequencing failed to generate results for 70 samples for core promoter region and 61 samples for precore region.

**Table 1** Demographic data for the study population

| | Patients with HCC (n = 248) | Control patients (n = 248) |
|---|---|---|
| Sex (M:F) | 199:49 | 199:49 |
| Age (years) | 57.5 (24.8–83.7) | 57.7 (24.8–81.8) |
| HBeAg:anti-HBe (%) | 61:187 (24.6%:75.4%) | 61:187 (24.6%:75.4%) |
| Albumin (g/l) | 37 (16–59)* | 43 (17–53)* |
| Bilirubin (μmol/l) | 17 (5–531)† | 12 (2–96)† |
| ALT (U/l) | 57 (4–1154)‡ | 46 (9–920)‡ |
| AFP (ng/ml) | 136.5 (1–1 060 000)§ | 5 (1–200)§ |
| Presence of cirrhosis (%) | 170 (68.5%)¶ | 87 (35.1%)¶ |

*,†,‡,§,¶p<0.001.
Continuous variables are expressed in median (range).
ALT, alanine aminotransferase; AFF, α-fetoprotein.

Results of core promoter mutations were successfully obtained in 194 (78.2%) samples of patients with HCC and in 232 (93.5%) samples of control patients. Patients with HCC had a higher prevalence of core promoter mutations compared to patients without HCC [173/194 (89.2%) vs. 155/232 (66.8%), respectively; p<0.001; OR, 4.1 (95% CI, 2.4 to 6.9)].

Results of precore mutations were successfully obtained by the direct sequencing in 198 (79.8%) samples of patients with HCC and in 237 (95.6%) samples of patients without HCC. There was no significant difference in the prevalence of precore mutations between patients with and without HCC [72/198 (36.4%) vs. 106/237 (44.7%), respectively; p = 0.10].

### Relationship between HBV genotypes and core promoter/precore mutations

Patients with genotype B had a higher chance of harbouring precore mutations compared to patients with genotype C [105/144 (72.9%) vs. 67/267 (25.1%), respectively; p<0.001; OR, 8.0; 95% CI, 5.1 to 12.7]. Patients with genotype C had a higher chance of harbouring core promoter mutations compared to patients with genotype B [237/264 (89.8%) vs. 76/141 (53.9%), respectively; p<0.001; OR, 7.5; 95% CI, 4.5 to 12.6].

### HBV DNA levels

To determine whether there is an exact HBV DNA level below which HCC is unlikely to occur, the HBV DNA levels of all the patients with or without HCC were entered in the ROC curve analysis (fig. 1). The ROC nearly overlapped with reference line with the area under the curve (AUC) of 0.51 (p = 0.75; 95% CI, 0.46 to 0.56) indicating that there existed no cut-off HBV DNA level that was associated with minimal risk of HCC. Further separate analysis of patients who had HBeAg seroconversion (anti-HBe positive) with less fluctuation of HBV DNA levels during the course of the disease was performed. The AUC was only 0.56 (p = 0.054; 95% CI, 0.50 to 0.62). This suboptimal value confirmed that there was no HBV DNA level that was associated with minimal risk of HCC even for anti-HBe-positive patients.

Though a "safe" lower limit of HBV DNA level could not be identified, a higher proportion of patients with HCC had high viral load defined by HBV DNA level $\geqslant 4$ $\log_{10}$ copies/ml compared to that of control patients [218/248 (87.9%) vs. 193/

248 (77.8%), respectively; p = 0.003; OR, 2.1; 95% CI, 1.3 to 3.4].

### T1653 and V1753 mutations

Results of T1653 and V1753 were successfully obtained by the direct sequencing in 133 (95%) out of 140 samples of patients with HCC and in 99 out of 100 (99%) samples of control patients. The reason for samples with no obtainable results for these two mutations was due to the failure of generation of sequence with good quality by direct sequencing. Patients with HCC had a significantly higher prevalence of T1653 mutations compared to control patients [19.5% (26/133) vs. 9.1% (9/99), respectively; p = 0.028; OR, 2.4; 95% CI, 1.1 to 5.4]. There was no difference in the prevalence of T1753 mutations between patients with HCC and control patients [42.1% (56/133) vs. 44.4% (44/99), respectively; p = 0.72].

### Multivariate analysis on the risk factors for HCC

HBV genotypes, core promoter mutations, T1653 mutations, HBV DNA levels and presence of cirrhosis were entered into the logistic regression analysis. Core promoter mutations, T1653 mutation, HBV DNA levels $\geqslant 4$ $\log_{10}$ copies/ml and presence of cirrhosis were shown to be independent factors associated with HCC (p = 0.015, 0.044, 0.048 and 0.005, respectively). Genotype C, identified as a significant risk factor in the univariate analysis was *not* an independent risk factor for HCC.

### Relationship between core promoter mutations, T1653 mutations, HBV DNA levels and cirrhosis

A higher proportion of patients with core promoter mutations had high viral load (HBV DNA $\geqslant 4$ $\log_{10}$ copies/ml) compared to that of patients without core promoter mutations [284/328 (86.6%) vs. 71/98 (72.4%), respectively; p = 0.001; OR, 2.6; 95% CI, 1.4 to 4.2]. There was no difference in the prevalence of T1653 mutation between patients with core promoter mutations and wild-type [28/174 (16.1%) vs. 6/43 (14.0%), respectively; p = 0.91]. Patients with core promoter mutations had a significantly higher prevalence of cirrhosis compared to patients with core promoter wild-type [177/328 (54.0%) vs. 39/98 (39.8%), respectively; p = 0.014; OR, 1.8; 95% CI, 1.1 to 2.8].

### Adjusted risks for patients with core promoter mutations stratified according to HBV DNA levels, 1653 mutations and cirrhosis

Stratifying core promoter mutations, 1653 mutations, HBV DNA levels and presence of cirrhosis to assess the combined risk for the development of HCC resulted in 16 different groups of patients with certain groups having fewer than five patients, thus precluding reliable statistical analysis. Therefore separate analyses were performed by stratifying (1) core promoter mutations according to HBV DNA levels, (2) core promoter mutations with or without concomitant 1653 mutations and (3) core promoter mutations according to presence or absence of cirrhosis. The adjusted odds ratios for the development of HCC are shown in tables 2, 3 and 4, respectively.

### DISCUSSION

To our knowledge, the present study is the largest study examining the individual role as well as the possible interacting effects of HBV genotypes, the two commonly occurring mutations (core promoter and precore mutations), mutations at the enhancer II (T1653) and at the more upstream core promoter region (V1753), HBV DNA levels, and liver cirrhosis
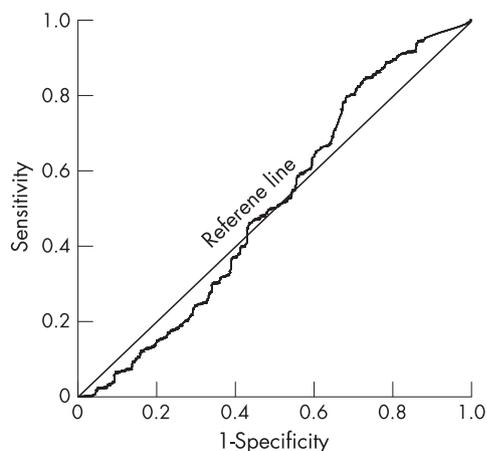


**Figure 1** Receiver operating characteristics curve (ROC) of HBV DNA levels and development of HCC (area under curve = 0.51) (p = 0.75; 95% CI, 0.46 to 0.56).

**Table 2** Adjusted odds ratios for HCC in patients with core promoter wild-type/mutations according to the HBV DNA levels

| Core promoter | HBV DNA ($\log_{10}$ copies/ml) | Number of patients | Odds ratio (95% CI) | p Value |
|---|---|---|---|---|
| Wild-type | <4 | 27 | Reference | – |
| Wild-type | ≥4 | 77 | 1.8 (0.6 to 6.0) | 0.33 |
| Mutant | <4 | 44 | 3.1 (0.9 to 10.6) | 0.07 |
| Mutant | ≥4 | 426 | 7.2 (2.4 to 21.4) | <0.001 |

CI, confidence interval.

**Table 4** Adjusted odds ratios for HCC in patients with core promoter wild-type/mutations according presence or absence of cirrhosis

| Core promoter | Cirrhosis | Number of patients | Odds ratio (95% CI) | p Value |
|---|---|---|---|---|
| Wild-type | No | 59 | Reference | – |
| Wild-type | Yes | 39 | 7.5 (2.5 to 23.0) | <0.001 |
| Mutant | No | 151 | 6.0 (2.3 to 15.9) | <0.001 |
| Mutant | Yes | 177 | 22.2 (8.4 to 58.4) | <0.001 |

CI, confidence interval.

on the development of HCC. This relatively large number of patients would allow any possible links or associations between these factors contributing to the development of HCC to be defined more unequivocally. One of the limitations of the present study is that the role of deletions in the pre-S region of HBV genome which have been recently shown to be associated with the development of HCC has not been studied.[24]

An epiphenomenon observed in the present study was the higher risk of HCC in patients with genotype C compared to patients with genotype B (all were subgenotype B2 in our locality according to our previous study).[25] This is apparently consistent with other studies.[3–5] However, genotype C was not found to be an independent factor for HCC when tested in the multivariate analysis. Core promoter mutations, T1653 mutations, high HBV DNA levels and presence of cirrhosis were independent risk factors for HCC. This is not an unusual finding because of the strong association of genotype C with core promoter mutations (89.8%), and genotype B with precore mutations (72.9%). Though it is well proven that patients with genotype B have an earlier HBeAg seroconversion,[21 26] it appears neither genotype B nor C has any major influential effects on the life-time risk of HCC, a finding in concordance with other studies.[6 27] We have recently shown that the earlier HBeAg seroconversion with genotype B is related to the more intense immunogenic stimulation during the immunoclearance phase.[28] The effects exerted by HBV genotypes B and C on the disease progression of CHB subsequent to HBeAg seroconversion appear to be similar.

However, there are at least two documented effects accompanying core promoter mutations on the development of HCC. Mutations in the core promoter region result in a shift change of the viral pregenomic secondary structure which may enhance the viral replication.[29] Viral replication can also be further enhanced by a second mechanism in which the transcription of the pregenomic RNA will be increased through the removal of the nuclear receptor binding site and creation of a hepatocytes nuclear binding factor.[30] These changes increase the core RNA transcription with enhanced core protein, DNA polymerase, pre-genomic RNA synthesis, but suppress the precore RNA transcription whose normal function is to decrease pregenomic RNA packaging.[31 32] This is in complete concordance with the finding of the present study and of Chauhan and

colleagues.[33] HBV DNA levels were higher in patients with core promoter mutations compared to those without core promoter mutations.

In the present study, by setting the patients without core promoter mutations and HBV DNA <4 $\log_{10}$ copies/ml as a reference, the adjusted odds ratio for HCC for patients with core promoter mutations at the same viraemic level was 3.1 (95% CI, 0.9 to 10.6), with a borderline p value of 0.07 (table 2). It is possible that the higher risk of HCC in patients with core promoter mutations may also be mediated through another additional pathway independent of the increase in viral replication. The possible carcinogenic mechanisms require further in vitro studies and functional analyses to delineate.

The present study demonstrated that the risk of HCC was substantially increased in patients harbouring core promoter mutations and having liver cirrhosis, a 22.2-fold increase when compared to patients with core promoter wild-type and without cirrhosis (table 4). Similarly, patients with core promoter mutations with high HBV DNA levels of ≥4 $\log_{10}$ copies/ml had a 7.2-fold increase risk of HCC when compared to patients with core promoter wild-type with HBV DNA levels <4 $\log_{10}$ copies/ml (table 2).

In the present study, we found that T1653 was an independent risk factor for the development of HCC. According to our previous studies,[20 34] T1653 mutation is associated with HCC in patients with genotype C. In the present study, we further confirmed with larger number of patients that T1653 was an independent risk factor for HCC irrespective of HBV genotypes. 1653 is located in the box alpha of the enhancer II region of HBV genome. The C to T mutation at 1653 converts histidine to tyrosine at amino acid 94 of the X protein which may explain its association with the hepatocarcinogenesis. According to Takahashi and colleagues, the frequency of T1653 mutation increases with the progression of liver disease from chronic hepatitis to cirrhosis.[19] It occurs later than, and independent of, core promoter mutations in chronic hepatitis B disease. However, when both viral mutations, that is, core promoter and T1653 mutations, co-existed, the risk of HCC was substantially increased to 9.9-fold when compared to patients with wild-type at both genomic regions (table 3).

Finally, the present study showed that there was no reliable cut-off HBV DNA level associated with low risk of HCC. This means that maximal viral suppression to the lowest possible HBV DNA levels should be the target for future management of CHB disease.

In conclusion, core promoter mutations, T1653 mutations, HBV DNA levels ≥4 $\log_{10}$ copies/ml and presence of cirrhosis were independent factors for the development of HCC. The risk increased substantially in patients who carried these factors in combination. Future studies should consider these factors in conjunction with age and gender of patients to formulate the risk of HCC in CHB patients.

**Competing interests:** None.

**Table 3** Adjusted odds ratios for HCC in patients with core promoter wild-type/mutations according mutations at 1653

| Core promoter | 1653 | Number of patients | Odds ratio (95% CI) | p Value |
|---|---|---|---|---|
| Wild-type | Wild-type | 37 | Reference | – |
| Wild-type | Mutant | 6 | 2.7 (0.5 to 15.6) | 0.27 |
| Mutant | Wild-type | 146 | 3.6 (1.6 to 7.9) | 0.02 |
| Mutant | Mutant | 28 | 9.9 (3.1 to 31.5) | <0.001 |

CI, confidence interval.

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