Genotype C hepatitis B virus infection is associated with an increased risk of hepatocellular carcinoma

H L-Y Chan, A Y Hui, M L Wong, A M-L Tse, L C-T Hung, V W-S Wong, J J-Y Sung

Background: Identification of risk factors for the development of hepatocellular carcinoma (HCC) is important for HCC surveillance in chronic hepatitis B virus (HBV) infection. Our aim was to study the independent risk factors and effect of HBV genotypes on HCC development in a prospective longitudinal cohort of chronic hepatitis B patients.

Patients and methods: Chronic hepatitis B patients recruited since 1997 were prospectively followed up for the development of HCC. HCC was diagnosed by a combination of α-fetoprotein, imaging, and histology. Liver cirrhosis was defined as ultrasonic features of cirrhosis together with hypersplenism, ascites, varices, and/or encephalopathy.

Results: In total, 426 patients were followed up for 1664 person years; median 225 (range 12–295) weeks. Forty nine (11%) patients had underlying clinical liver cirrhosis. A total of 242 (57%) and 179 (42%) patients had HBV genotypes C and B, respectively. Twenty five patients developed HCC in a median follow up of 121 (range 14–236) weeks. The overall incidence of HCC was 1502 cases per 100 000 person years. On multivariate analysis, clinical liver cirrhosis and HBV genotype C infection were independently associated with HCC development, with an adjusted relative risk of 10.24 (95% confidence interval 4.39–23.89; p = 0.001) and 2.84 (95% CI 1.05–7.72; p = 0.040), respectively. Patient age, sex, hepatitis B e antigen (HBeAg) status, alanine aminotransferase (ALT) levels, and basal core promoter mutations did not predict HCC development. Patients infected with HBV genotype C tended to have a persistently positive HBeAg or fluctuating HBeAg status and higher ALT levels during the follow up period.

Conclusion: Genotype C HBV infection is an independent risk factor for HCC development in addition to liver cirrhosis.

Epidemiological studies have shown a strong association between chronic hepatitis B virus (HBV) infection and hepatocellular carcinoma (HCC), which is a major cause of morbidity and mortality in areas where chronic hepatitis B is endemic. The annual incidence of HCC development in chronic HBV infected patients ranges from 0.2% to 0.8% in different centres. Surveillance of HCC by semi annual α-fetoprotein testing followed by ultrasound and/or computerised tomography imaging can detect HCC at an early resectable stage, which may be translated to prolongation of patient survival. As there are 350 million chronic HBV infected patients worldwide, population based HCC surveillance programmes would imply a huge demand on public health resources. This problem will be more serious in Southeast Asia where the prevalence of chronic hepatitis B is up to 15%. Identification of risk factors for HCC and stratification of patient risk is therefore important to guide future surveillance strategy.

Various risk factors for HCC development have been identified but their relationship is complicated. Older patient age and liver cirrhosis have been consistently found to be important risk factors for HCC in most studies. However, the incidence of liver cirrhosis also increases with patient age. A population based study in Taiwan found that positive hepatitis B e antigen (HBeAg) at recruitment was associated with a higher risk of HCC development after adjustment for the effect of age and sex after 10 years of follow up. However, the status of liver cirrhosis was not assessed in this study.

To date, eight different HBV genotypes (A–H) have been reported according to viral genomic heterogeneity. In Southeast Asia, HBV genotypes B and C are most prevalent. Case control studies found that HBV genotype C was associated with a higher risk of HCC than genotype B in Japan and Taiwan. Contradictory results were obtained from two large scaled cross sectional studies which found that the distributions of HBV genotypes B and C were similar among patients with and without HCC. All of these studies are potentially subject to sampling bias and the potential confounding effects of patient age, HBeAg status, and liver cirrhosis were not assessed. As HBV genotype C is associated with more aggressive disease than genotype B, HBeAg status and liver cirrhosis status must be taken into consideration before the true impact of HBV genotype on HCC development can be determined. Previous studies have suggested that basal core promoter (BCP) mutations (A to T at nucleotide 1762 and G to A at nucleotide 1764), which are commonly found in HBV genotype C, increase the risk of HCC development. However, the prevalence of BCP mutations increases with HBeAg seroconversion and their association with HCC has not been confirmed in longitudinal studies.

The primary aim of this study was to determine the independent risk factor(s) for HCC development in a prospective longitudinal cohort of chronic hepatitis B patients in Hong Kong. Potential risk factors, including patient demographics, liver cirrhosis, HBeAg status, BCP mutations, and viral genotypes were assessed. The secondary aim was to investigate disease progression and effect on HCC development among patients infected with different HBV genotypes.

Abbreviations: ALT, alanine aminotransferase; anti-HBe, antibody to hepatitis B e antigen; BCP, basal core promoter; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; PCR, polymerase chain reaction.
PATIENTS AND METHODS

Patients
Consecutive chronic hepatitis B patients of Chinese ethnicity recruited at the Hepatitis Clinic, Prince of Wales Hospital, from December 1997 to July 2000, were studied. No patient had a history of alcoholism (>20 g/week) or hepatitis C virus coinfection. Patients who had other possible causes of hepatitis or liver cirrhosis, including autoimmune liver disease, primary biliary cirrhosis, Wilson’s disease, and haemochromatosis, were also excluded. At the initial presentation, an ultrasound examination of the abdomen was performed to exclude any pre-existing HCC. Patients were prospectively followed up every six months or more frequently if clinically indicated, with monitoring of liver biochemistry, HBeAg, and antibody to hepatitis B e antigen (anti-HBe) status, as well as α-fetoprotein levels. Ultrasound of the abdomen, computerised tomography, hepatic angiogram, and/or liver biopsy were performed if α-fetoprotein levels were higher than 50 μg/L or demonstrated a rising trend over 20 μg/L to confirm the diagnosis of HCC. For patients with normal α-fetoprotein levels, ultrasound of the abdomen was performed every 1–2 years. Clinical liver cirrhosis was defined as ultrasonic features of liver cirrhosis plus evidence of hypersplenism (splenomegaly with a platelet count of less than 100 × 10^3/μL or white count less than 4 × 10^3/μL), clinical ascites, varices, and/or hepatic encephalopathy.

Serology assays
HBsAg and anti-hepatitis C virus antibodies (third generation assay) were tested using commercially available enzyme linked immunosorbant assay kits (Abbott GmbH Diagnostika, Wiesbaden-Delkenheim, Germany). HBeAg and anti-HBe were measured by enzyme linked immunosorbant assay kits (Abbott GmBH Diagnostika, Wiesbaden-Delkenheim, Germany). HBsAg status, alanine aminotransferase (ALT) level, BCP mutations, and HBV genotype. Categorical variables were compared by χ² test and continuous variables by the Mann-Whitney U test. Variables with a p value <0.1 on univariate analysis were analysed by backward stepwise multivariate analysis for independent risk factors associated with HCC development. The 95% confidence interval (95% CI) for the relative risks was also calculated. Cumulative probability of HCC development was determined by the Kaplan-Meier method and compared by log rank test. All statistical analyses were performed using the Statistical Package of Social Science (SPSS version 11.0). All statistical tests were two sided. Statistical significance was taken as p<0.05.

HBV genotyping
HBV genotyping was performed in the residual serum sample of each patient at the initial visit. Genotyping polymerase chain reaction (PCR) amplification and restriction fragment length polymorphism were performed as described previously. In short, 10 μL of extracted HBV DNA was amplified by PCR using primers flanking the HBV genome between nucleotides 256 to 796 (sense primer 5'-GAC TTC TCT CAA TTT TC and antisense primer 5'-GAC TCT CTA TTT TC and antisense primer 5'-GGG TA(A/T) AAA GGG ACT CA(A/C) GAT). The PCR product (10 μL) was then mixed and incubated with restriction enzymes Tsp 5091 (New England Biolabs, Beverly, Massachusetts, USA) and HinfI. The PCR product was taken as p<0.005.

RESULTS

Baseline characteristics
A total of 426 patients, including 278 males and 148 females (median age 41 (range 12–80) years) were followed up for 1644 person years (median 225 (range 12–295) weeks). Forty nine (11%) patients had underlying clinical liver cirrhosis. Twenty nine (59%) patients had Child’s A, 15 (31%) Child’s B, and five (10%) patients had Child’s C cirrhosis. Baseline clinical data are shown in table 1. Two hundred and forty two (57%) patients had HBV genotype C, 179 (42%) had genotype B, three had a mixed genotype B and C, one patient had genotype A, and one patient had negative PCR. A total of 215 (56%) patients had BCP mutations; among them two patients had deletion of nucleotides 1762 and 1764.

Risk factors of HCC
A total of 25 patients developed HCC in a median follow up time of 121 (range 14–236) weeks. The overall incidence of HCC was 1502 cases per 100 000 person years. The cumulative probability of HCC development was 1.4%, 2.9%, 4.5%, 5.9%, and 6.9% from the first to the fifth year.

Statistical analysis
The Cox proportional hazard model was used to estimate the relative risk of HCC development associated with age, sex, clinical liver cirrhosis, HBeAg status, alanine aminotransferase (ALT) level, BCP mutations, and HBV genotype. Categorical variables were compared by χ² test and continuous variables by the Mann-Whitney U test. Variables with a p value <0.1 on univariate analysis were analysed by backward stepwise multivariate analysis for independent risk factors associated with HCC development. The 95% confidence interval (95% CI) for the relative risks was also calculated. Cumulative probability of HCC development was determined by the Kaplan-Meier method and compared by log rank test. All statistical analyses were performed using the Statistical Package of Social Science (SPSS version 11.0). All statistical tests were two sided. Statistical significance was taken as p<0.05.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Univariate analysis for factors associated with development of hepatocellular carcinoma (HCC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>Overall</td>
</tr>
<tr>
<td>Age (y) (mean (SD))</td>
<td>426</td>
</tr>
<tr>
<td>Age &gt;40 (n (%))</td>
<td>228 (54%)</td>
</tr>
<tr>
<td>Male (n (%))</td>
<td>278 (65%)</td>
</tr>
<tr>
<td>Cirrhosis (n (%))</td>
<td>49 (11%)</td>
</tr>
<tr>
<td>HBeAg+ (n (%))</td>
<td>157 (37%)</td>
</tr>
<tr>
<td>ALT</td>
<td>50 (12–1072)</td>
</tr>
<tr>
<td>Genotype C (n (%))*</td>
<td>242 (57%)</td>
</tr>
<tr>
<td>BCP mutations (n (%))</td>
<td>215 (56%)</td>
</tr>
<tr>
<td>Follow up (weeks)</td>
<td>225 (12–295)</td>
</tr>
</tbody>
</table>

*Five patients were not evaluated; one patient who developed HCC had a mixed HBV genotype B and C. †Forty three patients (two had HCC) had a negative polymerase chain reaction (PCR) for BCP mutations and were not evaluated. Twenty patients had genotype B, 21 patients had genotype C, one patient had a mixed genotype B and C, and one patient had a negative PCR for HBV genotyping.
of follow up (fig 1). On univariate analysis of baseline variables, age >40 years, male sex, presence of clinical liver cirrhosis, BCP mutations, and HBV genotype C were associated with the development of HCC (p<0.1) (table 1). HBeAg positivity and ALT levels were not associated with HCC development.

On multivariate analysis, clinical liver cirrhosis and genotype C HBV infection were independently associated with HCC development. Clinical liver cirrhosis was the strongest predictor of HCC development with an adjusted relative risk of 10.24 (95% CI 4.39–23.89; p<0.001) whereas HBV genotype C has an adjusted relative risk of 2.84 (95% CI 1.05–7.72; p = 0.040). On subgroup analysis, the incidence of HCC development among patients with clinical cirrhosis was 1.1-fold higher in HBV genotype C (10 608 cases per 100 000 person years) versus HBV genotype B (5147 cases per 100 000 person years) infection (p = 0.21) (fig 2). Similarly, the incidence of HCC development among patients who had no clinical liver cirrhosis was 2.3-fold higher in HBV genotype C (1049 cases per 100 000 person years) versus HBV genotype B (322 cases per 100 000 person years) infection (p = 0.11) (fig 2). Differences in incidence of HCC development were however not statistically significant between HBV genotypes B and C with or without liver cirrhosis, probably due to the small number of events in each subgroup and the marginal significance of HBV genotype.

Mean (SD) age of HCC development was comparable between patients infected with HBV genotype B (49 (15)) and genotype C (53 (9)) (p = 0.51). Using age 40 years as the cut off, there was no difference in the proportion of patients infected with HBV genotype C for early (2/4 patients, 50%) and late (16/18 patients, 89%) onset HCC (p = 0.21).

**HCC in non-cirrhotic livers**

Among 11 patients who developed HCC but without clinical evidence of liver cirrhosis, nine had liver histology available. Seven (78%) patients had histological evidence of severe fibrosis or liver cirrhosis while the remaining two patients had mild to moderate liver fibrosis (not to the stage of liver cirrhosis). Hence at least 8% (2/25) of the HCC developed in our cohort were derived from a non-cirrhotic liver. These two patients were male, aged 36 and 49 years, respectively, infected with HBV genotype C. They had mildly elevated ALT (2–5 times the upper limit of the laboratory normal) and persistently negative HBeAg during a follow up period of 123 and 169 weeks, respectively, before HCC was diagnosed. Serum HBV DNA levels at initial visits were 1 770 000 and 800 copies/ml, respectively.

**Disease patterns of different HBV genotypes**

At baseline, patients infected with HBV genotype C were generally younger with a borderline female predominance and higher proportion of positive HBeAg compared with those infected with HBV genotype B (table 2). The prevalence of BCP mutations was higher among patients infected with genotype C. Otherwise, the percentage of clinical liver cirrhosis, median ALT levels, and duration of follow up were comparable between patients infected with HBV genotypes B and C.

On follow up, 87 of 242 (36%) patients infected with HBV genotype C versus 33 of 179 (18%) infected with HBV genotype B had persistently positive HBeAg or fluctuating HBeAg status (p<0.001) (table 2). In contrast 123 of 242 (51%) patients infected with HBV genotype C versus 127 of 179 (71%) infected with genotype B had persistently negative HBeAg (p<0.001) (table 2). A similar proportion of patients infected with both HBV genotypes underwent sustained HBeAg seroconversion. In general, HBV genotype C was associated with higher ALT levels than genotype B (p = 0.015) (table 2).

**DISCUSSION**

The results of this study confirm that HBV genotype C is associated with an increased risk of HCC, and is independent of the presence of liver cirrhosis. Patient age, sex, HBeAg positivity, and ALT levels had no independent impact on the cumulative probability of HCC development after five years of follow up.

The overall incidence of HCC in this study was 1052 cases per 100 000 person years. The high incidence of HCC in this study may be related to the 11% of patients who had clinical liver cirrhosis with a very high incidence of HCC (7998 cases per 100 000 person years). If only non-cirrhotic patients are considered, and incidence of HCC was 743 cases per 100 000 person years. This is comparable with the reported incidence in Taiwan (822 per 100 000 person years) but still higher than that reported in Toronto, Canada (470 per 100 000 person years). One reason may be related to the long
mutations are strongly associated with HBV genotype C,21 23 patients infected with HBV genotype C. Although BCP
the younger age and marginal female predominance among
was not as obvious on univariate analysis, probably due to
consideration. Therefore, in surveillance programmes for HCC, the impor-
tance of these two demo-
holds true in the case of HCC, as shown in the univariate
ence of liver cirrhosis is defined by clinical criteria, all
patients in this category have established liver cirrhosis while
patients with early liver cirrhosis may be classified as non-
cirrhotic. This explains the relatively high cumulative
incidence of HCC among cirrhotic patients in this study
compared with previous series among compensated cirrhotic
patients.18 The use of clinical criteria to classify liver cirrhosis enables an undisputable diagnosis of liver cirrhosis while the
diagnosis of early liver cirrhosis on histology may be affected
by biopsy size and interobserver variations.28 As liver biopsy
may not be a routine procedure for all chronic hepatitis B
patients, particularly among those who have normal liver
enzymes as in our centre, a clinical diagnosis is more feasible in
clinical practice.

Patients who have developed liver cirrhosis are usually
older, and male patients generally have more advanced liver
disease than female patients.9 29 The same association also
holds true in the case of HCC, as shown in the univariate
analysis. When liver cirrhosis was included in the multi-
variate analysis equation, the impact of these two demo-
graphic factors on HCC development became insignificant. Therefore, in surveillance programmes for HCC, the impor-
tance of patient age and sex should not be overemphasised as
long as the status of clinical liver cirrhosis is taken into
consideration.

HBV genotype C was found to be an independent risk
factor for HCC development, which concurs with results of
case control studies reported previously.15 16 This association
was not as obvious on univariate analysis, probably due to
the younger age and marginal female predominance among
patients infected with HBV genotype C. Although BCP
mutations are strongly associated with HBV genotype C,11 21 no association between BCP mutations and HCC develop-
ment was found in this study on multivariate analysis. More
patients infected with HBV genotype C had persistently
positive HBeAg and fluctuating HBeAg whereas more
patients infected with genotype B had persistently negative
HBeAg on follow up. This may reflect a relatively higher level of
viraemia among patients infected with HBV genotype C.30 31 One of two patients who developed HCC from a non-
cirrhotic liver in this study had elevated HBV DNA levels
despite persistently negative HBeAg on follow up. Viral
genotype and high viraemia may be an independent direct
cause of hepatocarcinogenesis in addition to liver cirrhosis, which is the result of continuous necroinflammation.32 The
direct carcinogenic mechanism may act through increasing δs
activation of the proto-oncogene, suppression of tumour
suppressor gene, or transactivation by HBV X protein.13 34

In contrast with the Taiwan report, a single HBeAg result
at baseline could not predict the risk of HCC development in
this study.31 One probable reason is that a significant proportion of HBeAg positive patients underwent HBeAg
seroconversion while some HBeAg negative patients had
HBeAg reversion during follow up. In fact, none of the 51
patients who underwent sustained HBeAg seroconversion
developed HCC in the present cohort. This concurs with previous reports that sustained HBeAg seroconversion is
associated with a favourable prognosis.22 In contrast, fluctuating HBeAg status may associate with more aggressive
disease progression although it was not confirmed in this
study (4/25 (11%) with HCC versus 42/401 (16%) without
HCC; p = 0.60).2 In this study, the impact of baseline HBV
DNA level on HCC development was not evaluated. As HBV
DNA is closely related to HBeAg status and tends to change
with time, particularly on HBeAg seroconversion, serial
monitoring of HBV DNA is necessary.10 31

In summary, HBV genotype C is an independent risk
factor for the development of HCC in addition to liver cirrhosis. Viral
genotype should therefore be determined on risk
stratification in future HCC surveillance programmes. The
exact reason for the higher risk of hepatocarcinogenesis
associated with HBV genotype C warrants further investiga-
tion but direct viral effects in addition to necroinflammation
and liver cirrhosis are possible.

ACKNOWLEDGEMENTS
We would like to thank the Cheng Suen Man Shook Foundation for the Study of Viral Hepatitis for support of this study.

<table>
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<tr>
<th>Genotype B</th>
<th>Genotype C</th>
<th>p Value</th>
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<tbody>
<tr>
<td>n</td>
<td>179</td>
<td>242</td>
</tr>
<tr>
<td>Age (y) (mean (SD))</td>
<td>43 (14)</td>
<td>39 (13)</td>
</tr>
<tr>
<td>Age &gt;40 y (n (%))</td>
<td>106 (60%)</td>
<td>116 (48%)</td>
</tr>
<tr>
<td>Male (n (%))</td>
<td>124 (71%)</td>
<td>150 (62%)</td>
</tr>
<tr>
<td>Cirrhosis (n (%))</td>
<td>22 (12%)</td>
<td>26 (11%)</td>
</tr>
<tr>
<td>HBeAg+ (n (%))</td>
<td>47 (27%)</td>
<td>110 (40%)</td>
</tr>
<tr>
<td>ALT</td>
<td>46 (12–1072)</td>
<td>54 (13–1011)</td>
</tr>
<tr>
<td>BCP mutations† (n (%))</td>
<td>62 (39%)</td>
<td>54 (13–1011)</td>
</tr>
<tr>
<td>Follow up (weeks)</td>
<td>226 (12–266)</td>
<td>224 (19–295)</td>
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<tr>
<td>HBeAg trend on follow up</td>
<td></td>
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<tr>
<td>Persistently positive HBeAg (n (%))</td>
<td>20 (11%)</td>
<td>54 (22%)</td>
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<tr>
<td>Fluctuating HBeAg status (n (%))</td>
<td>13 (7%)</td>
<td>33 (14%)</td>
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<td>Sustained HBeAg seroconversion (n (%))</td>
<td>19 (11%)</td>
<td>32 (13%)</td>
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<tr>
<td>Persistently negative HBeAg (n (%))</td>
<td>127 (71%)</td>
<td>123 (51%)</td>
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<td>Peak ALT on follow up (n (%))</td>
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<tr>
<td>&lt;2–ULN</td>
<td>105 (59%)</td>
<td>115 (47%)</td>
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<tr>
<td>≥2–5–ULN</td>
<td>59 (33%)</td>
<td>94 (39%)</td>
</tr>
<tr>
<td>&gt;5–ULN</td>
<td>15 (8%)</td>
<td>33 (14%)</td>
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</table>

BCP, basal core promoter; HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase; ULN, upper limit of normal.
†Twenty patients with genotype B and 21 patients with genotype C HBV infection had negative a polymerase chain
reaction for BCP mutations and were not evaluated.
REFERENCES


26. Chan HLY, Hussain M, Lok ASF. Different hepatitis B virus genotypes are associated with different mutations in the core promoter and core regions during hepatitis B e antigen seroconversion. Hepatology 1999;29:976-84.


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