Retrospective study investigating the prevalence and clinical significance of hepatitis B virus precore and basal core promoter variants

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BACKGROUND: Hepatitis B virus (HBV) precore (PC) and basal core promoter (BCP) variants are well known; however, their prevalence in North America is unclear, especially among hepatitis B e antigen-negative patients.

OBJECTIVE: To investigate the prevalence of PC/BCP mutations and their clinical significance.

METHODS: One hundred twenty-eight patients positive for both hepatitis B surface antigen and hepatitis B e antibody were selected, and PC/BCP mutations were identified using a line probe assay. The subjects’ charts were reviewed for race/ethnicity, HBV genotype, HBV viral load, sex, liver enzyme levels, imaging and biopsy results up to 10 years before the study.

RESULTS: The prevalence of PC and BCP variants were 47.6% and 62.5%, respectively. Older age was associated with aspartate aminotransferase-to-platelet index ratio (APRI) ≥0.7 (P=0.011) and abnormal imaging/biopsy results (P=0.0008). Although the presence of BCP variant(s) was associated with APRI ≥0.7 (P=0.029), it was not associated with abnormal imaging/biopsy results. The combination of age ≥50 years and the presence of BCP variant(s) was associated with abnormal imaging/biopsy results, suggestive of either cirrhosis or hepatocellular carcinoma (not observed with PC mutation). Neither sex or genotype, or median HBV viral load showed significant influence on any of these outcomes.

CONCLUSIONS: The present study suggests that the prevalence of PC and BCP mutations are higher than what has been previously reported. One potential explanation would be increased immigration in the past decade. Considering the potential public health and clinical implications of these variants, long-term multicentre and prospective studies could further unravel the uncertainty around these variants.

Key Words: Canada; Clinical significance; Precore mutations

Hepatitis B virus (HBV) contains a core gene that is divided into two parts – the precore (PC) and core regions. Mutations can occur in either region, culminating in a PC mutant or a basal core promoter (BCP) mutant, respectively. Mutations in the PC region (particularly at position G/A 1896) are primarily nonsense or frameshift mutations, or mutated initiation codons, whereas mutations in the BCP region (particularly dual mutations at positions 1762 and 1764, the so-called ‘TA change’) are primarily missense substitutions (1,2). These mutations lead to loss of expression of the hepatitis B e antigen (HBeAg) or a significant reduction in its production and secretion. The presence of HBeAg normally elicits an anti-HBeAg immune response. This immune response causes, for the most part, a desired reduction in HBV viral load, but selects variants that have little or no HBeAg expression such as those produced by PC or BCP mutations (1). To detect these mutations, a range of methodologies, including direct sequencing, restriction fragment length polymorphism (3) and line probe assay
(LiPA) (4,5), have been developed, the latter being more sensitive in detecting variants than direct sequencing (5). Studies have shown a significant geographical variation in the prevalence of PC and BCP mutants, with some of the lowest prevalence rates being found in North America (6). Prevalence rates for PC mutants range from 0% to 100%, with most approximately 60%. Prevalence rates for BCP mutations range from 50% to 77%; however, these studies were either meta-analyses or performed outside of North America (6-8). The most recent comprehensive study investigating PC/BCP variants in North America was conducted in the United States by the HBV Epidemiology Study Group and was published in 2003 (9). Since then, no thorough studies have been performed, particularly in anti-HBe-positive patients. This is despite the fact that significant immigration to North America from endemic countries has occurred since then and may have culminated in the current findings. To date, only one study has assessed the prevalence of PC mutations in Canada (10), and that study only examined 30 Canadian Inuit patients. To date, no studies have investigated the prevalence of BCP mutations in Canada; however, there is evidence to suggest that the presence of mutants affects the natural history of HBV infection. Studies have shown that PC and BCP mutations may be associated with poorer clinical outcomes including a higher risk for cirrhosis and hepatocellular carcinoma (HCC), although results have been conflicting (11-15).

The primary aim of the present study, therefore, was to determine the prevalence of PC and BCP mutations in Manitoba in individuals chronically infected with HBV and who are anti-HBe positive. The study also aimed to determine whether a correlation exists between the presence of a mutant and HBV disease severity, including progression to cirrhosis and development of HCC.

METHODS

Study design

In the present study, 128 residual plasma specimens that were submitted to Cadham Provincial Laboratory (CPL, Winnipeg, Manitoba) from September 2010 through May 2014 for HBV viral load testing as well as HBV genotyping were chosen. Using the residual specimens, the prevalence of PC and BCP mutations were determined, and this information was linked to the subjects’ other clinical and laboratory parameters. Most of subjects had a relatively long history of testing; however, past specimens were not available to be reamplified. CPL is the provincial public health laboratory in Manitoba that performs HBV viral load testing for the province, while referring out HBV genotyping requests to the National Microbiology Laboratory in Winnipeg, where genotyping is performed by either direct sequencing or INNO-LiPA HBV Genotyping kit (Innogenetics, Fujirebio, Japan). Thirty specimens had met inclusion criteria but did not have known HBV genotypes. These genotypes were determined using the INNO-LiPA HBV Genotyping kit (Innogenetics, Fujirebio). Briefly, HBV DNA was extracted from specimens (200 µL) using the easyMAG system (bioMérieux, France). Amplification was performed using Platinum Taq DNA Polymerase (Life Technologies, USA). The volume of water was adjusted because the MgCl₂ reagent is separate (final concentration 1.5 mM). Amplification used Platinum Taq DNA Polymerase (Life Technologies, USA). The dNTPs were combined in equal volumes to yield a dNTP mixture of 25 mM each. The master mixes and cycling parameters were followed as per product insert for outer and nested amplification. The thermocycler used was an Eppendorf AG Mastercycler ep (Eppendorf, USA). The LiPA was performed on a Med/Tec AutoBlot 3000H (Fujirebio, Japan) following the automation protocol for this instrument.

HBV PC/BCP mutations

HBV PC (G1896A) and BCP (A1762T, G1764A) mutation testing was performed using the INNO-LiPA HBV PreCore kit (Innogenetics, Fujirebio). Briefly, HBV DNA was extracted from samples (200 µL) using easyMAG system (bioMérieux); the final elution volume was 25 µL. Amplification used Platinum Taq DNA Polymerase (Life Technologies) and 100 mM dNTP set. The volume of water was adjusted because the MgCl₂ reagent is separate (1.5 mM final concentration 1.5 mM). The dNTPs were combined in equal volumes to yield a dNTP mixture of 25 mM each. The master mixes and cycling parameters were followed as per product insert for outer and nested amplification. The thermocycler used was an Eppendorf AG Mastercycler ep (Eppendorf). LiPA was performed on a Med/Tec AutoBlot 3000H (Fujirebio) following the automation protocol for this instrument.

Statistical analyses

Data were analyzed using Analyse-it (Analyse-it Software, Ltd, United Kingdom) for Excel version 2.0 (Microsoft Corporation, USA). Statistical analyses were performed using the Fisher’s exact test (including OR calculation and 95% CIs) and Mann-Whitney U test for categorical variables. Results were considered to be statistically significant at P<0.05.

RESULTS

One hundred twenty-eight specimens chosen from subjects who met the inclusion criteria were studied. Subjects belonged to three major ethnicities: Asian (71%), non-Hispanic white (10%) and African descent (19%). Of an initial 132 specimens, four were excluded because two did not have amplified DNA for genotyping and PC/BCP mutation testing and two were positive for HCCV or hepatitis D virus. The age range was 18 to 80 years, with a median age of 40 years. Fifty-five percent of subjects were male and 45% were female. Of eight known HBV genotypes (A through H) only five were present: A
TABLE 1
Demographics, laboratory abnormalities and any abnormal liver findings according to imaging or biopsy versus precore (PC)/basal core promoter (BCP) mutations

<table>
<thead>
<tr>
<th>Variable</th>
<th>PC</th>
<th>BCP</th>
<th>P</th>
<th>Statistical test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient population</td>
<td>61/128 (47.6)</td>
<td>80/128 (62.5)</td>
<td></td>
<td>Not applicable</td>
</tr>
<tr>
<td>Age, years</td>
<td>41.27±13.37</td>
<td>41.25±13.42</td>
<td>0.099</td>
<td>Mann-Whitney</td>
</tr>
<tr>
<td>Male sex</td>
<td>37/61 (60.6)</td>
<td>8/80 (60)</td>
<td>1.00</td>
<td>Fisher’s exact</td>
</tr>
<tr>
<td>Abnormal median ALT level</td>
<td>30/61 (49.1)</td>
<td>46/79 (58.2)</td>
<td>0.66</td>
<td>Fisher’s exact</td>
</tr>
<tr>
<td>Abnormal median AST level</td>
<td>16/61 (26.2)</td>
<td>26/79 (32.9)</td>
<td>0.65</td>
<td>Fisher’s exact</td>
</tr>
<tr>
<td>ALT level, &gt;1.5×upper limit of normal</td>
<td>29/61 (47.5)</td>
<td>44/79 (55.6)</td>
<td>0.69</td>
<td>Fisher’s exact</td>
</tr>
<tr>
<td>Platelet count, ×10^9/L</td>
<td>223±54</td>
<td>218±59</td>
<td>0.64</td>
<td>Mann-Whitney</td>
</tr>
<tr>
<td>APRI ≥0.7</td>
<td>8/61 (13.1)</td>
<td>17/78 (21.7)</td>
<td>0.37</td>
<td>Fisher’s exact</td>
</tr>
<tr>
<td>Abnormal liver imaging and/or biopsy</td>
<td>22/59 (37.2)</td>
<td>32/75 (42.6)</td>
<td>0.80</td>
<td>Fisher’s exact</td>
</tr>
<tr>
<td>Hepatitis B virus load, median, log_{10} IU/mL</td>
<td>3.49</td>
<td>3.49</td>
<td>Not applicable</td>
<td></td>
</tr>
</tbody>
</table>

Data presented as n/n (%) or mean ± SD unless otherwise indicated. Abnormal liver imaging was defined as a computed tomography scan, magnetic resonance imaging or an ultrasound radiology report of irregular hepatic surface/contour, or regenerative hepatic nodules with/without portal hypertension measured by Doppler. ALT Alanine aminotransferase; APRI Aspartate aminotransferase (AST)-to-platelet ratio index

Association of APRI ≥0.7 with age, sex and HBV genotype

An APRI ≥0.7 was chosen because those with APRI ≥0.7 had significantly more abnormal liver imaging and/or biopsy findings compared with those with APRI <0.7 (OR 2.4 [95% CI 1.1 to 5.7]; P=0.03). Additionally, in HCV-infected individuals, this cut-off has been deemed reasonable (16). Although 20.2% of males had an APRI ≥0.7, only 8.9% of females had an APRI ≥0.7; however, no statistical significance was found (OR 2.27 [95% CI 0.77 to 6.69]; P=0.20). HBV genotypes were compared for the presence of APRI ≥0.7 and no statistical significance was noted (Table 2). Age was an independent factor affecting APRI, with older age being associated with APRI ≥0.7 (median difference 10 years [95% CI 3.0 to 18.0]; P=0.011) (Table 2).

PC/BCP mutations and combination of age ≥50 years and BCP mutations

Individuals with a G1896A point mutation did not have a significantly higher proportion of APRI ≥0.7, but in fact showed a relative, but non-significant protective effect (OR 0.76 [95% CI 0.28 to 2.02]; P=0.76). However, those with a BCP dual mutation had a significantly higher...
HBV viral loads <4.3 log10 IU/mL, normal liver enzyme levels, and no BCP mutation (17). Individuals in the inactive phase tend to have biopsy results compared with those <50 years of age and no mutations showed statistical significance with abnormal liver imaging and/or biopsy results; however, the nonsignificance was related to abnormal liver imaging and/or biopsy results compared with those without the mutation (P=0.75). Those with BCP dual mutation had a higher proportion of APRI ≥0.7 (OR 5.12 [95% CI 1.13 to 23.16]; P=0.029), suggesting that a BCP dual mutation is an independent and relatively strong factor in elevating APRI. Finally, a combination of BCP dual mutation and age ≥50 years showed a borderline nonstatistical significance with APRI ≥0.7 (OR 6.67 [95% CI 0.79 to 56.42]; P=0.096) (Table 2).

Association of abnormal liver imaging and/or biopsy with age, sex and HBV genotype

Abnormal liver imaging and/or biopsy results were found in 45.4% of men, but only 20.3% of women had such results (OR 2.23 [95% CI 1.02 to 4.86]); however, the difference was nearly significant (P=0.059). Similar to what was observed with APRI ≥0.7, HBV genotypes did not affect liver imaging and/or biopsy results (Table 2). However, older age was associated with abnormal liver imaging and/or biopsy results, with a median difference of nine years (95% CI 4.0–14.0) (P=0.0008†) (Table 2).

| Table 3 |

<table>
<thead>
<tr>
<th>Factors associated with abnormal liver imaging suggestive of cirrhosis/hepatocellular carcinoma and/or abnormal finding on liver biopsy</th>
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<tbody>
<tr>
<td><strong>Factor</strong></td>
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<tr>
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<tr>
<td>Sex</td>
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<tr>
<td>Male</td>
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<tr>
<td>Female</td>
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<tr>
<td>Hepatitis B virus genotype</td>
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<tr>
<td>A</td>
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<td>B</td>
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<td>C</td>
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<td>Age*</td>
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<tr>
<td>PC mutation</td>
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<tr>
<td>BCP mutation</td>
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<td>Age ≥50 years AND BCP mutation</td>
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</table>

PROPORTION OF APRI ≥0.7 (OR 5.12 [95% CI 1.13 to 23.16]; P=0.029)

Association of abnormal liver imaging and/or biopsy with age, sex and HBV genotypes

Abnormal liver imaging and/or biopsy results were found in 45.4% of men, but only 20.3% of women had such results (OR 2.23 [95% CI 1.02 to 4.86]); however, the difference was nearly significant (P=0.059). Similar to what was observed with APRI ≥0.7, HBV genotypes did not affect liver imaging and/or biopsy results (Table 2). However, older age was associated with abnormal liver imaging and/or biopsy results, with a median difference of nine years (95% CI 4.0 to 14.0) (P=0.0008†) (Table 2).

Association of abnormal liver imaging and/or biopsy with PC/BCP mutations and combination of age ≥50 years and BCP mutations

Those with a G1896A point mutation did not have a higher proportion of abnormal liver imaging and/or biopsy results compared with those without the mutation (P=0.75). Those with BCP dual mutation also did not have a significantly higher proportion of abnormal liver imaging and/or biopsy results; however, the nonsignificance was relatively marginal (P=0.098), with OR 2.13 (95% CI 0.93 to 4.87). Finally, the combination of age ≥50 years and BCP dual mutation showed statistical significance with abnormal liver imaging and/or biopsy results compared with those <50 years of age and no mutations at postions 1762 and 1764 (P=0.041) (Table 3). Lower age cut-offs applied did not yield statistical significance (data not shown).
Interestingly, despite previous reports (15), median HBV viral load (as a measure of viremia over time in the present study) was not significantly associated with either mutation, suggesting that median HBV viral load cannot be used as a stand-alone parameter to predict adverse outcomes. The contrasting result may be somewhat due to the fact that in the Risk Evaluation of Viral Load Elevation and Associated Liver Disease/Cancer-Hepatitis B Virus (REVEAL) (15) study, the design was different because only those with HBV viral loads >3.23 log10 IU/mL were tested for PC/BCP mutation, while we did not use such an inclusion criterion. Additionally, their study was limited to a certain race/ethnicity and only two HBV genotypes (B and C). However, consistently detectable levels of HBV viral load may increase the theoretical risk for further development of mutations because the higher the viral propagation (which is error prone), the higher the probability of developing a mutation, thereby increasing its adverse outcomes indirectly. This, at least in part, was reflected in a higher (although not significant) proportion of APRI ≥ 0.7, and significantly higher proportion of imaging and/or biopsy abnormalities in those with both older age (≥ 50 years) and BCP mutation (Table 2).

Male sex is known to be associated with HCC (17). In our study, individuals with abnormal liver imaging and/or biopsy results suggest- ive of advanced fibrosis, cirrhosis or HCC were grouped together. This may explain why statistical significance was achieved with either of the two sexes; although male sex showed both higher percentage with APRI ≥ 0.7 and/or liver imaging/biopsy abnormalities. This may partly be due to a sex bias in referrals and in further investigations (including HBV viral load, genotype, ALT/AST assessments and imaging) as recommended per guidelines for HCC screening or could be due to immunological differences between the two sexes leading to T-helper (Th1/Th2) balance shifting toward Th1 in men. This could result in a greater chance of immune activation and liver tissue damage, which could lead to repair/regeneration and fibrosis as a consequence; however, this remains controversial (31-33).

Another interesting finding is the absence of an effect of HBV genotypes on clinical outcomes including genotype C, which has been shown to have worse outcomes (16). There are eight genotypes of HBV (A through H [34-36]). Initially the genotypes were somewhat geographically distinct, with genotype A focused in northern Europe, North America, South America, Australia and Sub-Saharan Africa; genotype B focused in West Africa, southeast Asia, China and Japan; genotype C focused in Southeast and East Asia, Pacific Islands, Australian Aboriginals and Alaska; genotype D focused in the Mediterranean, Middle East, Eastern Europe, Indian subcontinent, Central Asia, Mongolia, South Africa, Arctic region, Somalia, Papua-New Guinea, Australian Aboriginals and Indonesia; genotype E focused in West Africa; genotype F focused in Alaska, South America and Central America; genotype G focused in the Europe and North America; and genotype H focused in Central America (36,37). Two additional genotypes, I and J, have recently been proposed (38,39). However, with more frequent and widespread migration occurring, the geographical distribution of the genotypes is likely shifting. The largest study to date investigating genotype prevalence in North America was by Chu et al in 2003 (9). This study examined 694 patients with chronic HBV infection and found that while all genotypes from A to G were seen, A and C were the most common, with a slight variation in prevalence based on ethnicity. Although none of the HBV geno- types found in our study were directly associated with abnormal laboratory, imaging or biopsy results, because the distribution of PC and BCP mutations varies with HBV genotypes, HBV genotype could be an indirect measure of outcomes, especially if used in combination with PC or BCP mutations as previously shown (40).

Our study shows that the prevalence of PC and BCP mutations in the largest subpopulation of chronically HBV-infected patients (anti-HBe patients) is relatively high; this could be attributed to immigration over the past decade. The high prevalence has unique and challenging ramifications from both clinical and public health per- spective, requiring more attention and systematic monitoring. The present study was retrospective in nature, relatively small in size and limited in geographical span. Therefore, multicentre prospective observational studies could shed further light on the highly intricate relationship of HBV variants with their human host and may potentially benefit individual patient management.

**KEY POINTS**

- The prevalence of PC and BCP mutations in North America needs more recent studies in all jurisdictions.
- PC and BCP mutations are more common than previously known and tend to be, at least indirectly, associated with poor clinical outcomes.

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**REFERENCES**


