Acceleration to Death from Liver Cancer in People with Hepatitis B Viral Mutations Detected in Plasma by Mass Spectrometry

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Abstract

Liver cancer is the leading cause of cancer death in many regions of the world. With the goal to discover biomarkers that reflect subsets of high-risk individuals and their prognosis, we nested our study in a male cohort of 5,581 hepatitis B surface antigen carriers in Qidong, People's Republic of China, who were recruited starting in 1989. By December 2003, 667 liver cancer cases were diagnosed in this group and plasma samples collected at the initial screening at enrollment were available in 515 cases who had succumbed to liver cancer. Hepatitis B virus (HBV) DNA could be isolated in 355 (69%) of these samples. In 14%, 15%, 19%, 31%, and 22%, screening took place at $\leq 1.5$, $1.51$ to $3$, $3.01$ to $5$, $5.01$ to $9$, and $>9$ years before death, respectively; and 39% died at age below 45 years. The relation between mutations in HBV and time to death were determined by logistic regression for the odds of death and by survival analyses methods with age as the time scale. In 279 (79%) of these individuals, the samples contained a two-nucleotide 1762T/1764A HBV mutation. Sixteen samples lacking the 1762T/1764A mutation had novel mutations elsewhere in the 1761 to 1767 region of the HBV genome. There was a statistically significant difference ($P = 0.012$) for the high prevalence of the HBV mutations in the men who died from hepatocellular carcinoma under the age of 45 years relative to those who died after 55 years of age and HBV mutations accelerated death (relative hazard, 1.40; 95% confidence interval, 1.06-1.85) and that the effect was attenuated by age from 2.04 for age 35 years to 1.0 for age 65 years with the 90% confidence band being above 1 for ages $<50$ years. These findings provide a conceptual framework to explain the acceleration of mortality in individuals infected with HBV.

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Introduction

Hepatocellular carcinoma (HCC) is a major cause of cancer morbidity and mortality in many parts of the world, including Asia and sub-Saharan Africa, where there are upward of 500,000 new cases each year and more than 200,000 deaths annually in the People’s Republic of China alone (1, 2). The major etiologic factors associated with development of HCC in these regions are infection with hepatitis B virus (HBV) and/or hepatitis C virus and lifetime exposure to high levels of aflatoxin B1 in the diet (3, 4). Detailed knowledge of the etiology of HCC has spurred many mechanistic studies to understand the pathogenesis of this nearly always fatal disease, and this knowledge is beginning to be translated to preventive interventions in high-risk populations (3, 5).

HBV is a significant risk factor for HCC in the economically developing world where there are over 400 million viral carriers (2, 6, 7). The biology, mode of transmission, and epidemiology of this virus continues to be actively investigated and has been recently reviewed (6, 8, 9). The contribution of HBV to the pathogenesis of liver cancer is multifactorial and is complicated by the identification of mutant variants in HBV that modulate the carcinogenesis process (10, 11). The HBV genome encodes its essential genes with overlapping open-reading frames; therefore, a mutation in the HBV genome can alter the expression of multiple proteins. In many cases of HCC in China and Africa, a double mutation in the HBV genome, an adenine to thymine transversion at nucleotide 1762, and a guanine to adenine transition at nucleotide 1764 (1762A/1764A), has been found in tumors (12-14). The molecular basis for the formation of these nucleotide changes and the temporal relation for HBV infection is still a source of active investigation. Thus, whether these mutational changes can be acquired and transmitted from person to person is not completely understood. Nonetheless, this segment of the HBV genome contains an overlapping sequence for the base core promoter and the HBV X gene; therefore, the double mutation in codons 130 and 131 of the HBV X gene reported in human HCC is identical to the 1762 and 1764 nucleotide changes (15). The onset of these mutations have been also associated with the increasing severity of the HBV infection and cirrhosis (13, 14). Thus, the tracking of these polymorphisms with disease outcomes makes it a candidate biomarker for the early detection of HCC risk in individuals.

Several studies have now shown that DNA isolated from serum and plasma of cancer patients contains the same genetic aberrations as DNA isolated from an individual’s tumor (16-18). The process by which tumor DNA is released into circulating blood is unclear but may result from accelerated necrosis, apoptosis, or other processes (19). Recently, we have found that a specific HBV double 1762T/1764A mutation was not only detectable in plasma samples at the time of HCC diagnosis, but that it can be measured in some individuals at least 5 years before diagnosis (20). In the present study, we have extended the use of an electrospray ionization mass spectrometry–based method called short oligonucleotide mass spectrometry.
Mass spectrometry. High-performance liquid chromatography of a 50:50 (v/v) 0.8 mol/L 1,1,1,3,3,3-hexafluoro-2-propanol/methanol (pH 6.9) and solvent B was used in this investigation was chosen to examine the relation between the presence of these mutations and the age at death from liver cancer. The population studied was at elevated risk for liver cancer due to their defined HBV carrier status. Archived plasma samples collected during the initial screening of this cohort provided the opportunity to determine the extent to which these HBV mutations tracked with acceleration to liver cancer death within this high-risk cohort.

Materials and Methods

Case Materials. The plasma samples investigated in this report were obtained as part of an ongoing investigation of HCC and its risk factors in Qidong, People’s Republic of China (21). In this investigation, 36,382 men residing in Qidong were screened for hepatitis B surface antigen status starting in 1989. These individuals were followed up at a 6-month time point to determine carrier status and by 1992, a total of 5,581 of these men were identified as carriers and they were enrolled into a cohort study. All of the men were between the ages of 30 and 69 years at the time of the screening when a blood sample was taken and stored. These individuals have been followed through the Qidong Cancer Registry for occurrence of HCC and cause-specific deaths. By the end of December 2003, a total of 667 of these men had been diagnosed with HCC. In all cases, HCC diagnosis was made by one or more of the following means: (a) surgical biopsy, (b) elevated serum α-fetoprotein (levels >100 ng/mL) with consistent clinical and radiological history, (c) positive computerized axial tomography scan, and (d) ultrasonography with consistent clinical history. The accumulation of HCC cases in this cohort proceeded at a fairly constant rate of between 40 and 45 cases per year and the mean time from diagnosis of HCC to death was 6 months. This collaboration between the Qidong Liver Cancer Institute and Johns Hopkins University and the respective consent forms have been approved by each respective Institutional Review Board for Human Research.

Mutation Detection by Short Oligonucleotide Mass Analysis. DNA was isolated from plasma samples and short oligonucleotide mass analysis was done as previously described (20, 22). PCR was done on this reaction mix using the following primers: HBVx7F, 5′-TTGTGTTTAAAGACGCTG-GAGGCTGGAGGAGGATTTAGTTAAT-3′ and HBVx7R, 5′-TTGTTGCCAGCAACATTTATCTGAGGGCCTCTGATG-3′. The thermocycling conditions were 95°C for 2 min, then 40 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 30 s, followed by a final extension of 72°C for 2 min. Negative controls (no DNA added) were included for each set of PCR reactions. PCR product was purified by ethanol precipitation and digested with 8 units of BpmI (New England Biolabs) overnight at 37°C in a volume of 50 μL to release 8 bp internal fragments. A phenol-chloroform extraction followed by an ethanol precipitation in the presence of SeeDNA (Amersham Pharmacia) was done to purify samples for analysis by electrospray ionization mass spectrometry.

The digested fragments were resuspended in 10 μL of the high-performance liquid chromatography mobile phase [70:30 (v/v) solvent A solvent B, where solvent A was 0.4 mol/L 1,1,1,3,3,3-hexafluoro-2-propanol (pH 6.9) and solvent B was 50:50 (v/v) 0.8 mol/L 1,1,1,3,3,3-hexafluoro-2-propanol/methanol] and 8 μL was introduced into the high-performance liquid chromatography coupled to the electrospray ionization mass spectrometry. High-performance liquid chromatography was carried out at 30 μL/min using a 1×150 mm Luna C18, 5 μ reversed phase column (Phenomenex) and Surveyor pumps (ThermoFinnigan Corp.). The gradient conditions were 70% A/30% B isocratic 1 minute programme to 100% B in 3 min, where it was held for 2.5 min followed by a return to 70% A/30% B in 1.5 min and isocratic elution for the remaining 32 min of the chromatography.

Mass spectra were obtained with a LCQ Deca ion-trap mass spectrometer (ThermoFinnigan) equipped with an electrospray ionization source operated in the negative ionization mode. The spray voltage was set at −4.0 kV and the heated capillary was held at 240°C. Each of the oligonucleotide ions was isolated in turn and subjected to collision-induced dissociation at 30% collision energy. Full scan spectra of the resultant fragment ions from m/z 600 to 2,000 were acquired and signals from up to three specific fragment ions were summed as a function of time for each of the oligonucleotides. The mass spectrometer was programmed to acquire data in the centroid mode (1 μscan; 200 ms; isolation width 3 Da) using scan events monitoring each [M-2H]^− oligonucleotide individually. Scan event 1: WT-s [5′-AAGGTCCT-3′]; m/z 1,099.20→750-2,000. Scan event 2: WT-as [5′-ACCTTTA-3′]; m/z 1066.70→750-2,000. Scan event 3: Mut-s [5′-ATGATCT-3′]; m/z 1,086.70→750-2,000. Scan event 4: Mut-as [5′-ATCATTTA-3′]; m/z 1,078.70→750-2,000. The fragment ions used for each oligonucleotide were as follows. WT-s: m/z 803.78 + 1,132.22 + 1,243.27; WT-as: m/z 910.07 + 1,531.29; Mut-s: m/z 914.30 + 1,227.29; and Mut-as: m/z 1,084.0 + 1,252.0. A sample was considered positive when fragment masses were observed in either or both sense and antisense channels for the mutant allele in at least three scans across the peak.

Data Analysis. All samples were coded to mask identity before short oligonucleotide mass analysis. In addition, the codings of the samples was done to allow for randomization of the order of sample analysis and interspersion of control samples. The study population comprised the individuals who died from HCC and on whom DNA was isolated from plasma collected at the time the individuals were screened for enrollment into the cohort study.

To determine whether the presence of 1762T/1764A and other HBV mutations accelerated the time to death we first carried out a logistic regression to characterize the odds of having these HBV mutations according to (a) the years elapsed between the screening and the age at which the individuals died from HCC; and (b) the age at which individuals died. For this analysis, time from screening to death were handled as an ordinal variable taking values 0 to 4 for the ≤1.5, 1.51 to 3, 3.01 to 5, 5.01 to 9, and >9 years categories, respectively; and age at death was handled as a categorical variable with 45 and 55 years as cutoff values for three categories. To provide a graphical depiction of the frequency of 1762T/1764A and other HBV mutations according to the two variables of interest, we used the diamond plot as described by Li et al. (23).

To tightly control for the intrinsic association of age and death, we carried out a semiparametric regression analysis using age as the time scale. Specifically, the outcome for each individual was determined by the time period defined by the age at screening and the age at death. To incorporate the heterogeneity of ages at the time of screening, survival analysis methods for staggered entries were implemented to determine whether presence of HBV mutations accelerated the hazard of death in individuals of the same age. To allow for the putative effect of HBV mutations to differ according to age, we carried out a stratified analysis according to age being ≤45, 46 to 55, and >55 years. We also tested for an interaction of HBV mutations with age as a continuous variable. Specifically, if h(a) denotes the conditional hazard of dying at age a among deceased cases without HBV mutations, the hazard of those with the HBV mutations and at the same age was h(a)exp(α + β(a − 55)). In this model, exp(α) represented the relative
Table 1. HBV sequence from nucleotide 1761 to 1767 in 355 plasma samples (wild-type AAGGTCT)

<table>
<thead>
<tr>
<th>Sequence</th>
<th>No. samples (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type only (AAGGTCT)</td>
<td>60 (16.9%)</td>
</tr>
<tr>
<td>Mutations in 1762/1764 only (ATGATCT)</td>
<td>244 (68.7%)</td>
</tr>
<tr>
<td>Wild-type and 1764/1766 mutations</td>
<td>27 (7.6%)</td>
</tr>
<tr>
<td>Mutations in 1764/1766 only</td>
<td>11 (3.1%)</td>
</tr>
<tr>
<td>Mutations in 1762/1764 and 1764/1766</td>
<td>7 (2.0%)</td>
</tr>
<tr>
<td>Triple mutations in 1762/1763/1764</td>
<td>2 (0.6%)</td>
</tr>
<tr>
<td>Triple mutations in 1762/1763/1764 and 1762/1764</td>
<td>1 (0.3%)</td>
</tr>
<tr>
<td>Triple mutations in 1762/1764/1766</td>
<td>2 (0.6%)</td>
</tr>
<tr>
<td>Triple mutations in 1762/1764/1765</td>
<td>1 (0.3%)</td>
</tr>
</tbody>
</table>

hazard of death for those with the HBV mutations at 45 years of age; and \( \exp(\beta) \) represented the modification of the effect of the mutation according to age.

Results

Of the 5,581 hepatitis B surface antigen carriers enrolled into this cohort study, 667 cases of HCC were diagnosed by December 31, 2003. A review of the specimen collection repository revealed that 536 of these cases had sufficient serum remaining for DNA isolation. Because the primary objective was to characterize the presence of 1762T/1764A and other HBV mutations in relation to when individuals died from HCC, the relevant subgroup selected consisted of the 515 individuals who died from liver cancer before December 31, 2003. HBV DNA was isolated from 355 (69%) of the 515 samples and this is consistent with prior experience with serum/plasma banked specimens (20, 22). Using the mass spectrometry technique, short oligonucleotide mass analysis, for the measurement of a double 1762T/1764A mutation in the HBV genome, 279 (79%) of these samples had this specific mutation. This level of positive samples compares well with the 75% positive rate for the presence of this mutation in liver tumor tissue samples from Qidong (20).

Table 1 shows the range of HBV wild-type sequence and mutations detected from nucleotides 1761 to 1767 in these samples. Only 60 of the 355 informative samples (16.9%) contained only wild-type sequence HBV. Collectively, 279 samples (78.6% of the total number) had mutations in HBV at nucleotides 1762 and 1764. Of these 279 samples, 244 contained only the 1762T/1764A mutation, whereas 27 samples carrying the 1762/1764A mutation had detectable wild-type HBV. The eight remaining 1762T/1764A mutation containing samples carried additional detectable mutations in the 1761 to 1767 nucleotide region. Thus, collectively, 295 of the 355 samples (83.1%) having amplifiable HBV DNA contained one or more mutations in this seven-nucleotide region. Seven of these samples had a 1764A mutation, but at nucleotide 1766 the mutation was a cytosine to thymine transition. In one case, the 1762/1764A mutation was detected but at nucleotide 1763 there was a guanine to cytosine transition resulting in a three base change in the viral genome. Eleven of the cases only had a common 1764 adenine mutation followed by a cytosine to thymine transition at nucleotide 1766. Five other variants independent of the 1762T/1764A mutation were also detected. The mean age of death for those individuals with a 1762T/1764A mutation was 48.7 years, but the mean age increased to 54.3 years in the 37 cases containing the other HBV alterations.

Discussion

The pathogenesis of HCC is complex and the disease progresses through a multistage process with a majority of cases involving liver cirrhosis (24). The worldwide geographic distribution of HCC illustrates that areas where viral hepatitis infection with HBV is endemic also have a high incidence of disease (9). Unfortunately, the effect of HBV has resulted in 400 million HBV carriers worldwide who are at elevated risk for liver cancer (6). Because there is a less than 20% chronicity rate for HBV infection, there are more than 2 billion people who are or have been exposed to this virus at some point during their lifetime. In hyperendemic areas, such as in regions of Korea and China, infection rates of HBV have exceeded 50% by age 30 years (25). The other major viral factor in liver cancer is HCV but to date in China, HCV is found in less than 5% of HCC cases (26). Collectively, the need to identify high-risk individuals well before clinical diagnosis of liver cancer would be an aid in the development of prevention and intervention strategies. The detection and validation of new HBV biomarkers offers to be an important contribution to attain this goal.

Table 2. Descriptive statistics according to presence of HBV mutation of 355 men who died from HCC and whose DNA was isolated from plasma

<table>
<thead>
<tr>
<th>Years to death from screening (y)</th>
<th>Overall (N = 355)</th>
<th>HBV mutation Present (n = 295)</th>
<th>HBV mutation Absent (n = 60)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median (IQR)</td>
<td>5.5 (2.6-8.5)</td>
<td>5.0 (2.5-8.2)</td>
<td>7.6 (3.2-10.1)</td>
</tr>
<tr>
<td>≤1.5</td>
<td>50 (14%)</td>
<td>44 (15%)</td>
<td>6 (10%)</td>
</tr>
<tr>
<td>1.51-3</td>
<td>52 (15%)</td>
<td>45 (15%)</td>
<td>7 (12%)</td>
</tr>
<tr>
<td>3.01-5</td>
<td>67 (19%)</td>
<td>62 (21%)</td>
<td>5 (8%)</td>
</tr>
<tr>
<td>5.01-9</td>
<td>109 (31%)</td>
<td>86 (29%)</td>
<td>23 (38%)</td>
</tr>
<tr>
<td>Median (IQR)</td>
<td>77 (22%)</td>
<td>58 (20%)</td>
<td>19 (32%)</td>
</tr>
</tbody>
</table>

Abbreviation: IQR, interquartile range.
Death from Liver Cancer in People with HBV Mutations

The prevalence rates of mutation (p) are calibrated to the lowest (65%) and the largest (93%) according to (p – 65) / (93 – 65). The value of each calibrated rate is depicted by a polygon inside each cell where the areas of each polygon (relative to the area of each cell) and the middle vertical length of the polygon (relative to the vertical diagonal of each cell) represent the value of the calibrated rate. For example, for the rate 85 for those with age <45 y and time to death from screening >9 y, the calibrated rate corresponds to 71.4% = 100(85 – 65) / (93 – 65); therefore, the polygon in that cell represents 71.4% of the area of the cell.

The development of new biomarkers for predicting an individual’s risk for HCC after HBV infection is predicated upon an understanding of the molecular pathways through which the virus mediates its effects (4, 12, 27). The pathology of HBV infection is also modulated through the selection and expression of a number of common viral mutants that affect a number of key viral proteins (28-30). One of these common mutations is HBV 1762 T/1764A that affects the expression of both the hepatitis B e antigen, because the mutation lies in the basic core promoter, and the X gene (31). This mutation was originally characterized in HBV e antigen–negative people (32). The 1762 T/1764A double mutation occurs more frequently in people infected with the genotype C strains of HBV, which is the most common genotype found in East Asian patients (33-35). This double mutation tracks with an increase in the rate of HBV genome replication was found (10, 37). Thus, the alterations of the disease.

The HBV 1762 T/1764A double mutation also affects the amino acid sequence of the HBV X gene because it resides in codons 130 and 131, thereby inducing lysine to methionine and valine to isoleucine alterations, respectively (15). The X gene protein has been found to have numerous biological activities; however, its specific role and that of this mutant protein in the pathogenesis of liver cancer have yet to be elucidated (27). Recent data have also shown that there is a sequential accumulation of these mutations in people during the course of the progression to cancer (38). Finally, the downstream effects of the formation of the double mutation at nucleotides 1762 and 1764 are being explored in model systems and recent data have shown that this alteration affects the binding of several transcription factors (HNF1 and HNF4) that are liver specific (39). This may serve as yet another biological basis for mutant viral selection in the liver.

The development and validation of biomarkers for early detection of disease or for the identification of high-risk individuals is a major translational effort in cancer research. In liver cancer, the early detection of HCC is well established using α-fetoprotein biomarker levels in plasma or serum (40). Although the use of α-fetoprotein as a HCC diagnostic marker is widely used in high-risk areas, because of its ease of use and low cost, this marker does suffer from low specificity due to its occurrence in diseases other than liver cancer. This lack of specificity has contributed to the desire to identify of other molecular biomarkers that are possibly more mechanistically associated with HCC development, including hypermethylation of the p16 gene, p53, GSTP1 promoter regions, and codon 249 mutations in the p53 gene (41-44). Results from investigations of p16, p53, GSTP1 promoter hypermethylation, and p53 mutations indicate that these markers are prevalent in HCC, but there is as of yet limited information on the temporality of these genetic changes before clinical diagnosis.

In this current investigation, we examined the temporality of detecting the HBV 1762 T/1764A mutation and other mutations in the nucleotide 1761 to 1767 region in plasma before death from HCC. This study was facilitated by the availability of a large cohort of hepatitis B surface antigen carriers that have been prospectively followed in Qidong, People’s Republic of China. Overall, there was an inverse relation between HBV mutations and age at death revealing that cases under the age of 45 years were statistically significantly (P = 0.012) more likely to have these changes than in men who died after age 55 years. Among the cases with the mutation, 39% died at an age younger than 45 years but only 23% did so among those without the mutation. Thus, these detectable mutations are potential biomarkers of exposure and risk of HCC. The underlying mechanism for the formation and selection of the HBV 1762 T/1764A double mutation and other changes is unknown at this time. Clearly, inflammatory responses in cells produce large quantities of reactive oxygen species that are known to modify DNA and induce mutations (45). Inflammatory responses also lead to the activation of a group of cytidine deaminases of the APOBEC3 family and this activation process has also been found to induce hypermutations in this part of the HBV genome coding region (46). Further, HBV genome replication has been shown to be a very error prone process that can lead to increased guanine to adenine transition mutations (47). However, the underlying mechanism(s) for the formation of these changes in the HBV sequence remains unclear and needs further investigation.

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Deceased</th>
<th>Relative hazard (95% confidence interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall</td>
<td>355</td>
<td>355</td>
<td>1.40 (1.06-1.85)</td>
</tr>
<tr>
<td>Age ≤ 45 y</td>
<td>220</td>
<td>137</td>
<td>2.04 (1.37-3.54)</td>
</tr>
<tr>
<td>45 y &lt; Age ≤ 55 y</td>
<td>179</td>
<td>121</td>
<td>1.17 (0.74-1.84)</td>
</tr>
<tr>
<td>Age &gt; 55 y</td>
<td>97</td>
<td>97</td>
<td>1.21 (0.76-1.95)</td>
</tr>
</tbody>
</table>

NOTE: A person can cross over age categories.

* Includes all people who reached age 45 yrs alive.
† Includes all people who reached age 55 yrs alive.
Figure 2. Relative hazard of death for HBV mutations by age. Dashed lines, 90% confidence bands.

epidemiologic and molecular studies. The striking differences in the age of onset of HCC in China when Beijing and Qidong are compared suggest that factors such as the HBV alterations and exposures to environmental agents such as aflatoxins could be playing a significant role (3).

Parenthetically, the striking sequence-specific binding and induction of mutations by aflatoxin at codon 249 of p53 in HCC may be an impact into the formation of these alterations. The wild-type sense strand sequence of p53 at codon 249 is AGG and this codon is bracketed by two purines and two pyrimidines when reading from 5' to 3', resulting in a GGA-GGCC sequence. Interestingly, the HBV 1762T/1764A double mutation lies in a sequence of AAGGTC. The AGG is also bracketed by two purines and two pyrimidines when reading from 5' to 3'. Thus, it is intriguing to speculate whether this common pattern shared by both p53 and HBV might be a mutational hotspot targeted by aflatoxin or some other chemical agent. Because both changes are induced mutations that contribute in some fraction to the pathogenesis of HCC, these biomarkers could be used in the early detection of disease.

The etiology of HCC in some of the highest risk regions is further affected by the interplay between environmental chemical agents and HBV infection. Two major cohort studies have shown the strong multiplicative interaction between aflatoxin B1 exposure and HBV in the development of HCC (48-50). Collectively, these results strongly support a causal, amplifying relationship between two major HCC risk factors. These findings have encouraged the development and validation of both aflatoxin and HBV biomarkers that can be used to identify high-risk individuals before HCC diagnosis. Thus, a combined use of exposure and genetic biomarkers might reveal the subset of high-risk people within a population who would most benefit from targeted, mechanism-based interventions.

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