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Prospective evaluation of hepatitis B 1762^{T}/1764^{A} mutations on hepatocellular carcinoma development in Shanghai, China

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Abstract

Chronic infection with the hepatitis B virus (HBV) is the most important risk factor for hepatocellular carcinoma (HCC). However, determinants of HCC risk in infected individuals are not well understood. We prospectively evaluated the association between acquired HBV 1762^{T}/1764^{A} double mutations and HCC risk among 49 incident HCC cases and 97 controls with seropositive hepatitis B surface antigen (HBsAg) at baseline from a cohort of 18,244 men in Shanghai, China, enrolled during 1986−1989. Compared with HBV carriers without the mutations, chronic HBV carriers with the HBV 1762^{T}/1764^{A} double mutations experienced an elevated risk of HCC (odds ratio=2.47, 95% confidence interval=1.04 to 5.85, *P*=0.04). Risk increased with increasing copies of the double mutations; men with 500 or more copies per μL serum had an odds ratio of 14.57 (95% confidence interval=2.41 to 87.98) relative to those without the double mutations (P_{trend}=0.004). Thus, the HBV 1762^{T}/1764^{A} double mutation is a co-determinant of HCC risk for people chronically infected with HBV.

Keywords

hepatitis B; viral mutation; liver cancer

Introduction

Chronic infection with the hepatitis B virus (HBV) is the most important risk factor for hepatocellular carcinoma (HCC) in humans. Given that only a fraction of persons chronically infected with HBV will eventually develop HCC, viral characteristics may contribute to the HBV-induced hepatocarcinogenesis. A double mutation in the HBV genome, an adenine to thymine transversion at nucleotide 1762, and a guanine to adenine transition at nucleotide 1764 (1762^{T}/1764^{A}), has been found in liver tumors (1-3). Recently, we have found that a specific

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CONFLICT OF INTEREST STATEMENT
All authors declare that they have no conflict of interest.

HBV double $1762^{T}/1764^{A}$ 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 cancer diagnosis (4). HBV carriers with these HBV double mutations experienced an earlier age at death from HCC than HBV carriers without the mutations (5). Also, these HBV double mutations were associated with an approximately 70% increased risk of HCC among individuals with total HBV DNA level of 10,000 or more copies/mL plasma at baseline (6). Here, we evaluated the role of HBV $1762^{T}/1764^{A}$ double mutations in the development of HCC in a prospective cohort of 18,244 men in Shanghai, China.

Materials and Methods

The design of the Shanghai Cohort Study has been described in detail elsewhere (7,8). Briefly, 18,244 men (about 80% of eligible subjects) between 45 and 64 years of age were enrolled in the study between January 1986 and September 1989. In addition to in-person interviews soliciting information on use of tobacco and alcohol, and medical history, we collected a 10-ml blood sample from each participant at baseline. The Institutional Review Boards at the University of Minnesota and the Shanghai Cancer Institute have approved this study.

Identification of incident cancer cases and deaths among cohort participants has been accomplished through record linkage of our cohort dataset with those from the Shanghai Cancer Registry and the Shanghai Municipal Vital Statistics Office, and by annual re-contact of all surviving cohort members. Cumulatively, 422 (2.3%) cohort participants had been lost to follow-up. As of December 31, 2001, 214 cohort participants developed HCC. In our original study (i.e., the parent study of the present one), we randomly chose 5−10 control subjects for each case among all cohort members, who were individually matched to the index cases by date of birth (±2 years), month and year of blood collection, and neighborhood of residence at recruitment. We tested serum samples of all 214 cases and the 1100 matched controls for the presence of hepatitis B surface antigen (HBsAg), using a standard radioimmunoassay (AUSRIA; Abbott Laboratories, Abbott Park, IL), and 131 cases and 106 controls were positive for HBsAg.

For the present study, we randomly chose 50 cases from the 131 HBsAg-positive HCC patients to preserve precious pre-diagnostic serum samples for other on-going research projects. All 106 identified control subjects with seropositive HBsAg were included in the present study given the relatively low prevalence of HBV double mutations in other Asian populations (6, 9). We included an additional 20 HBsAg-negative serum samples (10 from HCC cases and 10 from controls) as negative controls in the laboratory measurements. The determination of HBV $1762^{T}/1764^{A}$ double mutations was done with some modification as previously described (4, 5). Briefly, DNA was isolated from serum. PCR was done on this reaction mix using the following primers: HBVx-7F, 5-TTTGTTTAAAGACTGGGAGGACTGGAGGGAGGAGA TTAGGTTA-3 and HBVx7R, 5-TGGTGCGCAGACCAATTTATGCTGGAGGCCTCCTA GTACAA-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.

The copy numbers of HBV DNA with the $1762^{T}/1764^{A}$ double mutations were quantified from 200 μl serum and determined by quantitative real-time (RT) PCR as described previously (5). The RT PCR was done using an ABI 7300 Real-Time PCR System utilizing wildtype and mutant-specific fluorogenic probes (Applied Biosystems, Foster City, CA). Real-time PCR

was performed on 2 μL of serum-isolated template DNA. Real-time PCR was performed on 5ng template DNA from cell pellet samples and 100pg template DNA from media samples. Primer pair and wildtype and mutant-specific probe sequences for HBV $1762^{T}/1764^{A}$ are 5'-CCG ACC TTG AGG CAT ACT TCA -3' for the forward primer and 5'- CCA ATT TAT GCC TAC AGC CTC CTA -3' for the reverse primer. The probes were VIC-AGG TTA AAG GTC TTT GTA C and 6FAM-AGG TTA ATG ATC TTT GTA C for the wild-type and mutants, respectively. The Real-time PCR was done in a total volume of 25 μL consisting of 1× Taqman Universal Master Mix (Applied Biosystems), 900nM each primer, 200nM each probe and template DNA. Thermal cycler conditions were as follows: 50°C for 2 minutes, then 95°C for 5 minutes, followed by 45 cycles of 95°C for 30 seconds and 60°C for 1 minute. Each sample was run in duplicate. Serial dilutions of HBV nts1762/ 1764 wildtype and mutant plasmid DNA standards were included on every 96-well plate analyzed. Also, HepG2 cell DNA (an HBV-negative control) and no DNA added controls were included on each 96-well plate analyzed. All laboratory staff were masked to case status during the analysis and interpretation of mutation data.

All 20 HBsAg-negative serum samples tested were absent for HBV $1762^{T}/1764^{A}$ mutation. Serum samples from one case and nine controls were insufficient for the assay. Thus, the present study included 49 cases and 97 control subjects.

Chi-square test was used to examine the difference in distribution by year of blood draw, neighborhood of residence at enrollment, smoking, alcohol drinking, and serum retinol levels in tertile, and *t*-test for the difference in mean age between cases and controls. Although we used an individually matched case-control study design in the parent study, we broke the matched case-control sets to maximize the sample size for the present statistical analysis since HBsAg serologic status was not a matching factor. We used standard statistical methods for analyses of unmatched case-control data (10). Unconditional logistic regression models were used to calculate odds ratios (ORs) for HCC and their corresponding 95% confidence intervals (CIs) and *P* values associated with the presence of HBV $1762^{T}/1764^{A}$ double mutations. In addition to original matching factors (age, year of blood draw, and neighborhood of residence at recruitment), cigarette smoking (never, ever), heavy alcohol consumption (nondrinkers or <4 drinkers per day, 4 or more drinkers per day), and serum concentration of retinol in tertile were included in all unconditional logistic regression models as covariates.

Statistical computing was conducted using the SAS version 9.1 (SAS Institute Inc., NC) and Epilog windows version 1.0 (Epicenter Software, CA) statistical software packages. All *P* values quoted are two-sided. Two-sided *P* values less than 0.05 were considered statistically significant.

Results

The mean ages (±standard deviation) of cases (n=49) at HCC diagnosis was 58.0 (±4.2). The average time interval between blood draw and cancer diagnosis among cases was 3.1 (±1.8) years (range, 3.4 month to 6.5 years).

Cases were comparable with controls in terms of mean baseline age and body mass index (weight in kg divided by height in meters squared), and frequencies by year of blood draw and neighborhood of residence at recruitment (Table 1). The prevalence of cigarette smoking and heavy alcohol consumption (4 or more drinks per day) was higher in cases than controls. As reported previously (11), individuals who developed HCC during follow-up had significantly lower level of serum retinol at baseline than control subjects who remained free of HCC (Table 1). All 49 cases and 97 controls with seropositive HBsAg reported a history of physician-diagnosed hepatitis and/or cirrhosis during the in-person interviews at baseline.

Twenty-two (44.9%) of 49 HCC cases and 21 (21.6%) of 97 controls tested positive for HBV $1762^{T}/1764^{A}$ double mutations, yielding an OR of 2.47 (95% CI=1.04−5.85). The OR increased with increasing copies of the $1762^{T}/1764^{A}$ mutation (*P* for trend=0.004). Men with 500 or more copies per μL serum had an OR of 14.57 (95% CI=2.41−87.98) (Table 2). We repeated statistical analysis after excluding 6 cases of HCC that were diagnosed within one year of the blood draw. The results became slightly stronger. Compared with those negative for the HBV double mutations, men with the double mutations had an OR of 2.66 (95% CI=1.08 −6.59). The OR was 16.31 (95% CI=2.35−113.00) for men with 500 or more copies per μl serum (Data not shown).

To evaluate the potential differential effect of HBV mutation on risk of HCC among individuals with different length of follow-up, we examined the association between the HBV double $1762^{T}/1764^{A}$ mutation and HCC risk stratified by the time interval (years) between baseline sample collection and date of cancer diagnosis among HCC cases. The HBV mutation-HCC risk association was similar for cases with short-term to those with relative long-term follow-up. ORs (95% CIs) was 2.35 (0.83−6.66) in cases diagnosed within 3 years post-enrollment and 2.81 (0.87−9.06) in cases with longer follow-up.

Discussion

Although the majority (61%) of HCC cases in this cohort of 18,244 middle-aged and older Chinese men tested positive for HBsAg, HCC is not an inevitable consequence of chronic infection with HBV. Only a fraction (approximately 15%) of HBV carriers develop HCC over their lifetime, suggesting that other factors may modify the risk of developing HCC among HBV carriers. Previously, we have demonstrated significant modifying effects of dietary aflatoxin and retinol exposures on HBV-related HCC risk in this study population (7,11,12). We also showed that genetic mutations in the cytokine genes played an important role in determining an individual's susceptibility to HBV-related HCC (13).

The role of HBV DNA mutation in hepatocarcinogenesis continues to be of great interest. The prevalence of HBV $1762^{T}/1764^{A}$ double mutations in this middle-aged male population in China was 21.6%, consistent with studies in other Asian populations (6,9). A recent Taiwan study reported that 28.6% of HBV carriers possessing at least 10,000 copies/mL plasma of total HBV DNA tested positive for these HBV double mutations (6). Our present finding of a statistically significant, 2.5-fold increased risk of HCC associated with the $1762^{T}/1764^{A}$ double mutations after adjustment for potential confounders also is consistent with the 1.7-fold increased relative risk noted in the Taiwan study (6). A novel finding of the present study is a strong, dose-response relationship between the mutant copy numbers of the HBV $1762^{T}/1764^{A}$ double mutations and HCC risk. HBV carriers with 500 or more mutant copies per μL serum experienced an approximately 15-fold increased risk of HCC relative to HBV carriers without the double mutations.

The HBV genome encodes its essential genes with overlapping open reading frames; therefore, the $1762^{T}/1764^{A}$ double mutation in the HBV genome can alter the expression of multiple proteins. The molecular basis for the formation of the double mutations 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.

Several mechanisms of hepatocarcinogenesis relating to the HBV $1762^{T}/1764^{A}$ double mutations have been hypothesized. The $1762^{T}/1764^{A}$ double mutation may enhance HBV virulence by increasing the host's immune response (14,15), increasing viral replication (16-18), or altering the coding region for the X antigen (3,19). The $1762^{T}/1764^{A}$ double

mutation appears to enhance the efficiency of viral replication either by modulating the relative levels of the precore and core RNAs or by creating a hepatocyte nuclear factor 1 transcription factor binding site (20). In cellular studies, the $1762^{T}/1764^{A}$ double mutation increased the replication of the viral genome by two times; in the case of some of the rarer triple mutations, an 8-fold increase in genome replication was found (21,22). The X antigen of HBV has potential transactivation activity for both viral and host genes, and it has been shown to interact directly with p53 and the DNA repair enzyme XAP-1 (23). Mutations in the basal core promoter region, which overlaps the coding sequence for the X antigen of HBV, may result in amino acid changes in the X antigen. The HBV $1762^{T}/1764^{A}$ 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 (24). These amino acid changes may interfere with cell growth control and DNA repair and may cause HCC (23,25).

A strength of this study is that the HBV $1762^{T}/1764^{A}$ double mutations were determined in serum collected before the development of HCC from participants who were not treated with any anti-HBV drugs, thus ruling out the possibility that the HBV double mutations were caused by the malignant transformation of hepatocytes or clinical treatment for HCC or hepatitis B. The present study has some limitations. Our analysis of HBV mutants was based on a single serum sample obtained at study entry; thus, the changes in HBV mutation status over time could not be assessed. In the present study, we used, for the first time, real-time PCR assay for $1762^{T}/1764^{A}$. The assay did not allow for the quantification of total HBV DNA levels. An earlier report based on a cohort study in Taiwan showed a statistically significant association between HBV DNA level and HCC risk (26). However, this positive association became statistically non-significant after adjustment for the HBV $1762^{T}/1764^{A}$ double mutants and other potential confounding factors while the association between the HBV DNA double mutations and HCC risk remained statistically significant after adjustment for HBV DNA load (6). These data indicated that the role of HBV DNA double mutations in the development of HCC is independent of and more important than total HBV DNA level.

In summary, the present study demonstrated that HBV $1762^{T}/1764^{A}$ mutation is a co-determinant of HCC risk among HBV carriers. This genetic feature of HBV, in addition to other environmental and host factors, may lead to better identification of HBV carriers at very high risk of HCC, and targeting of aggressive anti-viral therapy in this subgroup of infected individuals.

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Table 1

Distribution of risk factors for hepatocellular carcinoma in cancer patients and control subjects among subjects who were tested positive for hepatitis B surface antigen in serum, Shanghai Cohort Study

	No. of cancer patients(%)	No. of control subjects (%)	2-sided *P* *
Age (years)	57.98±4.23†	56.66±4.94†	0.11
Body mass index (kg/m^2)	21.11±3.04†	21.80±2.87†	0.18
Year of blood draw			
1986	19 (38.8)	33 (34.0)	0.52
1987	4 (8.2)	16 (16.5)	
1988	14 (28.5)	29 (30.0)	
1989	12 (24.5)	19 (19.5)	
Neighborhood of residence			
No. 1	19 (38.8)	31 (32.0)	0.72
No. 2	7 (14.3)	11 (11.3)	
No. 3	16 (32.6)	40 (41.2)	
No. 4	7 (14.3)	15 (15.5)	
Smoking status			
Never smokers	14 (28.6)	44 (45.4)	0.05
Ever smokers	35 (71.4)	53 (54.6)	
Alcohol drinking			
Non-drinkers	31 (63.3)	55 (56.7)	0.03
<4 drinks/day	12 (24.5)	39 (40.2)	
4+ drinkers/day	6 (12.2)	3 (3.1)	
Serum retinol concentration in tertile (μl/dL)			
1st (0−34.65)	31 (63.3)	32 (33.3)	0.0003
2nd (34.66−46.59)	15 (30.6)	33 (34.4)	
3rd (≥46.60)	3 (6.1)	31 (32.3)	

**P*-values were derived from *t*-test (for means) or X^2 (for frequencies) statistics.

†mean ± standard deviation.

Table 2

Hepatitis B virus (HBV) DNA double mutation ($1762^{T}/1764^{A}$) in relation to risk of developing hepatocellular carcinoma among subjects who were tested positive for hepatitis B surface antigen in serum, Shanghai Cohort Study

HBV $1762^{T}/1764^{A}$ mutation in serum	No. of cancer patients	No. of control subjects	Age-adjusted OR (95% CI)	Multivariate-adjusted OR (95% CI)*
Mutant status				
Negative	27	76	1.00	1.00
Positive	22	21	3.10 (1.45−6.59)	2.47 (1.04−5.85)
No. of copies/μL†				
Negative	27	76	1.00	1.00
<10	3	9	0.89 (0.22−3.62)	0.85 (0.19−3.86)
10 to <500	6	9	2.11 (0.67−6.61)	1.73 (0.50−5.96)
≥500	13	3	13.28 (3.42−51.55)	14.57 (2.41−87.98)
P for trend			0.0001	0.004

*Adjusted for age at recruitment, years between blood draw and measurement of HBV DNA double mutation, neighborhood of residence at recruitment, cigarette smoking (never, ever), heavy alcohol consumption (nondrinkers or <4 drinkers per day, 4 or more drinkers per day), and serum concentration of retinol in tertile; OR, odds ratio; CI, confidence interval.

†Number of copies of HBV DNA harboring the $1762^{T}/1764^{A}$ double mutations.