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ORIGINAL ARTICLE

Retrospective Cohort Study

Hepatitis B virus persistent infection-related single nucleotide polymorphisms in HLA regions are associated with viral load in hepatoma families

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Abstract

BACKGROUND

Genome-wide association studies from Asia indicate that HLA-DP and HLA-DQ loci are important in persistent hepatitis B virus (HBV) infections. One of the key elements for HBV-related carcinogenesis is persistent viral replication and inflammation.

AIM

To examine genetic and nongenetic factors with persistent HBV infection and viral load in families with hepatocellular carcinoma (HCC).

METHODS

The HCC families included 301 hepatitis B surface antigen (HBsAg) carriers and 424 noncarriers born before the nationwide vaccination program was initiated in 1984. Five HBV-related single nucleotide polymorphisms (SNPs) — rs477515, rs9272105, rs9276370, rs7756516, and rs9277535 — were genotyped. Factors associated with persistent HBV infection and viral load were analyzed by a generalized estimating equation.

RESULTS

In the first-stage persistent HBV study, all SNPs except rs9272105 were associated with persistent infection. A significantly higher area under the reciprocal operating characteristic curve for nongenetic factors *vs* genetic factors ($P < 0.001$)

suggests that the former play a major role in persistent HBV infection. In the second-stage viral load study, we added 8 HBsAg carriers born after 1984. The 309 HBsAg carriers were divided into low (n = 162) and high viral load (n = 147) groups with an HBV DNA cutoff of 10^5 cps/mL. Sex, relationship to the index case, rs477515, rs9272105, and rs7756516 were associated with viral load. Based on the receiver operating characteristic curve analysis, genetic and nongenetic factors affected viral load equally in the HCC family cohort (P = 0.3117).

CONCLUSION

In these east Asian adults, the mechanism of persistent HBV infection-related SNPs was a prolonged viral replication phase.

Key Words: Generalized estimating equation; Genetic polymorphism; Genome-wide association study; Hepatitis B surface antigen; Hepatitis B virus; Replication

Core Tip: Hepatitis B virus (HBV)-related single nucleotide polymorphisms (SNPs) have been identified in East Asians. We evaluated five SNPs and nongenetic factors associated with HBV infection in a hepatocellular carcinoma family cohort. The factors were correlated with hepatitis B surface antigen (HBsAg) in the first-stage and with HBV viral load in the second-stage. The SNPs, sex, generation, and index case HBsAg contributed to persistent HBV infection. Neonatal tolerance and SNPs in the HLA loci were both independently associated with persistent HBV infection. A prolonged HBV replication phase in parents could be the main mechanism of persistent HBV infection in children in East Asia.

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INTRODUCTION

Chronic hepatitis B is a global disease, with the highest prevalence in Africa and Asia [1,2]. Hepatitis B virus (HBV) is highly infectious[3,4], and those who are infected early in life are likely to develop a persistent infection[5-7]. Intra-familial spread of infection is common, resulting in the clustering of chronic hepatitis B surface antigen (HBsAg) carriers and hepatocellular carcinoma (HCC) in families[8-10]. Recent genome-wide association studies (GWASs) in Japan, Korea, Saudi Arabia, China, and Taiwan have consistently shown that single nucleotide polymorphisms (SNPs) at the HLA-DP and HLA-DQ loci play important roles in persistent HBV infection[11-19]. However, risk alleles of HBV-related SNPs are not present in the majority of Africans[20,21], so the high prevalence of HBsAg carriers in Africa cannot be completely explained by the SNPs.

It is well known that clearance of the hepatitis B e antigen (HBeAg) occurs earlier in African than in Asian HBsAg carriers[22-25]. In east Asia, the annual HBeAg seroconversion rate is < 2% in children younger than 3 years of age and around 5% in children older than 3 years of age[22,23]. On the contrary, an HBeAg annual clearance rate of 14%-16% has been found in Euro-Mediterranean and African children[24,25]. HBeAg clearance is associated with a decreased viral load and results in a decrease of perinatal infections and the development of chronic persistent HBV infection[7,23]. We propose that persistent HBV infection-related SNPs may be one of the reasons for the prolonged HBV replication phase in east Asians. To evaluate this hypothesis, we analyzed the HBV-related SNP and demographic data obtained from HCC families. HCC families are known to have higher perinatal transmission and a longer HBV replication phase than the general population[9,10]. We expect that the genetic and nongenetic factors characteristic of HCC families may help us to understand the

and data comparisons were carried out in compliance with relevant laws and guidelines, and in accord with the ethical standards of the Declaration of Helsinki.

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nature of persistent HBV infection.

MATERIALS AND METHODS

Ethics statement

Our study was approved by the institutional review board of Chang Gung Memorial Hospital, Taiwan (IRB 104-2596). Written informed consent was obtained from all participants. All experiments and data comparisons were carried out in compliance with relevant laws and guidelines, and complied with the ethical standards of the Declaration of Helsinki.

Study participants

Patients with HCC who were diagnosed at Chang Gung Memorial Hospital, Lin-Kou Medical Center were included as index cases. From 2003 to 2007, relatives of the patients were prospectively invited to complete a liver disease survey. The details of the survey can be seen in our previous report[10]. Briefly, after confirmation of their relation to the index HCC patient, the relatives received a structured questionnaire and underwent assessments of their liver biochemistry, alpha-fetoprotein, viral markers, and HBV genotyping. Peripheral blood samples were collected for host genome analysis.

Study size

We calculated sample sizes and statistical power to detect genetic effects in the study. The calculation considered the impact the minor allele frequency (MAF, from 0.1 to 0.4), odds ratio (OR, from 1.05 to 3), statistical power (from 0.5 to 0.9) and measurement error (type I error = 0.05) have on sample size. Power calculations were performed with QUANTO power calculator, version 1.2.4 (https://preventivemedicine.usc.edu/download-quanto/).

SNP selection and genotyping

Four genetic variants (rs477515, rs9276370, rs7756516, rs9277535) associated with persistent HBV infection that were previously identified[17] were included in the analysis. One additional HCC-related SNP (rs9272105) previously identified in China was also included[26]. Genomic DNA was extracted from peripheral blood cells using MagNA Pure LC DNA isolation kits with automated DNA isolation instruments (MagNA Pure LC II; Roche Diagnostics, Mannheim, Germany). Triple-SNP (rs477515, rs9272105, rs9277535) genotyping was performed with *Taq*Man Genotyping assays (Applied Biosystems, Foster City, CA, United States). Two SNPs (rs7756516, rs9276370) were genotyped with a Sequenom MassARRAY System (Sequenom, San Diego, CA, United States). The *Taq*Man assays were carried out by Vita Genomics (New Taipei City, Taiwan), and the Sequenom MassARRAY assays were performed by the Academia Sinica National Genotyping Center (Taipei, Taiwan). The overall genotype call rate was > 95%.

Statistical analysis

The statistical analyses were performed with SAS version 8.2 for UNIX (SAS Institute, Cary, NC, United States), PLINK (http://zzz.bwh.harvard.edu/plink/) (http://zzz.bwh.harvard.edu/plink/summary.shtml), R 2.15.1 (http://www.r-project.org/), and the Family-Based Association Test software (http://www.biostat.harvard.edu/~fbat/fbat.htm)[27]. A two-tailed P value < 0.05 was considered statistically significant. All associations were controlled for confounding factors. SNP data was quality controlled using the following criteria: (1) Call rate > 0.95; (2) MAF > 0.01; and (3) Deviation from Hardy-Weinberg equilibrium P > 0.001.

Individual locus analysis: We assessed the association of SNPs with persistent HBV infection or viral load in an additive genetic model using univariate and multivariate logistic regression of the data from unrelated male participants. In the family analysis, relatives included individuals living in the same household. First- and second-stage analyses were conducted with a generalized estimating equation (GEE) that included data correlated with a binary response (*e.g.*, to HBsAg status and HBV DNA level) using an exchangeable working correlation structure[28,29]. Univariate and multivariate analysis of the first- and second-stage results were assessed using the GEE method combined with the PROC GENMOD procedure in SAS 9.3 (SAS Institute). ORs were reported with 95% confidence intervals (CIs).

Weighted genetic risk score calculation: The weighted genetic risk score (WGRS) was calculated for the SNPs that were significantly associated with persistent infection or viral load. We assumed that each SNP was independently associated with risk according to an additive genetic model. The WGRS was calculated by multiplying the number of risk alleles at each polymorphic locus (0, 1, or 2) by each person for the corresponding relative logarithm of the OR (w_i) from the multivariate individual locus analysis and rescaling it with the factor $m/\sum_i w_i$, as follows: $WGRS = (m/\sum_i w_i) \cdot \sum w_i n_i$, where m is the number of statistically significant SNPs and n_i is the number of risk alleles for SNPi[30]. We divided the continuous WGRS into quartiles (Q1-4) and compared the risks among them.

Evaluation of genetic and nongenetic factors: We analyzed factors associated with persistent HBV infection or viral load using the logistic regression model unrelated participants and the GEE method for family data. Three prediction models were used: (1) The genetic model included only SNPs and WGRS; (2) The nongenetic model included only demographic data; and (3) The mixed model included both genetic and nongenetic variables. The contribution of the WGRS was evaluated using the area under the receiver operating characteristic curve (AUC), net reclassification improvement (NRI) method[31], and integrated discrimination improvement (IDI)[32] with the prediction model with and without the WGRS. To assess the demographic impact of including the WGRS in the model, an AUC of 0.5 indicated no discrimination and an AUC of 1 indicated perfect discrimination. The NRI indicated the proportion of subjects reclassified correctly (NRI > 0) or incorrectly (NRI < 0) into the various risk categories. An IDI > 0 indicated a statistically significant prediction of improvement as a result of adding variables to the model.

RESULTS

The HCC family cohort included 835 participants (Figure 1), of whom 301 HBsAg-positive and 424 of HBsAg-negative family members were selected for the first-stage HBV infection-persistence analysis. We excluded those born after the nationwide vaccination program was initiated in 1984. In the second-stage viral load study, we added 8 HBsAg carriers born after 1984 (Figure 1). A cohort of 309 HBsAg carriers was divided into high (n = 147) and low (n = 162) viral load groups using an HBV DNA cutoff of 10^5 cps/mL.

First stage: Factors associated with persistent HBV infection

Risk factors associated with being an HBsAg carrier were identified in the first-stage analysis. Demographic factors, which included age, sex, index case sex, relation to the index case, index HBsAg, and maternal HBsAg, are shown in Table 1. Age (OR = 1.018, P = 0.0013), sex (OR = 1.641, P = 0.0001), relation to the index case (OR = 3.203, P < 0.0001; index generation compared with children and grandchildren), index HBsAg (OR = 4.913, P < 0.0001), maternal HBsAg (OR = 3.31, P < 0.0001), and serum glutamic pyruvic transaminase (SGPT) (OR = 1.017, P < 0.0001) were significantly associated with persistent HBV infection. The associations remained significant after controlling for sex and age.

The SNPs rs477515 (OR = 1.377, P = 0.0274), rs9276370 (OR = 1.790, P = 0.0012), rs7756516 (OR = 1.654, P = 0.0048), and rs9277535 (OR = 1.519, P = 0.0004) were significantly associated with chronic HBV infection (Table 1). The ORs remained statistically significant after controlling for sex and age. HCC families carrying more risk alleles had an increased OR (Table 2, upper panel). Compared with participants with a WGRS in Q1, those with scores in Q2 and Q3–4 had higher risks of HBsAg positivity (Q2 OR = 1.878, P = 0.0014; Q3-4 OR = 2.538, P < 0.0001).

Results of the multivariate GEE analysis of the risk factors associated with persistent HBV infection are shown in Table 3. In the nongenetic model, sex, index generation, and index and maternal index HBsAg were associated with persistent HBV infection. In the genetic model, rs9277535 and WGRS were associated with persistent HBV infection. In the mixed model, all the risk factors were significant (male sex P = 0.0205; index generation P = 0.0001; index HBsAg P < 0.0001; maternal HBsAg P = 0.0072; rs9277535 P = 0.0029; WGRS P = 0.0012; Table 3).

The AUC for persistent HBV infection (Table 3) was 0.786 (P < 0.0001) in the nongenetic model and 0.620 (P < 0.0001) in the genetic model. Although the SNPs were identified by GWAS in unrelated subjects, the AUC data suggest that nongenetic factors were more important than genetic factors for the development of persistent

Table 1 Factors associated with persistent hepatitis B virus infection in the hepatocellular carcinoma family cohort

	HBsAg					
Category	**Positive**	**Negative**	**OR (95%CI)**	**Adjusted OR (95%CI)[1]**	***P* value**	**Adjusted *P* value[2]**
Total family members, *n*	301	424				
Age in yr, mean ± SD	44.23 ± 13.84	41.25 ± 14.97	1.018 (1.007-1.03)	1.017 (1.006-1.028)	0.0013	0.0030
Sex, *n* (%)						
Male	182 (60.47)	203 (47.88)	1.641 (1.279-2.107)	1.57 (1.225-2.011)	0.0001	0.0004
Female	119 (39.53)	221 (52.12)	1	1		
Index sex, *n* (%)						
Male	226 (75.08)	309 (72.88)	1.207 (0.726-2.006)	1.147 (0.681-1.93)	0.4685	0.6061
Female	75 (24.92)	115 (27.12)	1	1		
Relation to index, *n* (%)						
Children and grandchildren	146 (48.50)	319 (75.24)	1	1		
Parent generation	7 (2.33)	15 (3.54)	0.7 (0.302-1.622)	1.472 (0.533-4.065)	0.4059	0.4559
Index generation	148 (49.17)	90 (21.23)	3.203 (2.282-4.498)	4.861 (2.923-8.083)	< 0.0001	< 0.0001
Index status, *n* (%)						
HBsAg-	70 (23.26)	257 (60.61)	1	1		
HBsAg+	231 (76.74)	167 (39.39)	4.913 (3.209-7.522)	5.928 (3.747-9.377)	< 0.0001	< 0.0001
Mother's status, *n* (%)						
HBsAg-	85 (28.24)	239 (56.37)	1	1		
HBsAg+	91 (30.23)	45 (10.61)	3.31 (1.894-5.783)	3.296 (1.891-5.746)	< 0.0001	< 0.0001
Unknown	125 (41.53)	140 (33.02)	2.305 (1.568-3.39)	1.87 (1.202-2.91)	<.0001	0.0055
SGPT, mean ± SD	49.98 ± 65.39	25.83 ± 24.92	1.017 (1.011-1.022)	1.015 (1.009-1.020)	< 0.0001	< 0.0001
rs477515 (MAF = 0.1552) Chr6: 32601914[1]						
TT (reference)	5 (1.66)	20 (4.72)				
TC	59 (19.60)	116 (27.36)				
CC	237 (78.74)	288 (67.92)	1.377 (1.036-1.831)	1.38 (1.034-1.842)	0.0274	0.0285
rs9272105 (MAF = 0.4282) Chr6: 32632222[1]						
GG (reference)	54 (17.94)	84 (19.86)				
GA	137 (45.51)	207 (48.94)				
AA	110 (36.54)	132 (31.21)	1.054 (0.859-1.293)	1.031 (0.844-1.261)	0.6126	0.7639
rs9276370 (MAF = 0.1159) Chr6: 32739518[1]						
GG (reference)	3 (1.00)	13 (3.07)				
GT	39 (12.96)	97 (22.88)				
TT	259 (86.05)	314 (74.06)	1.790 (1.258-2.547)	1.759 (1.228-2.519)	0.0012	0.0021
rs7756516 (MAF = 0.1166) Chr6: 32756140[1]						
CC (reference)	3 (1.00)	13 (3.07)				
CT	42 (13.95)	95 (22.41)				
TT	256 (85.05)	316 (74.53)	1.654 (1.166-2.346)	1.612 (1.123-2.313)	0.0048	0.0096
rs9277535 (MAF = 0.3234) Chr6: 33087084[1]						
AA (reference)	21 (6.98)	61 (14.39)				
AG	114 (37.87)	191 (45.05)				
GG	166 (55.15)	172 (40.57)	1.519 (1.204-1.916)	1.493 (1.182-1.886)	0.0004	0.0008

[1]Genome build GRCH38.
[2]Adjusted for sex and age. HBsAg: Hepatitis B surface antigen; MAF: Minor allele frequency; SGPT: Serum glutamic pyruvic transaminase; CI: Confidence interval; OR: Odds ratio.

Table 2 Cumulative effect of the genetic-risk alleles associated with hepatitis B viral load or persistent hepatitis B virus infection

Study	WGRS quartile	OR (95%CI)	*P* value
Family first stage: Persistent HBV infection	Q1 (WGRS ≤ 6.166)	1	
	Q2 (WGRS = 6.166-7.083)	1.878 (1.277-2.762)	0.0014
	Q3,4 (WGRS > 7.083)[1]	2.538 (1.742-3.698)	< 0.0001
	Cochran-Armitage trend test		< 0.0001
Family second stage: Viral load	Q1 (WGRS ≤ 4.583)	1	
	Q2 (WGRS = 4.583-5.291)	2.204 (1.253-3.878)	0.0061
	Q3,4 (WGRS > 5.291)[2]	3.156 (1.780-5.595)	< 0.0001
	Cochran-Armitage trend test		< 0.0001

[1]The number of hepatitis B surface antigen negative individuals in Q4 was < 5, so Q3 and Q4 were combined.
[2]The number of individuals with hepatitis B virus DNA < 10^5 cps/mL in Q4 was < 1, so Q3 and Q4 were combined. The cumulative effect was calculated from: Four single nucleotide polymorphisms (SNPs) (rs9272105, rs9276370, rs7756516, and s9277535) in unrelated male hepatitis B surface antigen (HBsAg) carriers; four SNPs (rs477515, rs9276370, rs7756516, and rs9277535) in the first-stage hepatocellular carcinoma (HCC) family cohort analysis; and three SNPs (rs477515, rs9272105, and rs7756516) in HBsAg-positive carriers in the second-stage HCC family cohort analysis. CI: Confidence interval; OR: Odds ratio; Q: Quartile; WGRS: Weighted genetic risk score; HBV: Hepatitis B virus.

HBV infection (P < 0.0001; Figure 2). The combination of genetic and nongenetic factors resulted in an AUC of 0.795 (P < 0.0001; Figure 2 and Table 3). The IDI was 0.017 (95%CI: 0.009-0.026, P < 0.0001) and the NRI was 0.330 (95%CI: 0.192-0.467, P < 0.0001). The IDI and NRI values indicated statistically significant predicted improvement in the mixed, relative to the nongenetic model (Table 3).

Second stage: Factors associated with HBV viral load in HBsAg-positive HCC families

Factors associated with the HBV viral load were evaluated in HBsAg-positive families (Table 4). In that group, male sex (OR = 1.922, P = 0.0078), relation to the index case (OR = 2.033, P = 0.0029), index HBsAg (OR = 2.508, P = 0.0036), and SGPT (OR = 1.010, P = 0.0105) were significantly associated with the HBV viral load. The associations remained statistically significant after controlling for sex. HBV genotypes were also evaluated in HCC families, and of the participants with known HBV genotypes, the prevalence of genotype C was higher in those with high viral loads (41/143, 28.7%) than in those with low viral loads (15/90, 16.7%, P = 0.0431). The difference was marginally significant in multivariate analysis (P = 0.0515; Table 4).

Of the five SNPs included in the analysis, rs477515 (OR = 3.107, P = 0.0002), rs9272105 (OR = 1.747, P = 0.0009), and rs7756516 (OR = 1.951, P = 0.0272) were significantly associated with HBV viral load. The associations remained significant after controlling for sex (Table 4). Participants carrying more risk alleles had higher ORs for HBV viral load (Table 2, lower panel) and compared with patients having a WGRS in Q1, those in Q2 (OR = 2.204, P = 0.0061) and Q3-4 (OR = 3.156, P < 0.0001) had higher odds of having an HBV viral load.

The results of multivariate GEE analysis of factors associated with the HBV viral load in the genetic, nongenetic, and mixed models are shown in Table 5. In the nongenetic model, the risk of HBV viral load was higher in males (OR = 1.955, P = 0.0162) and in those with index HBsAg positivity (OR = 2.219, P = 0.0187). In the genetic model, the risk allele rs477515 (OR = 2.246, P = 0.0159) and the WGRS (OR = 1.644, P < 0.0001) were significantly different between the groups with high and low viral loads. In the mixed model, sex, rs477515, and WGRS were significantly different in the groups with high and low viral loads (Table 5).

The AUC of the HBV viral load was 0.674 (P < 0.0001) for the nongenetic model, 0.632 (P < 0.0001) for the genetic model, and 0.704 (P < 0.0001) for the mixed model (Figure 3 and Table 5). The results suggest that both genetic and nongenetic factors

Table 3 Multivariate generalized estimating equation and area under the curve for hepatitis B surface antigen status in the hepatocellular carcinoma family cohort

	Nongenetic model		Mixed model	
Variable	**OR (95%CI)**	***P* value**	**OR (95%CI)**	***P* value**
Sex, male	1.458 (1.048-2.027)	0.0250	1.514 (1.075-2.133)[1]/1.487 (1.063-2.081)[2]	0.0177[1]/0.0205[2]
Index sex, male	0.915 (0.532-1.573)	0.7475	0.853 (0.496-1.466)[1]/0.838 (0.488-1.442)[2]	0.5648[1]/0.5238[2]
Age in yr	1.001 (0.984-1.018)	0.9392	1.000 (0.982-1.017)[1]/0.998 (0.981-1.016)[2]	0.9608[1]/0.8582[2]
Relation to index				
Parent generation	0.523 (0.176-1.555)	0.2434	0.560 (0.182-1.719)[1]/0.603 (0.198-1.833)[2]	0.3108[1]/0.3726[2]
Index generation	3.385 (1.836-6.239)	< 0.0001	3.344 (1.766-6.331)[1]/3.493 (1.860-6.559)[2]	0.0002[1]/0.0001[2]
Index's HBsAg+	5.077 (3.103-8.308)	< 0.0001	4.919 (2.980-8.119)[1]/4.756 (2.912-7.766)[2]	< 0.0001[1]/< 0.0001[2]
Mother's status				
HBsAg+	2.597 (1.332-5.064)	0.0051	2.459 (1.270-4.760)[1]/2.517 (1.284-4.933)[2]	0.0076[1]/0.0072[2]
Unknown	1.395 (0.788-2.469)	0.2538	1.412 (0.790-2.522)[1]/1.413 (0.795-2.511)[2]	0.2444[1]/0.2387[2]
AUC (95%CI)	0.786 (0.752-0.820)	< 0.0001		
	Genetic model			
rs477515	1.303 (0.969-1.753)	0.0802	1.121 (0.770-1.631)	0.5507
rs9276370	2.741 (0.766-9.812)	0.1211	3.040 (0.623-14.839)	0.1693
rs7756516	0.592 (0.171-2.042)	0.4064	0.516 (0.104-2.554)	0.4177
rs9277535	1.575 (1.244-1.995)	0.0002	1.535 (1.157-2.035)	0.0029
AUC (95%CI)	0.632 (0.593-0.671)	< 0.0001	0.798 (0.765-0.831)	< 0.0001
WGRS	1.322 (1.162-1.505)	< 0.0001	1.269 (1.099-1.465)	0.0012
AUC (95%CI)	0.620 (0.580-0.660)	< 0.0001	0.795 (0.762-0.829)	< 0.0001
IDI (95%CI)			0.017 (0.009-0.026)	< 0.0001
NRI (95%CI)			0.330 (0.192-0.467)	< 0.0001

[1]Each single nucleotide polymorphism was included in the mixed model.
[2]The weighted genetic risk score was added in the mixed model. AUC: Area under the receiver operating characteristic curve; CI: Confidence interval; OR: Odds ratio; GEE: Generalized estimating equation; IDI: Integrated discrimination improvement; NRI: Net reclassification improvement; WGRS: Weighted genetic risk score.

had an effect on HBV viral load. Both the IDI (0.042, 95%CI: 0.019-0.065, $P = 0.0003$) and the NRI (0.440, 95%CI: 0.236–0.644, $P < 0.0001$) indicated that the mixed model represented a significant improvement (Table 5).

DISCUSSION

In this HCC family cohort, we found that both genetic and nongenetic factors were significantly associated with persistent HBV infection. In addition, HBV-related SNPs in the HLA-DP and HLA-DQ regions were associated with HBV viral load. GWASs conducted in diverse Asian populations have revealed that the HLA-DP and -DP loci play roles in persistent HBV infection[10-19]. We evaluated persistent HBV infection in the first-stage HCC family study. Expression of four of the five HBV-related SNPs differed significantly between the HBsAg carriers and the noncarriers. When only the risk alleles of the four SNPs were included in the univariate analysis, the OR for

Table 4 Factors associated with hepatitis B viral load in a hepatitis B surface antigen-positive hepatocellular carcinoma family cohort

	HBV DNA					
Category	**≥ 10^5 cps/mL**	**< 10^5 cps/mL**	**OR (95%CI)**	**Adjusted OR (95% CI)[2]**	***P* value**	**Adjusted *P* value[2]**
Total members	147	162				
Age in yr, mean ± SD	45.03 ± 14.18	41.82 ± 14.21	1.017 (1-1.035)	1.017 (0.999-1.035)	0.0538	0.0668
Sex, *n* (%)						
Male	100 (68.03)	88 (54.32)	1.922 (1.188-3.111)	1.914 (1.187-3.087)	0.0078	0.0078
Female	47 (31.97)	74 (45.68)	1	1		
Relation to index, *n* (%)						
Children and grandchildren generation	58 (39.46)	95 (58.64)	1	1		
Parent generation	5 (3.4)	2 (1.23)	3.683 (0.866-15.656)	5.056 (1.259-20.3)	0.0775	0.0223
Index generation	84 (57.14)	65 (40.12)	2.033 (1.274-3.246)	1.845 (1.144-2.977)	0.0029	0.0121
Index's status, *n* (%)						
HBsAg-	21 (14.29)	49 (30.25)	1	1		
HBsAg+	126 (85.71)	113 (69.75)	2.508 (1.351-4.657)	2.492 (1.324-4.692)	0.0036	0.0047
Mother's status, *n* (%)						
HBsAg-	43 (29.25)	43 (26.54)	1	1		
HBsAg+	46 (31.29)	51 (31.48)	0.874 (0.467-1.634)	0.91 (0.485-1.707)	0.6724	0.7693
Unknown	58 (39.46)	68 (41.98)	0.855 (0.491-1.49)	0.857 (0.488-1.503)	0.5804	0.5898
HBV genotype (BGT230), *n* (%)						
Unknown[3]	3 (2.05)	72 (44.44)	0.03 (0.009-0.104)	0.029 (0.008-0.1)	< 0.0001	< 0.0001
B	102 (69.86)	75 (46.3)	1	1		
C	41 (28.08)	15 (9.26)	2.042 (1.023-4.079)	2.066 (0.995-4.288)	0.0431	0.0515
SGPT, mean ± SD	63.92 ± 79.63	37.02 ± 45.22	1.010 (1.002-1.018)	1.009 (1.001-1.017)	0.0105	0.0260
rs477515 (MAF = 0.1149) Chr6: 32601914[1]						
TT (reference)	1 (0.68)	4 (2.47)				
TC	15 (10.2)	46 (28.4)				
CC	131 (89.12)	112 (69.14)	3.107 (1.708-5.653)	3.195 (1.746-5.847)	0.0002	0.0002
rs9272105 (MAF = 0.4078) Chr6: 32632222[1]						
GG (reference)	20 (13.61)	36 (22.22)				
GA	59 (40.14)	81 (50)				
AA	68 (46.26)	45 (27.78)	1.747 (1.256-2.428)	1.75 (1.247-2.456)	0.0009	0.0012
rs9276370 (MAF = 0.07605) Chr6: 32739518[1]						
GG (reference)	1 (0.68)	3 (1.85)				
GT	14 (9.52)	25 (15.43)				
TT	132 (89.8)	134 (82.72)	1.747 (0.933-3.272)	1.679 (0.901-3.131)	0.0811	0.1029
rs7756516 (MAF = 0.08091) Chr6: 32756140[1]						
CC (reference)	0 (0)	4 (2.47)				
CT	16 (10.88)	26 (16.05)				
TT	131 (89.12)	132 (81.48)	1.951 (1.078-3.53)	1.875 (1.029-3.417)	0.0272	0.0400
rs9277535 (MAF = 0.2589) Chr6: 33087084[1]						
AA (reference)	13 (8.84)	8 (4.94)				
AG	45 (30.61)	73 (45.06)				

GG	89 (60.54)	81 (50)	1.235 (0.849-1.797)	1.303 (0.888-1.911)	0.2703	0.1767

[1]Genome Build ARCH38.
[2]Adjusted by sex.
[3]Eight cases not tested. CI: Confidence interval; HBsAg: Hepatitis B surface antigen; OR: Odds ratio; HBV: Hepatitis B virus.

Table 5 Multivariate generalized estimating equation and area under the curve hepatitis B viral loads in a hepatocellular family cohort

	Nongenetic model		Mixed model	
Variable	**OR (95%CI)**	***P* value**	**OR (95%CI)**	***P* value**
SNP				
Sex, male	1.955 (1.132-3.376)	0.0162	1.918 (1.101-3.341)[1]/1.911 (1.097-3.328)[2]	0.0214[1]/0.0223[2]
Index sex, male	0.903 (0.481-1.699)	0.7527	0.914 (0.488-1.713)[1]/0.912 (0.482-1.725)[2]	0.7786[1]/0.7771[2]
Age in yr	1.012 (0.987-1.037)	0.3363	1.007 (0.983-1.032)[1]/1.008 (0.983-1.033)[2]	0.5658[1]/0.5426[2]
Relation to index				
Parent generation	4.182 (0.68-25.731)	0.1228	4.343 (0.81-23.285)[1]/4.091 (0.75-22.316)[2]	0.0866[1]/0.1036[2]
Index generation	1.7 (0.844-3.423)	0.1372	1.851 (0.912-3.756)[1]/1.797 (0.894-3.611)[2]	0.0881[1]/0.0999[2]
Index HBsAg+	2.219 (1.142-4.31)	0.0187	1.734 (0.853-3.526)[1]/1.816 (0.907-3.636)[2]	0.1283[1]/0.0918[2]
Mother's status				
HBsAg+	0.828 (0.407-1.684)	0.6021	0.766 (0.361-1.623)[1]/0.763 (0.360-1.619)[2]	0.4862[1]/0.4814[2]
Unknown	0.537 (0.275-1.045)	0.0673	0.549 (0.272-1.107)[1]/0.559 (0.278-1.125)[2]	0.094[1]/0.1030[2]
AUC (95%CI)	0.674 (0.614-0.734)	< 0.0001		
	Genetic model			
rs477515	2.246 (1.164-4.333)	0.0159	2.242 (1.113-4.515)	0.0238
rs9272105	1.386 (0.965-1.991)	0.0775	1.266 (0.866-1.849)	0.2232
rs7756516	1.385 (0.765-2.509)	0.2826	1.379 (0.753-2.524)	0.2977
AUC (95%CI)	0.638 (0.579-0.698)	< 0.0001	0.705 (0.648-0.763)	< 0.0001
WGRS	1.644 (1.317-2.052)	< 0.0001	1.567 (1.250-1.965)	< 0.0001
AUC (95%CI)	0.632 (0.573-0.692)	< 0.0001	0.704 (0.646-0.761)	< 0.0001
IDI (95%CI)			0.042 (0.019-0.065)	0.0003
NRI (95%CI)			0.440 (0.236-0.644)	< 0.0001

[1]Each single nucleotide polymorphism was added in the mixed model.
[2]The weighted genetic risk score was added in the mixed model. AUC: Area under the receiver operating characteristic curve; GEE: Generalized estimating equation; IDI: Integrated discrimination improvement; NRI: Net reclassification improvement; CI: Confidence interval; HBsAg: Hepatitis B surface antigen; OR: Odds ratio.

persistence was significant if the WGRS was > 7 (Table 2, upper panel). In the genetic model, multivariate GEE analysis found that expression of one SNP (rs9277535) and the WGRS were significantly different between HBsAg carriers and noncarriers, and the differences remained significant in the presence of nongenetic factors (Table 3). Regression analysis showed that HBV-related SNPs were associated with persistent HBV infection in these HCC families. This is the first study to confirm that SNPs identified by GWAS were associated with persistent HBV infection in a family cohort.

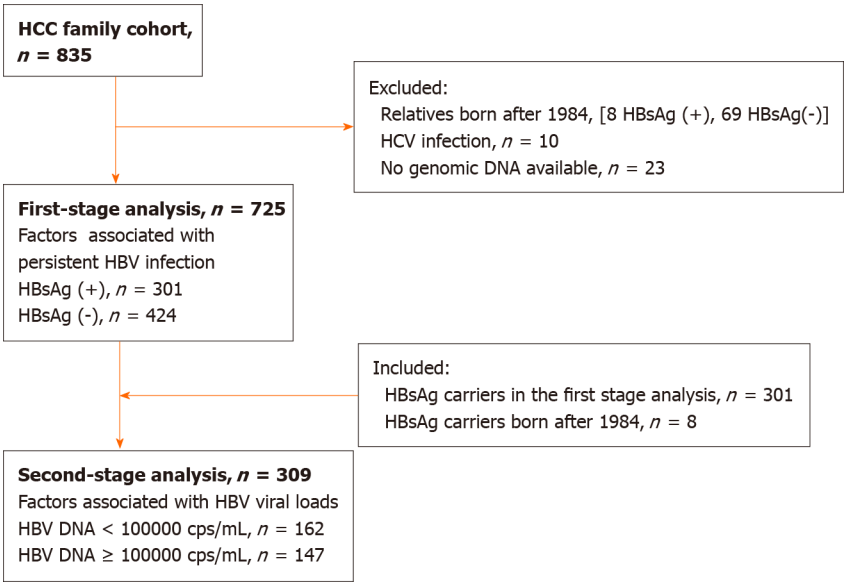

Figure 1 Study flow chart. Hepatitis B virus persistent infection and viral load were analyzed in a hepatocellular carcinoma family cohort. HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HBsAg: Hepatitis B surface antigen.

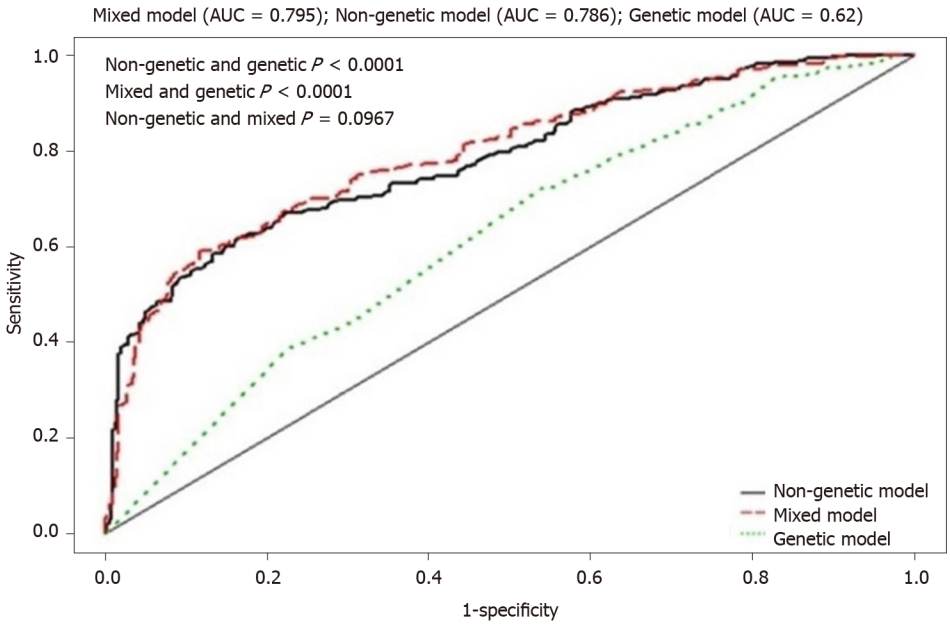

Figure 2 First-stage persistent hepatitis B virus infection. Genetic, nongenetic, and combined risk factors for persistent hepatitis B virus (HBV) infection were evaluated by area under the receiver operating characteristic curves derived from generalized estimating equation regression models. Significantly higher areas under the curve for nongenetic compared with genetic factors ($P < 0.001$) suggest that nongenetic factors played a major role in persistent HBV infection. AUC: Area under the receiver operating characteristic curve.

Nongenetic factors also affected persistent HBV infection. Age, sex, generation, index HBsAg status, and maternal HBsAg status all differed significantly between HBsAg carriers and noncarriers (Table 1). The AUC was 0.786 in the nongenetic model, 0.620 in the genetic model, and 0.795 in the mixed model (Table 3). The ROC analysis thus implied that nongenetic factors contributed more to persistent HBV infection than genetic factors did (genetic *vs* nongenetic factors $P < 0.0001$ and mixed *vs* nongenetic factors $P < 0.0001$; Figure 2). The results are consistent with exposure to HBV early in life and an important influence on the persistence of HBV infection[5-7]. Our overall findings indicate that the HCC family members may have been exposed to HBV early in life because of the high HBsAg prevalence in the index cases and/or their mothers. Accounting for both genetic and nongenetic cofactors, the prevalence of HBsAg was 41.5% (301/725) in this HCC family cohort.

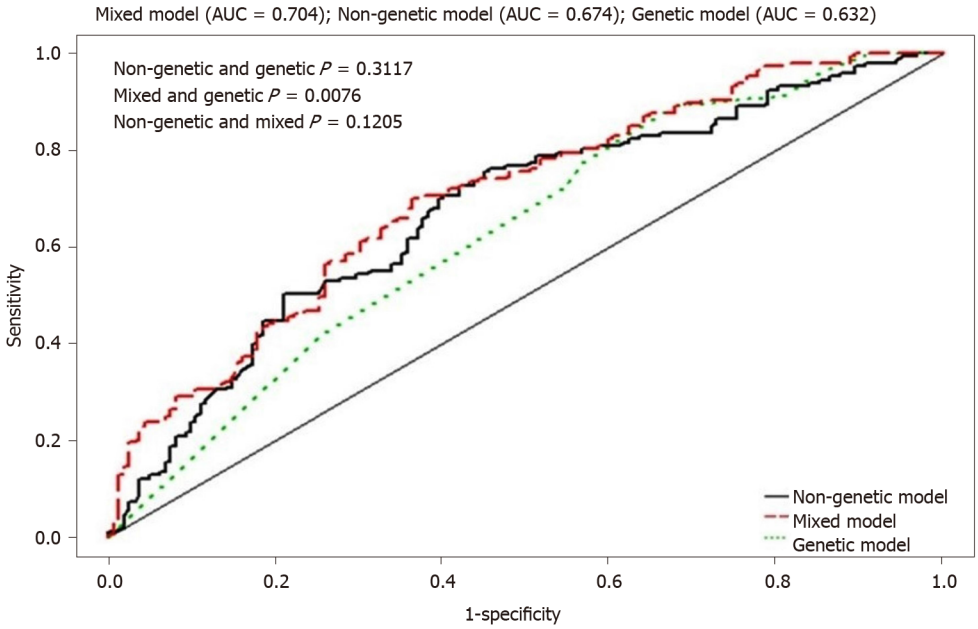

Figure 3 Second-stage hepatitis B virus viral load. The genetic, nongenetic, and combined risk factors for hepatitis B virus (HBV) viral load were evaluated by area under the receiver operating characteristic curves derived from generalized estimating equation regression models. The difference between the receiver operating characteristic curves of genetic and nongenetic factors was not significant (P = 0.3117). The finding suggests that both factors contributed to the HBV viral load. AUC: Area under the receiver operating characteristic curve.

In the presence of SNPs identified in a GWAS, nongenetic factors remain important in persistent HBV infection. The persistence of infection induced by the SNPs might depend on a delay in clearance of the HBeAg. It is known that in HBsAg carriers, HBeAg clearance occurs earlier in African than in Asian populations[22-25]. That means East Asians of reproductive age are likely to have higher HBV viral loads and a higher rate of perinatal HBV infection of their babies[7,10,22,23]. Perinatal infection usually persists as a chronic infection[7,23]. As African women usually clear HBeAg before reproductive age[24,25], the viral load during pregnancy is likely to be lower than that in East Asians, which would decrease the chance of perinatal HBV infection [24]. We suspect that a prolonged HBV replication phase in parents could be the mechanism of persistent HBV infection associated with SNPs.

Univariate analysis of the factors associated with HBV viral load in the HCC family cohort revealed that three of the five SNPs (rs477515, rs9272105, rs7756516) differed significantly between the high and low viral load groups (Table 4). The cumulative effect of the WGRS was also greater in the high viral load group (Table 3, lower panel). Multivariate GEE analysis found that the rs477515 SNP (OR = 2.242, P = 0.0238) and WGRS (OR = 1.567, P < 0.0001) were independently associated with a high viral load in the mixed model (Table 5). Our data thus support the prevailing view that the SNPs associated with persistent HBV infection promote persistent HBV replication. The mean ages of our study groups ranged from 41.25-45.03 years (Tables 1 and 4). Persistent high viral loads in these age groups were likely to have resulted in perinatal transmission of chronic HBV infection during the reproductive age.

Our previous study demonstrated that nongenetic factors influenced the HBV viral load in HCC families[10]. In this study, we observed that sex, generation, and index HBsAg cases were associated with a high viral load in the nongenetic model (Table 4). We also compared the relative contributions of genetic and nongenetic factors associated with viral load in the HCC family cohort. The AUCs of the viral load were 0.674 in the nongenetic model and 0.632 in the genetic model. The AUC in the mixed model was up to 0.704 (Table 5). Therefore, both genetic and nongenetic factors were associated with HBV viral load in the HCC family cohort. It should be noted that we included only SNPs in the HLA region. The association of other loci, such as polymorphisms of interferon gamma, complement factor B, CD40, and INST10, which have also been reported to be associated with HBV viral load, was not investigated[33-35].

One of the five SNPs we evaluated, rs9277535, was reported by Tao *et al*[36] to be associated with more aggressive liver disease, but it was reported by Li *et al*[37] not to be associated with disease progression. Our previous GWAS revealed that rs9276370 was associated with HBV therapeutic response[17]. Univariate analysis found that the

two SNPs were not significantly associated with viral load in this HCC family cohort. Two previous studies found that rs477515 was associated with HBV vaccine response [38,39], and that SNP was found to be associated with viral load in this cohort. Li *et al* [26] reported that rs9272105 was associated with HCC in a GWAS, and univariate analysis found that it was associated with viral load in this HCC family cohort. All these previous reports suggest that a single SNP provides a small contribution to HBV viral loads. Persistent HBV replication seems to be determined by multiple genetic and nongenetic risk factors.

This study provides information that may help to establish more accurate models of disease through the incorporation of genetic and nongenetic factors, but it was limited by the relatively small number of HCC families. Another limitation was that HBV genotype studies were not available in patients with low viral loads. HBV genotype C has been associated with a lower HBeAg clearance rate than genotype B[40]. We found a high adjusted OR (2.066, P = 0.0515) for the association of genotype C with a high viral load relative to a low viral load in this HCC family cohort (Table 4).

CONCLUSION

We conclude that SNPs associated with persistent HBV infection prolong the replication phase in the parent generation and increase the burden of persistent infection in the offspring generation.

ARTICLE HIGHLIGHTS

Research background

Genome-wide association studies (GWASs) in Asian populations indicate that the HLA-DP and HLA-DQ loci are involved in the persistence of hepatitis B virus (HBV) infections. Persistent viral replication and inflammation are key influencers in HBV-related carcinogenesis.

Research motivation

HBV-related single nucleotide polymorphisms (SNPs) have been identified in east Asian populations but are uncommon in African populations. Different mechanisms may drive persistent infection in those regions.

Research objectives

We examined genetic and nongenetic factors associated with persistent HBV infection and viral load in families with hepatocellular carcinoma (HCC).

Research methods

HCC families were enrolled. Five HBV-related SNPs (rs477515, rs9272105, rs9276370, rs7756516, and rs9277535) were genotyped. Factors associated with persistent HBV infection and viral load were identified with the use of generalized estimating equations.

Research results

In the first-stage persistent HBV study, all SNPs except rs9272105 were associated with persistent infection. A significantly higher contribution of nongenetic than genetic factors (P < 0.001) to persistent HBV infection was found. In the second-stage viral load study, sex, relationship with index case, rs477515, rs9272105, and rs7756516 were associated with viral load. Receiver operating characteristic curve, and genetic and nongenetic factors had equal effects on viral load in the HCC family cohort (P = 0.3117).

Research conclusions

GWAS identified SNPs that have roles in persistent HBV infection and HBV viral loads in an HCC family cohort. Nongenetic factors were more important than genetic factors in persistent HBV infection but had equal contributions to HBV viral load. HBV-related SNPs resulting in high viral loads in parents may drive persistent infection in East Asian populations. The mechanism of persistent HBV infection-related SNPs involves a prolonged viral replication phase in East Asian adults.

Research perspectives

Termination of the HBV replication phase before pregnancy will be a therapeutic goal in East Asian countries.

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