Hepatology International (2022) 16:789–798
https://doi.org/10.1007/s12072-022-10350-7

ORIGINAL ARTICLE

Genetic variants of NTCP gene and hepatitis B vaccine failure in Taiwanese children of hepatitis B e antigen positive mothers

Ya-Hui Chen[1] · Daw-Jen Tsuei[1] · Ming-Wei Lai[2] · Wan-Hsin Wen[3,4] · Cheng-Lun Chiang[1] · Jia-Feng Wu[1] · Huey-Ling Chen[1] · Hong-Yuan Hsu[1] · Yen-Hsuan Ni[1] · Mei-Hwei Chang[1,5]

Received: 22 February 2022 / Accepted: 25 April 2022 / Published online: 30 May 2022

Abstract

Background and aims Hepatitis B virus (HBV) vaccine failure remains a hurdle to the global elimination of HBV infections in the vaccination era. We aimed to elucidate the relationships between HBV entry receptor sodium taurocholate co-transporting polypeptide (NTCP) and vaccine failure in children born to highly infectious mothers.

Methods The genetic variants rs7154439, rs4646285, rs4646287, and rs2296651 were genotyped in 170 children with chronic HBV infections and 138 control children of mothers positive for hepatitis B e antigen (HBeAg). All children received hepatitis B immunoglobulin and complete HBV vaccination. Total RNAs from 82 adult non-tumor liver tissues were quantified for NTCP, type I interferons and interferon-induced transmembrane protein 3 (IFITM3) levels.

Results A higher rate of the GA/AA genotype (28.3% vs. 15.3%, $p=0.006$) of the genetic variant rs4646287 in intron 1 of the NTCP gene was detected in control children compared to the carrier children. The rs4646287 G > A genotype was associated with younger ages at which spontaneous HBeAg seroconversion occurred (10.8 ± 8.4 vs. 14.6 ± 8.7 years, $p=0.003$) in chronic HBV-infected children. Unique correlation patterns of NTCP and innate immunity-related genes (type I interferons and IFITM3) were found in HBV-infected liver tissues with the rs4646287 G > A genotype.

Conclusion The rs4646287 G > A genotype of the NTCP gene may be associated with lower risk for HBV vaccine failure in children born to highly infectious mothers. The protective effect of rs4646287 G > A was also present in carrier children, evidenced by earlier spontaneous HBeAg seroconversion.

Keywords Chronic infection · Genetic variant · Hepatitis B virus · HBeAg seroconversion · HBsAg · IFITM3 · NTCP · rs4646287 G > A · Type I interferons · Vaccine failure

Ya-Hui Chen and Daw-Jen Tsuei contributed equally to this work.

✉ Mei-Hwei Chang
changmh@ntu.edu.tw

[1] Department of Pediatrics, National Taiwan University Hospital and College of Medicine, National Taiwan University, Taipei, Taiwan

[2] Division of Pediatric Gastroenterology, Department of Pediatrics, Liver Research Center, Chang Gung Memorial Hospital, Linkou Branch, and Chang Gung University College of Medicine, Taoyuan, Taiwan

[3] Department of Pediatrics, Cardinal Tien Hospital, New Taipei City, Taiwan

[4] School of Medicine, College of Medicine, Fu-Jen Catholic University, New Taipei City, Taiwan

[5] Department of Pediatrics, College of Medicine and Children's Hospital, National Taiwan University, 17F, No. 8, Chung-Shan South Road, Taipei 100226, Taiwan

Abbreviations

ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AST	Aspartate aminotransferase
HBeAg	Hepatitis B e antigen
HBsAg	Hepatitis B surface antigen
HBIG	Hepatitis B immunoglobulin
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
IFITM3	Interferon-induced transmembrane protein 3
IFN	Interferon
LD	Linkage disequilibrium
MAF	Minor allele frequency
NBNC	Non-HBV/non-HCV infection
NTCP	Sodium taurocholate co-transporting polypeptide
RFLP	Restriction fragment length polymorphism

SLC10A1	Solute carrier family 10 member 1 gene
SNP	Single nucleotide polymorphism
WBC	White blood cell

Introduction

Chronic hepatitis B virus (HBV) infection affects approximately 250 million people worldwide [1]. The implementation of a universal hepatitis B immunization program in Taiwan in 1984 has successfully decreased the sero-prevalence of HBV surface antigen (HBsAg) in vaccinated birth cohorts from 13.7% to less than 1% [2, 3]. Nevertheless, approximately 10% of infants born to highly infectious mothers experience breakthrough HBV infections in spite of hepatitis B immunoglobulin (HBIG) within 24 h after birth and complete HBV vaccine in infancy [3]. Mother-to-infant transmission of HBV accounted for almost 40% of all new HBV infections before the HBV immunization program [4], and have accounted for 75–90% of all infections since then [5, 6]. Hepatitis e antigen (HBeAg) is a marker for active HBV replication and high infectivity [7]. HBeAg positivity [8] or high maternal viral load [9], vaccine-escape mutants [10], and intrauterine infections [11] are the main causes of HBV vaccine failure. Complete eradication of HBV infections requires identification of vaccine failure factors in infants born to highly infectious mothers.

The sodium taurocholate co-transporting polypeptide (NTCP), encoded by the solute carrier family 10 member 1 gene (*SLC10A1*), is the major hepatocellular sodium-dependent uptake system for conjugated bile acids in human livers [12]. It binds and transfers bile acids from the blood to liver parenchymal cells, and acts as a hepatocellular entry receptor for HBV by interacting with the N-terminus of the HBV large-envelope protein [13]. Its expression at 14–23 weeks of gestation tends to be low (2–4% of adults) [14, 15]. Nevertheless, about 12% of human fetus primary hepatocytes can be infected with HBV [16]. It remains unclear whether NTCP expression in fetal/neonatal livers determines the risk for mother-to-infant HBV transmission.

The association between single nucleotide polymorphisms (SNP) in the NTCP gene and HBV susceptibility and disease progression has been studied extensively. An association between the TT genotype of the SNP rs7154439 (c.-1956 C > T) at 5′ upstream non-coding region of the NTCP gene and HBV clearance has been reported in southwestern Chinese Han individuals [17], but not in central Chinese Han [18], Tibetans, or Uyghurs [19]. The SNP rs4646285 AA genotype (c.225 G > A, p.T75T) in exon 1 of the NTCP gene is more prevalent in individuals who spontaneously recovered from HBV infections [20]. High linkage disequilibrium (LD) between rs7154439 and rs4646285 has been observed in Chinese Han individuals [17]. It has also been reported that the A allele of the SNP rs4646287 (c.356 + 702 G > A) in intron 1 of the NTCP gene is common in East Asians (11.31% and 12.35% in Chinese and Japanese populations, respectively) but absent in Africans and Europeans [21]. A protective effect of the rs4646287 GA/AA genotype against HBV infections has been suggested in eastern Chinese Han [21]. Notably, a protective role of Asian-specific SNP rs2296651 (c.800 C > T, p.S267F) against chronic HBV infections, acute-on-chronic liver failure, and HCC has been suggested [22–24]. However, the role of NTCP and its genetic variants in HBV vaccine failure has not been addressed yet. In this study, we selected subjects born to HBsAg and HBeAg double-positive mothers, and investigated their genotypes for *SLC10A1*. We investigated the association between NTCP gene polymorphisms and HBV vaccine failure and the long-term consequences of chronic HBV infection.

Methods

Subjects

White blood cell (WBC) DNA samples were obtained from two cohorts to study NTCP genetic variants: 170 carrier children with chronic HBV infection and 138 non-carrier controls. Children in both cohorts were born to HBsAg and HBeAg double-positive mothers. Table 1 lists the characteristics of the studied subjects and the maternal HBV status. All of the children received HBIG at birth and three to four doses of HBV vaccine before reaching the age of 7 months. Chronic HBV-infected patients were followed up longitudinally every 6 months, or every 1–3 months in case of elevated serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). The study protocol was approved by the Institutional Review Board of National Taiwan University Hospital (NTUH IRB 201512129INB). All of the specimens were collected with parental and/or patient consent.

Specimens

For gene expression analyses, NTCP genetic variants were detected in adult WBC DNA samples from non-HCC patients with chronic HBV infection ($N = 100$) or non-HBV/non-hepatitis C virus (HCV) infection (NBNC) ($N = 100$). A total of 82 RNA samples, paired to the genotyped WBC DNA samples, were extracted from the non-tumor liver tissues of non-HCC patients (those with hemangiomas, focal nodular hyperplasia, angiomyolipomas, cirrhosis, or other liver diseases). WBC DNA and total RNA samples were provided by Taiwan Liver Cancer Network, National Health Research Institutes Biobank.

Table 1 Characteristics of the non-carrier control and HBV-infected carrier children of HBsAg and HBeAg double-positive mothers

Characteristics	Non-carrier controls ($N=138$)	HBV-infected carriers ($N=170$)
Male sex, N (%)	68 (49.3)	105 (61.8)
Calendar year of birth, years		
Median (IQR)	2013 (2011–2014)	2001 (1992–2009)
Age at enrollment, years		
Median (IQR)	0.51 (0.00–1.07)	2.83 (0.50–9.55)
Follow-up duration, years		
Median (IQR)	1.01 (0.44–1.05)	13.02 (7.22–17.52)
Maternal HBV status		
HBsAg	Positive	Positive
HBeAg	Positive	Positive
Childbearing age, years		
Median (IQR)	33.46 (31.12–35.94)	29.84 (26.55–33.11)

HBeAg hepatitis B e antigen, *HBsAg* hepatitis B surface antigen, *IQR* interquartile range

Selected SNPs and genotyping by restriction fragment length polymorphism (RFLP) and sequencing

Four SNPs of the NTCP gene, rs7154439 (in the 5′ upstream region), rs4646285 (in exon 1), rs4646287 (in intron 1), and rs2296651 (in exon 4), were selected for this study because a high minor allele frequency (MAF) among Asians and an association with HBV persistence have been reported. Genomic DNA was prepared from human peripheral blood cells using the Puregene Blood Kit (QIAGEN, GmbH, Hilden, Germany) and quantified on a NanoDrop 1000 spectrophotometer (NanoDrop, Wilmington, DE, USA).

Four selected SNPs were genotyped by PCR–RFLP. WBC DNA was amplified and digested with restriction enzymes *Bsm*AI (for rs7154439 C > T, 218 bp), *Eag*I (for rs4646285 G > A, 116 bp), *Cac*8I (for rs4646287 G > A, 118 bp), and *Hph*I (for rs2296651 C > T, 120 + 84 bp). Agarose gel electrophoresis was used to separate the wild type, heterozygous, and homozygous genetic variants (Supplementary Figure S1). The RFLP-identified variants were all confirmed by direct sequencing. Primers and restriction enzymes (New England Biolabs, Ipswich, MA, USA) are shown in Supplementary Table S1.

Quantitative RT-PCR

Total RNAs were prepared from non-tumor liver tissues of non-HCC patients. Complementary DNA was synthesized using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Waltham, MA, USA). Real-time PCR was performed using the SYBR Green PCR Master Mix and ABI Prism 7500 Fast System (Applied Biosystems). The primers used were human glyceraldehyde 3-phosphate dehydrogenase forward 5′-GAAGGTGAAGGTCGGAGT-3′ and reverse 5′-CATGGGTGGAATCATATTGGAA-3′; human NTCP forward 5′-TGACCACCTGCTCCACCTTC-3′ and reverse 5′-GAATGAGAACCAGGACCAGTGAT-3′; human pan-interferon α (IFNα) forward 5′-CACACAGGCTTCCAG GCATTC-3′ and reverse 5′-TCTTCAGCACAAAGGACT CATCTG-3′; human interferon β (IFNβ) forward 5′-TGCTCT CCTGTTGTGCTTCTCCAC-3′ and reverse 5′-ATAGATGGT CAATGCGGCGTCC-3′; and IFITM3 forward 5′-ATGAAT CACACTGTCCAAACCTTCT-3′ and reverse 5′-CTATCC ATGAGGCCTGGAAGATCAG-3′. The relative standard curve method was used for the calculation of fold changes in gene expression [25].

Statistical analyses

Pearson's chi-square (χ^2) test was used to analyze differences in the genotype frequencies and the p value was adjusted by Bonferroni correction. Pairwise linkage disequilibrium (LD) was estimated between four SNPs of *SLC10A1* based on D prime (D') and R square (r^2) values using Haploview software version 4.2. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated on the basis of conditional logistic regression analysis. The Haldane–Anscombe correction was applied to groups with zero individuals to calculate ORs. The Student's t test was used to analyze ALT and AST activities and NTCP transcription levels. Kaplan–Meier survival curves were used to analyze HBeAg seroconversion ages. A two-tailed $p < 0.05$ was used as the criterion for statistical significance. One-way analysis of variance (ANOVA) and the Pearson correlation test were used to analyze the quantitative RT-PCR results.

Results

Baseline characteristics

The control cohort included 138 children born to HBsAg and HBeAg double-positive mothers with high viral loads. The median maternal viral load was 8.01 $\log_{10}$ IU/mL (interquartile range: 7.14–8.38, $N = 125$). The vaccine failure cohort included 170 chronic HBV-infected patients, born to HBsAg and HBeAg double-positive mothers (viral loads were mostly not determined or unavailable). The carrier children were followed up longitudinally for a median of 13.02 years (interquartile range: 7.22–17.52) (Table 1). The mothers had no records of receiving antiviral treatments or of liver diseases, except chronic hepatitis, during the pregnancy.

NTCP genetic variants and HBV vaccine failure (Table 2)

The four selected SNPs of the NTCP gene were characterized by PCR–RFLP, and the identified variants were confirmed by direct sequencing. There was a significantly higher frequency of the rs4646287 GA/AA

Table 2 Comparison of the NTCP genetic variants in non-carrier control and HBV vaccine failure carrier children of HBsAg and HBeAg double-positive mothers

SNP	Position amino acid change	Genotype	Non-carrier controls ($N = 138$)	HBV-infected carriers ($N = 170$)	p value*
rs7154439	5′ upstream	c.-1956 C > T			
		CC	71.0% (98)	79.4% (135)	
		CT	27.5% (38)	20.6% (35)	
		TT	1.4% (2)	0	
		CT + TT	29.0% (40)	20.6% (35)	0.115
		C	84.8% (234)	89.7% (305)	
		T	15.2% (42)	10.3% (35)	0.086
rs4646285	Exon 1 synonymous	c.225 G > A			
		GG	71.7% (99)	79.4% (135)	
		GA	26.8% (37)	20.6% (35)	
		AA	1.4% (2)	0	
		GA + AA	28.3% (39)	20.6% (35)	0.152
		G	85.1% (235)	89.7% (305)	
		A	14.9% (41)	10.3% (35)	0.112
rs4646287	Intron 1	c.356 + 702 G > A			
		GG	71.7% (99)	84.7% (144)	
		GA	28.3% (39)	14.7% (25)	
		AA	0	0.6% (1)	
		GA + AA	28.3% (39)	15.3% (26)	**0.006**
		G	85.9% (237)	92.1% (313)	
		A	14.1% (39)	7.9% (27)	0.014
rs2296651	Exon 4	c.800 C > T			
	S267F	CC	92.0% (127)	91.2% (155)	
		CT	8.0% (11)	8.8% (15)	0.839
		C	96.0% (265)	95.6% (325)	
		T	4.0% (11)	4.4% (15)	0.843
Triple mutant variant					
rs7154439	5′ upstream	c.-1956 C > T			
rs4646285	Exon 1	c.225 G > A			
rs4646287	Intron 1	c.356 + 702 G > A			
		CC + GG + GG	93.5% (129)	100% (170)	
		CT + GA + GA	6.5% (9)	0% (0)	**< 0.001**

Bold type for p value less than 0.013 was considered significant after Bonferroni adjustment (0.05/4 SNPs)

*p values were calculated using the chi-square test for comparisons between non-carrier control and HBV carrier children

genotype in the non-carrier control children compared to chronic HBV carrier children (28.3% [39/138] vs. 15.3% [26/170], $p=0.006$). The MAF of rs4646287-A was 14.1% (39/276) in controls compared to 7.9% (27/340) in carriers ($p=0.014$). Genetic variant rs7154439 C > T coexisted with rs4646285 G > A, and had a trend of higher frequency in controls (15.2% [42/276] vs. 10.3% [35/340], $p=0.086$). Pairwise LD analysis showed that four SNPs were in weak LD ($r^2<0.1$), except the pair of rs7154439 C > T and rs4646285 G > A (Fig. 1). Significantly strong LD of rs7154439 C > T and rs4646285 G > A was found in carriers ($D'=0.968$, $r^2=0.937$) and controls ($D'=1$, $r^2=0.972$).

The frequency of the triple mutant variant carrying rs7154439 C > T, rs4646285 G > A, and rs4646287 G > A genotype was significantly higher in the control group (6.5% [9/138] vs. 0% [0/170], $p<0.001$). Conditional logistic regression analysis showed that higher frequencies and lower risk of vaccine failure (OR: 0.06, 95% CI: 0.003 – 1.09, $p=0.009$) of rs7154439 C > T and rs4646285 G > A were only found in controls bearing rs4646287 G > A (Table 3). The differences of rs7154439 C > T and rs4646285 G > A between controls and carriers disappeared when they coexisted with wild-type rs4646287-GG, indicating minor effect of rs7154439 C > T and rs4646285 G > A on HBV vaccine failure. The results further support a unique association of rs4646287 G > A with HBV vaccine failure. The prevalence of rs2296651 C > T was similar between the two groups, which suggests a minor association between this Asian-specific SNP and HBV vaccine failure.

NTCP genetic variants and clinical parameters in chronic HBV carriers

The impact of the four SNPs of the NTCP gene on clinical progression was characterized. Peak levels of ALT and AST in carrier children who had achieved HBeAg seroconversion ($N=71$) were analyzed (Fig. 2). Compared to wild-type rs4646287-GG, there was a significant correlation between rs4646287 G > A and higher peak ALT (588.6 ± 557.1 [$N=11$] vs. 247.1 ± 202.8 [$N=60$], $p<0.001$) and AST (378.4 ± 339.4 [$N=11$] vs. 160.9 ± 145.8 [$N=60$], $p<0.001$) levels. Among the subgroups divided according to sex and age, higher ALT levels stayed significantly associated with rs4646287 G > A (Supplementary Table S2). The other three SNPs (rs7154439, rs4646285, and rs2296651) showed no significant association with peak ALT and AST levels. Our results suggest that chronic HBV-infected children with the rs4646287 G > A genotype of the NTCP gene may have stronger immune responses to HBV infections.

Kaplan–Meier survival analyses were performed for the spontaneous HBeAg seroconversion ages in chronic HBV carrier children who did not receive antiviral treatment. Carrier children with wild-type NTCP gene (no genetic variance at the 4 SNPs, $N=82$) were compared to those with variants rs7154439 C > T/rs4646285 G > A ($N=31$), rs2296651 C > T ($N=15$), and rs4646287 G > A ($N=22$). The Kaplan Meier survival curves for HBeAg seroconversion separated HBV carriers with wild-type NTCP gene from those with NTCP genetic variants ($p=0.029$). Compared to the wild-type NTCP (14.6 ± 8.7 years), rs4646287 G > A was significantly associated with an earlier

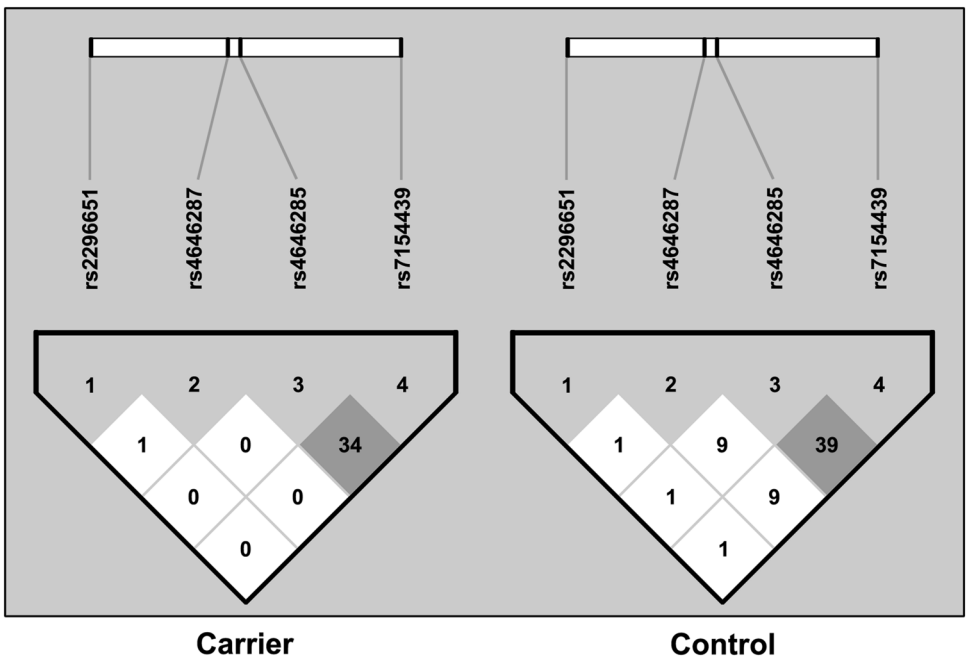

Fig. 1 Linkage disequilibrium map for four SNPs of the NTCP gene in 170 chronic HBV carriers and 138 control individuals. The LD plot shows r^2 values between each pair of SNPs; white squares indicate $r^2<0.1$ and dark squares indicate $r^2>0.9$

Table 3 Association between the selected polymorphisms in NTCP gene and HBV vaccine failure in non-carrier control and chronic HBV carrier children separated by rs4646287 genotype

SNP	Genotype	rs4646287 G > A ($N=65$)		OR (95% CI)	p value*	rs4646287-GG ($N=243$)		OR (95% CI)	p value
		Controls	Carriers			Controls	Carriers		
rs7154439	CC	30 (46.2)	26 (40.0)	1		68 (28.0)	109 (44.9)	1	
	CT	9 (13.9)	0			29 (11.9)	35 (14.4)		
	TT	0	0			2 (0.8)	0		
	CT + TT	9 (13.9)	0	0.06 (0.003–1.09)[a]	**0.009**	31 (12.8)	35 (14.4)	0.70 (0.40–1.25)	0.243
rs4646285	GG	30 (46.2)	26 (40.0)	1		69 (28.4)	109 (44.9)	1	
	GA	9 (13.9)	0			28 (11.5)	35 (14.4)		
	AA	0	0			2 (0.8)	0		
	GA + AA	9 (13.9)	0	0.06 (0.003–1.09)[a]	**0.009**	30 (12.4)	35 (14.4)	0.74 (0.42–1.31)	0.306

Bold type for p value less than 0.05 was considered significant

Data are presented as number (percentage). Differences in the genotype frequencies were analyzed by rs4646287 genotype (G > A or wild-type GG). OR, CI, and P values were calculated using conditional regression analysis

OR odds ratio, *CI* confidence interval

[a]Calculations were adjusted by Haldane–Anscombe correction due to 0 individuals in carrier group

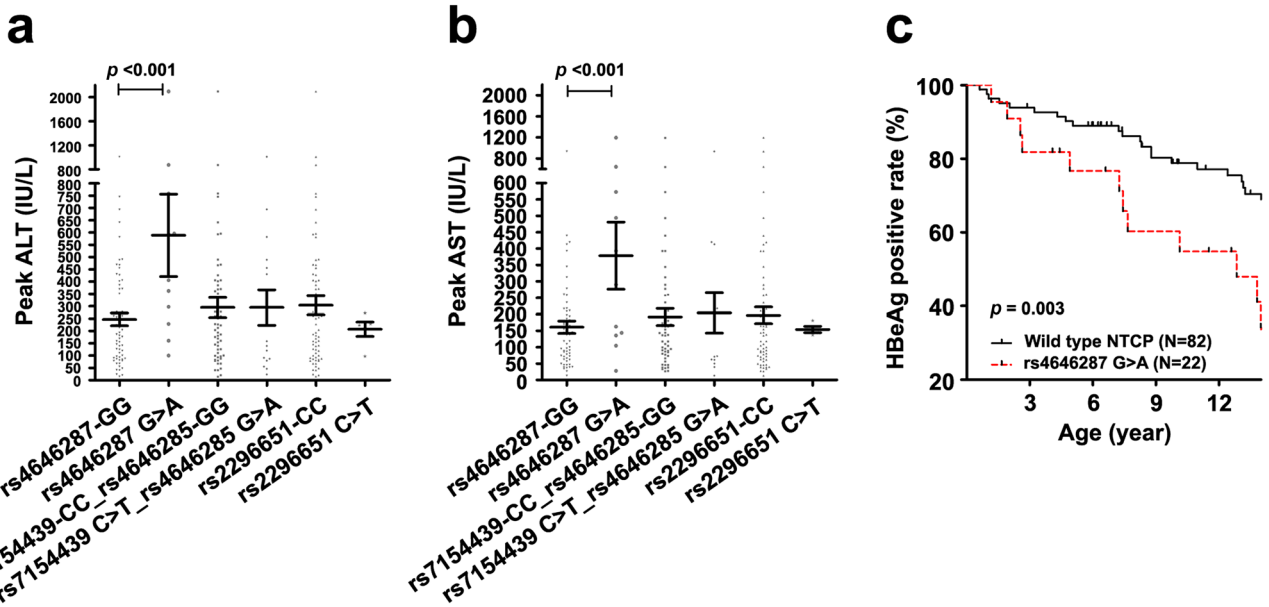

Fig. 2 Associations between NTCP genetic variants and clinical parameters in carrier children of chronic HBV. Peak alanine aminotransferase (ALT) (**a**) and aspartate aminotransferase (AST) (**b**) levels in carrier children with HBeAg seroconversion ($N=71$) were compared between different NTCP variants. **c** The spontaneous HBeAg seroconversion ages of carrier children without antiviral treatments were compared between NTCP genetic variants and the wild-type genotype. p values were determined using Student's t-test for peak ALT and AST levels, and Kaplan Meier analyses (log-rank test) for HBeAg seroconversion ages

HBeAg seroconversion age (10.8 ± 8.4 years, $p=0.003$) (Fig. 2c). The impact of rs7154439 C > T/rs4646285 G > A (13.07 ± 9.2 years, $p=0.423$) and rs2296651 C > T (11.2 ± 7.8 years, $p=0.151$) on spontaneous HBeAg seroconversion was not significant.

Correlation between the NTCP rs4646287 genotype and innate immunity

To further characterize the protective role of rs4646287 G > A, we investigated hepatic expression of NTCP and genes related to innate immunity. The four NTCP SNPs

were genotyped by PCR–RFLP in WBC DNAs from 100 HBV-infected non-HCC patients and 100 NBNC non-HCC patients. The MAFs of rs4646287-A allele were 15% and 24% in HBV-infected and NBNC patients, respectively. A total of 82 non-tumor liver RNA samples from those genotyped non-HCC patients were further analyzed and classified into four groups: HBV infection with the rs4646287 G > A ($N = 9$) or -GG ($N = 25$) genotype; NBNC with the rs4646287 G > A ($N = 19$) or -GG ($N = 29$) genotype.

Quantitative RT-PCR was used to detect the transcription levels of NTCP, type I interferons, and IFITM3. ANOVA indicated differences in NTCP mRNA levels between the four groups (Supplementary Figure S2). NBNC liver tissues with rs4646287 G > A had higher NTCP levels compared to the -GG genotype ($p = 0.065$) and compared to HBV-infected liver tissues with either the rs4646287 G > A ($p = 0.039$) or -GG genotype ($p = 0.017$). A significant difference in IFNα mRNA levels was found between the groups ($p < 0.0001$). IFNα levels were significantly reduced in HBV-infected liver tissues. No significant differences in IFNβ or IFITM3 levels were found.

Pearson correlation analyses (Fig. 3) showed an inverse correlation between NTCP and IFITM3 levels only in HBV-infected livers (rs4646287 G > A, $r = -0.672$, $p = 0.047$; rs4646287-GG, $r = -0.571$, $p = 0.003$). A trend of positive correlation between NTCP and IFNα was detected only in HBV-infected rs4646287 G > A liver tissues ($r = 0.638$, $p = 0.064$). An inverse correlation between type I interferons and IFITM3 was detected in HBV-infected liver tissues with rs4646287-GG (IFNα: $r = -0.530$, $p = 0.006$; IFNβ: $r = -0.664$, $p < 0.001$) and NBNC liver tissues with the rs4646287 G > A (IFNα: $r = -0.562$, $p = 0.012$; IFNβ: $r = -0.588$, $p = 0.008$) or -GG genotype (IFNα: $r = -0.639$, $p < 0.001$; IFNβ: $r = -0.615$, $p < 0.001$), but not in HBV-infected liver tissues with the rs4646287 G > A genotype. The unique correlation patterns of NTCP, type I interferons, and IFITM3 expressions were only found in HBV-infected liver cells carrying the rs4646287 G > A genotype.

Discussion

An association of NTCP genetic variants with HBV susceptibility and disease progression has been reported in different populations [18, 19, 26]. In this study, we demonstrated that rs4646287 (c.356 + 702) G > A variant located in intron 1 of the NTCP gene is associated with a reduced risk for HBV vaccine failure in children of HBeAg-positive mothers. Among children of HBsAg and HBeAg double-positive mothers, the frequency of the rs4646287 GA/AA genotype in the non-carrier children (28.3%) was significantly higher than in vaccine failure chronic HBV carrier children (15.3%). HBeAg is a marker of high infectivity and is associated with higher vaccine failure rates [5]. Our results suggest that the NTCP genetic variant rs4646287 G > A might have a protective effect against HBV vaccine failure under high maternal viral loads.

The SNP rs4646287 (c.356 + 702) G > A variant in intron 1 is protective for the host in response to HBV infections [21]. G > A may introduce a new DNA-binding site for the transcription co-activator p300. Protein p300 is an adenoviral E1A-binding protein and functions as a transcriptional co-factor and histone acetyltransferase. Lower NTCP mRNA levels have been reported in HBV-infected HCC tissues with rs4646287 GA/AA compared to the wild-type genotype, but no difference has been detected in paired non-tumor tissues [21]. Our study also found no significant differences in NTCP levels between HBV-infected non-tumor liver tissues with the wild-type genotype and those with rs4646287 G > A. The NBNC non-tumor liver tissues with rs4646287 G > A expressed the highest NTCP levels. Whether the genetic variant rs4646287 G > A affected NTCP transcription in liver tissues requires further investigation.

HBeAg seroconversion is indicative of reduced HBV replication and hepatitis activity in patients with chronic HBV [27]. Vaccine failure and maternal HBeAg are associated with a prolonged period of HBeAg seroconversion in such patients [28, 29]. In this study, SNP rs4646287 G > A of the NTCP gene was significantly associated with higher peak ALT levels in HBV carrier children who achieved HBeAg seroconversion. Besides, HBV carrier children with rs4646287 G > A achieved spontaneous HBeAg seroconversion at younger age. This suggests a stronger immune response in livers carrying the rs4646287 G > A genotype, which may have a protective effect on clinical progression in chronic HBV carrier children. Protective effects of the other three SNPs, namely, rs7154439 (in the 5′ upstream region) [17], rs4646285 (in exon 1) [20], and rs2296651 (in exon 4) [22, 23], against chronic HBV infections have been reported. In our study, these three SNPs showed little impact on HBV vaccine failure, peak ALT levels, and spontaneous HBeAg seroconversion.

The association between NTCP and innate immunity was examined by quantifying the expression of NTCP, type I interferons, and IFITM3 in non-tumor liver tissues of non-HCC patients. IFNα expression was significantly reduced in HBV-infected liver tissues compared to NBNC livers. HBV infections have a reported association with a lack of induction [30] and even suppression of innate immune responses [31, 32]. Our results indicate that chronic HBV infections may reduce the intrahepatic expression of IFNα but not IFNβ.

Pearson correlation analyses showed that NBNC liver tissues with the wild-type genotype or rs4646287 G > A displayed similar gene correlation patterns. However, different correlation patterns were detected in HBV-infected

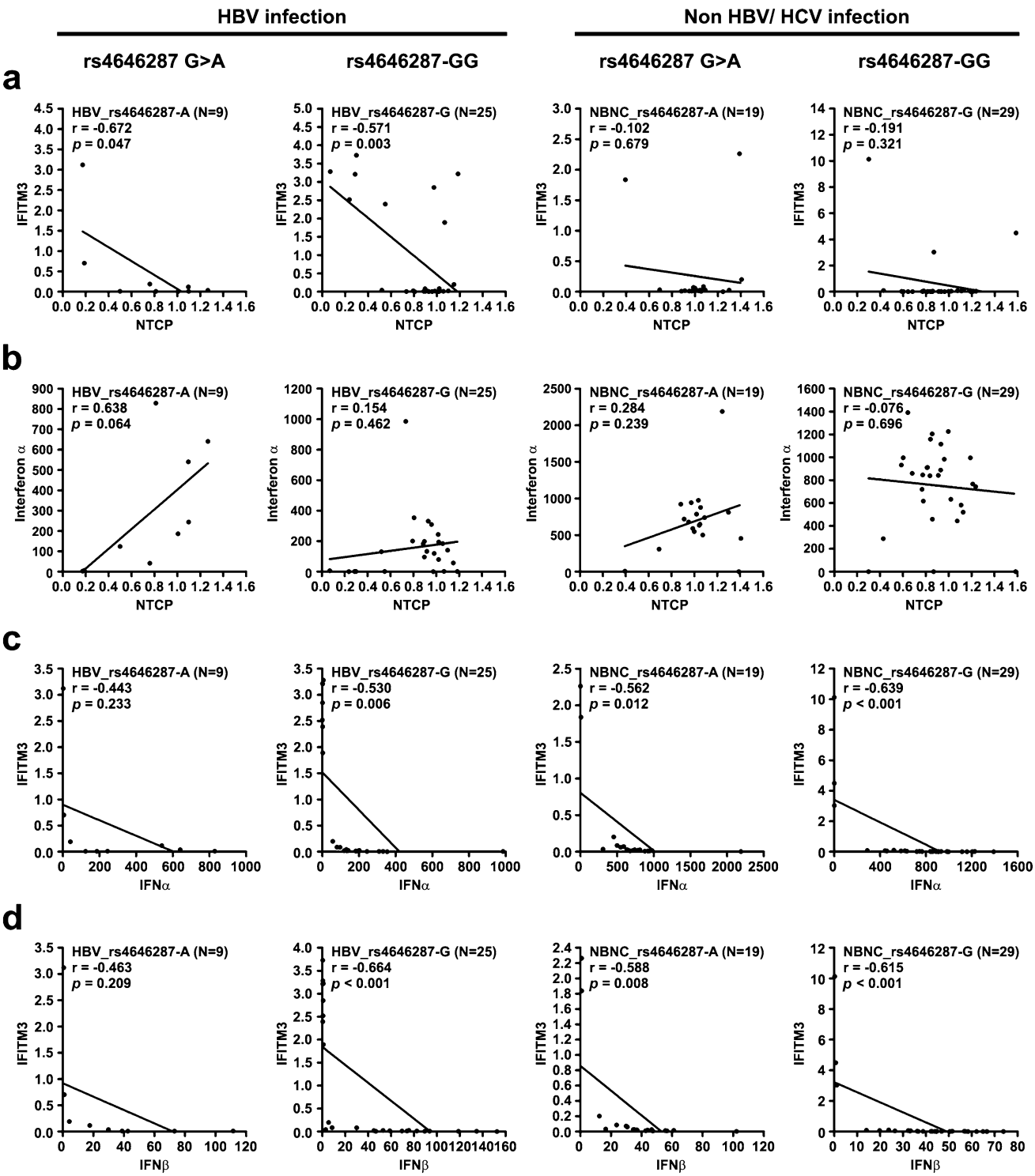

Fig. 3 Correlations between the rs4646287 genotype and NTCP, IFITM3, IFNα, and IFNβ expression. Pearson correlation tests were performed to determine the relationships between IFITM3 and NTCP (**a**), IFNα and NTCP (**b**), IFITM3 and IFNα (**c**), and IFITM3 and IFNβ (**d**) transcripts in non-tumor liver tissues from non-HCC patients classified into four groups: HBV infections with the NTCP genotype rs4646287 G > A ($N = 9$) or wild-type -GG ($N = 25$); and non-HBV/non-HCV infection (NBNC) with the rs4646287 G > A ($N = 19$) or wild-type -GG ($N = 29$). Pearson correlation coefficients r and p values are shown in each scatter plot

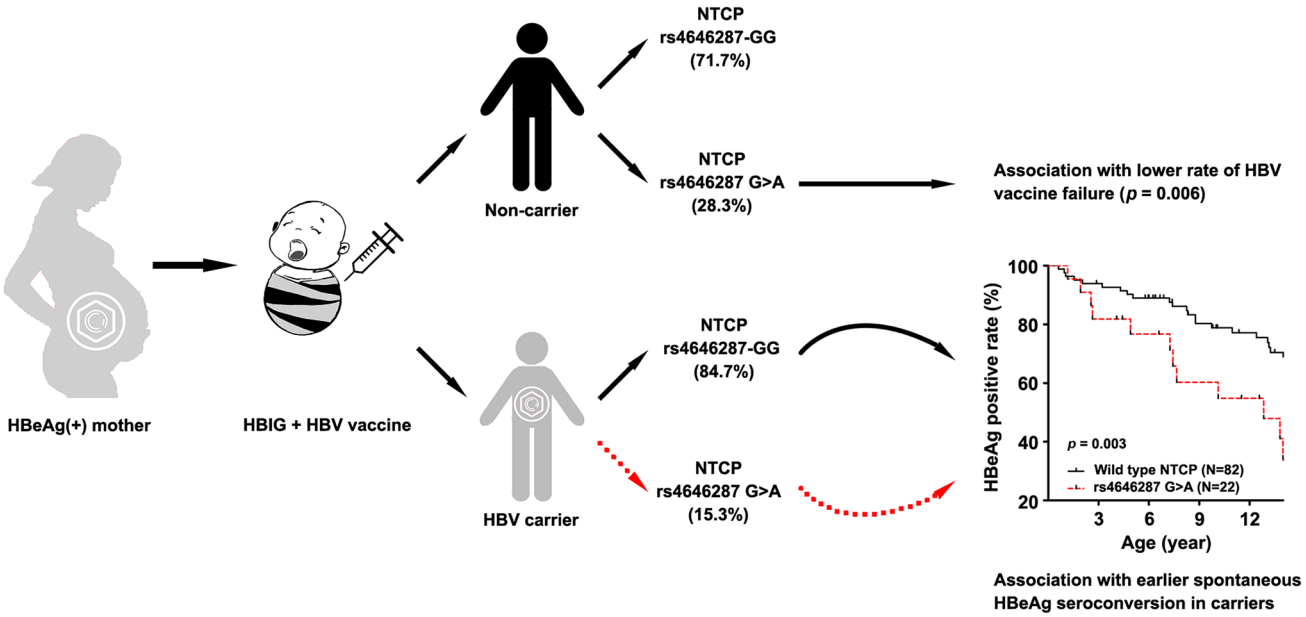

Fig. 4 Protective effects of the NTCP genetic variant rs4646287 G > A in children born to HBeAg-positive mothers. There was a higher frequency of rs4646287 G > A in non-carrier children compared to chronic HBV carriers. Lower spontaneous HBeAg seroconversion ages were associated with the rs4646287 G > A genotype in HBV carriers

liver tissues. A trend of positive correlation between NTCP and IFNα was present in HBV-infected livers with rs4646287 G > A ($r = 0.638$, $p = 0.064$) but not those with the wild-type genotype. In addition, there was an inverse correlation between type I interferons and IFITM3 in HBV-infected livers with the wild-type genotype but not those with rs4646287 G > A. Whether these negative correlations between type I interferons and IFITM3 contribute to the protective effects of rs4646287 G > A requires further investigation.

In conclusion, the genetic variant rs4646287 (c. 356 + 702) G > A in intron 1 of the NTCP gene is associated with a lower risk of vaccine failure in children born to HBeAg-positive mothers and with younger spontaneous HBeAg seroconversion ages in chronic HBV carrier children (Fig. 4). HBV-infected liver cells bearing the NTCP rs4646287 G > A genotype had unique innate immune responses that might be protective against HBV infections.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12072-022-10350-7.

Acknowledgements The authors express their gratitude to Dr. Jacob Shujui Hsu of the Graduate Institute of Medical Genomics and Proteomics, National Taiwan University for performing linkage disequilibrium analysis, and Dr. Ming-Fu Chang of the Institute of Biochemistry and Molecular Biology, National Taiwan University College of Medicine for valuable discussions. We also thank Taiwan Liver Cancer Network, National Health Research Institutes Biobank for providing the WBC DNAs, non-tumorous liver tissue RNAs, and related clinical data (kept anonymous) for our research. NHRI Biobank is supported by grants from Ministry of Science and Technology and National Health Research Institutes, Taiwan. We thank the staff of the Department of Medical Research, National Taiwan University Hospital for the Integrated Medical Database (NTUH-iMD).

Author contributions All authors contributed either to research design (MHC), acquisition (all authors), analysis (YHC, DJT, MHC), or interpretation (all authors) of data. YHC and DJT drafted the manuscript, which was read and critically revised by all other authors.

Funding This work was financially supported by the Ministry of Science and Technology, Executive Yuan, Taiwan (research grant MOST 105–2314-B-002–018-MY3).

Data availability statement The majority of results of the study are included in the manuscript or uploaded as supplementary information. Other data will be made available on request from the corresponding author.

Declarations

Conflict of interest Ya-Hui Chen, Daw-Jen Tsuei, Ming-Wei Lai, Wan-Hsin Wen, Cheng-Lun Chiang, Jia-Feng Wu, Huey-Ling Chen, Hong-Yuan Hsu, Yen-Hsuan Ni, Mei-Hwei Chang have no conflicts of interest to declare.

Ethical approval The study was approved by the Institutional Review Board of National Taiwan University Hospital (NTUH IRB 201512129INB).

References

1. Schweitzer A, Horn J, Mikolajczyk RT, Krause G, Ott JJ. Estimations of worldwide prevalence of chronic hepatitis B virus infection: a systematic review of data published between 1965 and 2013. Lancet. 2015;386(10003):1546–1555
2. Chen CL, Yang JY, Lin SF, Sun CA, Bai CH, You SL, et al. Slow decline of hepatitis B burden in general population: Results from a population-based survey and longitudinal follow-up study in Taiwan. J Hepatol. 2015;63(2):354–363
3. Ni YH, Huang LM, Chang MH, Yen CJ, Lu CY, You SL, et al. Two decades of universal hepatitis B vaccination in Taiwan: impact and implication for future strategies. Gastroenterology. 2007;132(4):1287–1293
4. Stevens CE, Beasley RP, Tsui J, Lee WC. Vertical transmission of hepatitis B antigen in Taiwan. N Engl J Med. 1975;292(15):771–774
5. Chen HL, Lin LH, Hu FC, Lee JT, Lin WT, Yang YJ, et al. Effects of maternal screening and universal immunization to prevent mother-to-infant transmission of HBV. Gastroenterology. 2012;142(4):773-781e772
6. Wen WH, Chang MH, Chen HL. Reply to: Mother-to-infant transmission of hepatitis B virus infection: significance of maternal viral load and strategies for intervention. J Hepatol. 2013;59(2):401
7. Liang TJ, Ghany M. Hepatitis B e antigen–the dangerous endgame of hepatitis B. N Engl J Med. 2002;347(3):208–210
8. Lin HH, Chang MH, Chen DS, Sung JL, Hong KH, Young YC, et al. Early predictor of the efficacy of immunoprophylaxis against perinatal hepatitis B transmission: analysis of prophylaxis failure. Vaccine. 1991;9(6):457–460
9. Burk RD, Hwang LY, Ho GY, Shafritz DA, Beasley RP. Outcome of perinatal hepatitis B virus exposure is dependent on maternal virus load. J Infect Dis. 1994;170(6):1418–1423
10. Hsu HY, Chang MH, Ni YH, Chen HL. Survey of hepatitis B surface variant infection in children 15 years after a nationwide vaccination programme in Taiwan. Gut. 2004;53(10):1499–1503
11. Tang JR, Hsu HY, Lin HH, Ni YH, Chang MH. Hepatitis B surface antigenemia at birth: a long-term follow-up study. J Pediatr. 1998;133(3):374–377
12. Hagenbuch BMP. Molecular cloning, chromosomal localization, and functional characterization of a human liver Na+-bile acid cotransporter. J Clin Investig. 1994;93(3):1326–1331
13. Yan H, Zhong G, Xu G, He W, Jing Z, Gao Z, et al. Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. Elife. 2012;1:1–80. https://doi.org/10.7554/eLife.00049
14. Chen HL, Chen HL, Liu YJ, Feng CH, Wu CY, Shyu MK, et al. Developmental expression of canalicular transporter genes in human liver. J Hepatol. 2005;43(3):472–477
15. Sharma S, Ellis EC, Gramignoli R, Dorko K, Tahan V, Hansel M, et al. Hepatobiliary disposition of 17-OHPC and taurocholate in fetal human hepatocytes: a comparison with adult human hepatocytes. Drug Metab Dispos. 2013;41(2):296–304
16. Ochiya T, Tsurimoto T, Ueda K, Okubo K, Shiozawa M, Matsubara K. An in vitro system for infection with hepatitis B virus that uses primary human fetal hepatocytes. Proc Natl Acad Sci USA. 1989;86(6):1875–1879
17. Su Z, Li Y, Liao Y, Cai B, Chen J, Zhang J, et al. Association of the gene polymorphisms in sodium taurocholate cotransporting polypeptide with the outcomes of hepatitis B infection in Chinese Han population. Infect Genet Evol. 2014;27:77–82
18. Chen X, Wang Y, Chen X, Cheng K, Li J, Lou J, et al. Genetic variants in the regulatory region of SLC10A1 are not associated with the risk of hepatitis B virus infection and clearance. Infect Genet Evol. 2016;44:495–500
19. Su Z, Li Y, Liao Y, Cai B, Chen J, Zhang J, et al. Polymorphisms in sodium taurocholate cotransporting polypeptide are not associated with hepatitis B virus clearance in Chinese Tibetans and Uygurs. Infect Genet Evol. 2016;41:128–134
20. Wu W, Zeng Y, Lin J, Wu Y, Chen T, Xun Z, et al. Genetic variants in NTCP exon gene are associated with HBV infection status in a Chinese Han population. Hepatol Res. 2018;48(5):364–372
21. Yang J, Yang Y, Xia M, Wang L, Zhou W, Yang Y, et al. A genetic variant of the NTCP gene is associated with HBV infection status in a Chinese population. BMC Cancer. 2016;16:211
22. Peng L, Zhao Q, Li Q, Li M, Li C, Xu T, et al. The p.Ser267Phe variant in SLC10A1 is associated with resistance to chronic hepatitis B. Hepatology. 2015;61(4):1251–1260
23. Hu HH, Liu J, Lin YL, Luo WS, Chu YJ, Chang CL, et al. The rs2296651 (S267F) variant on NTCP (SLC10A1) is inversely associated with chronic hepatitis B and progression to cirrhosis and hepatocellular carcinoma in patients with chronic hepatitis B. Gut. 2016;65(9):1514–1521
24. An P, Zeng Z, Winkler CA. The loss-of-function S267F variant in HBV receptor NTCP reduces human risk for HBV infection and disease progression. J Infect Dis. 2018;218(9):1404–1410
25. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001;29(9): e45
26. Zhang Y, Li Y, Wu M, Cao P, Liu X, Ren Q, et al. Comprehensive assessment showed no associations of variants at the SLC10A1 locus with susceptibility to persistent HBV infection among Southern Chinese. Sci Rep. 2017;7:46490
27. Wu JF, Chiu YC, Chang KC, Chen HL, Ni YH, Hsu HY, et al. Predictors of hepatitis B e antigen-negative hepatitis in chronic hepatitis B virus-infected patients from childhood to adulthood. Hepatology. 2016;63(1):74–82
28. Tai CS, Wu JF, Chen HL, Ni YH, Hsu HY, Chang MH. The impact of hepatitis B vaccine failure on long-term natural course of chronic hepatitis B virus infection in hepatitis B e antigen-seropositive children. J Infect Dis. 2017;216(6):662–669
29. Tseng YR, Wu JF, Ni YH, Chen HL, Chen CC, Wen WH, et al. Long-term effect of maternal HBeAg on delayed HBeAg seroconversion in offspring with chronic hepatitis B infection. Liver Int. 2011;31(9):1373–1380
30. Suslov A, Boldanova T, Wang X, Wieland S, Heim MH. Hepatitis B virus does not interfere with innate immune responses in the human liver. Gastroenterology. 2018;154(6):1778–1790
31. Wieland S, Thimme R, Purcell RH, Chisari FV. Genomic analysis of the host response to hepatitis B virus infection. Proc Natl Acad Sci USA. 2004;101(17):6669–6674
32. Lebosse F, Testoni B, Fresquet J, Facchetti F, Galmozzi E, Fournier M, et al. Intrahepatic innate immune response pathways are downregulated in untreated chronic hepatitis B. J Hepatol. 2017;66(5):897–909