CTLA-4 haplotype predicts HBsAg and HBcrAg levels and HBeAg seroconversion age in children with chronic HBV infection

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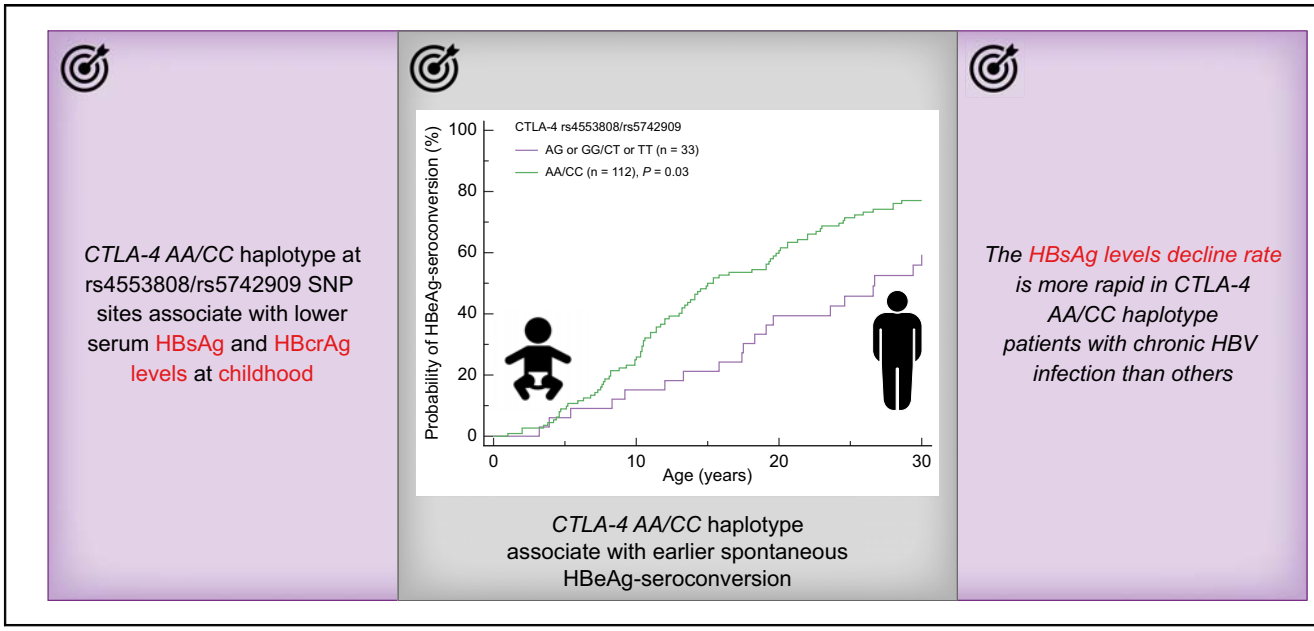
Graphical abstract



Highlights

- CTLA-4 modulates the disease course of chronic HBV infection.
- Chronic HBV-infected patients with a *CTLA-4* AA/CC haplotype have lower HBsAg and HBcrAg levels in childhood.
- These patients also experience earlier spontaneous HBeAg seroconversion and a more rapid annual decline in HBsAg levels.

Impact and implications

The role of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) in chronic HBV-infected children has not been studied previously. In a very long-term cohort followed from childhood to adulthood, we showed that *CTLA-4* haplotypes are associated with HBV biomarker levels in childhood and are correlated with the clinical course of chronic HBV infection. CTLA-4 pathway may serve as a future target for the development of therapeutic agents against HBV infection.

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Short communication

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CTLA-4 haplotype predicts HBsAg and HBcrAg levels and HBeAg seroconversion age in children with chronic HBV infection

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Background & Aim: Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) attenuates cytotoxic T lymphocyte (CTL) activation. This study was performed to examine the relationships between *CTLA-4* genotypes/haplotypes, hepatitis B surface antigen (HBsAg), and hepatitis B core-related antigen (HBcrAg) levels, and their potential impact on the clinical course of chronic HBV infection.
Methods: We recruited 145 treatment-naïve patients with genotype B or C chronic HBV infection who were initially hepatitis B e-antigen (HBeAg)-positive and had been followed from a mean age of 7.08 years for a total of 4,787 person-years in the study cohort. We also recruited another 69 treatment-naïve adults with genotype B or C chronic HBV infection as a validation cohort. We assessed the *CTLA-4* gene single nucleotide polymorphisms rs4553808 (–A1661G)/rs5742909 (–C318T) in both cohorts, and the serum HBsAg and HBcrAg levels in the study cohort.
Results: *CTLA-4* promoter haplotypes were associated with HBsAg and HBcrAg levels at 10 and 15 years of age in the study cohort. Patients with the *CTLA-4* AA/CC haplotype showed earlier spontaneous HBeAg seroconversion (hazard ratio = 1.58; p = 0.02), and a more rapid annual decline in the serum HBsAg level than other patients (0.09 *vs.* 0.03 $\log_{10}$ IU/ml/year, p = 0.02). The *CTLA-4* AA/CC haplotype was also predictive of HBeAg seroconversion in the validation cohort (p = 0.01).
Conclusions: Chronic HBV-infected patients with a *CTLA-4* AA/CC haplotype had lower serum HBsAg and HBcrAg levels in childhood and earlier spontaneous HBeAg seroconversion.
Impact and implications: The role of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) in chronic HBV-infected children has not been studied previously. In a very long-term cohort followed from childhood to adulthood, we showed that *CTLA-4* haplotypes are associated with HBV biomarker levels in childhood and are correlated with the clinical course of chronic HBV infection. CTLA-4 pathway may serve as a future target for the development of therapeutic agents against HBV infection.

Introduction

HBV remains a major global health threat, and the current goal is to develop therapeutic agents that can eradicate HBV cccDNA (covalently closed circular DNA).[1,2] Several serum biomarkers may serve as surrogates of HBV cccDNA, including hepatitis B surface antigen (HBsAg) and hepatitis B core-related antigen (HBcrAg).[3–6] Recent data demonstrate that the HBsAg level in childhood may predict the risk of advanced liver fibrosis in adulthood.[3] Previous studies have also shown that the HBsAg level may predict both hepatitis B e-antigen (HBeAg)- and HBsAg seroconversion, and the course of HBV infection is known to be associated with cytotoxic T lymphocyte (CTL) response.[7–9] CTL-associated protein 4 (CTLA-4) may transmit inhibitory signals to attenuate CTL activation.[10,11] A previous study demonstrated the role of *CTLA-4* single nucleotide polymorphisms (SNPs) in the outcome of acute HBV infection.[12] The expression pattern of the protein encoded by the *CTLA-4* gene was also altered by the promoter SNP rs4553808 (–A1661G) and rs5742909 (–C318T) of the *CTLA-4* gene.[13] The impacts of these functional SNPs of the *CTLA-4* gene on serum levels of HBsAg and HBcrAg, and clinical outcomes in patients with chronic HBV infection have not been reported. Thus, we explored the relationships among *CTLA-4* genotypes and the long-term clinical course of chronic HBV infection in this study.

Keywords: covalently closed circular DNA; hepatitis B core-related antigen; hepatitis B surface antigen; liver stiffness measurement; transient elastography.
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Materials and methods

Patients and clinical data

Our long-term cohort included 597 patients with chronic HBV infection who initially tested positive for HBeAg and were recruited from the pediatric outpatient clinic of National Taiwan University Hospital, six cross-sectional surveys of HBV prevalence in children in Taiwan conducted every 5 years from 1984 to 2009, and prospective screening programs for infants of HBsAg-positive mothers enrolled as part of a prospective study. From

this retrospective analysis, 145 patients (82 male and 63 female patients) with an initial follow-up age of less than 10 years were recruited to the study cohort. The patients were followed up at our institute from a mean age of 7.08 ± 2.76 years for a mean duration of 33.01 ± 4.37 years. These patients underwent 4,787 person-years of follow-up. The exclusion criteria of the study cohort were evidence of HIV, HCV or HDV coinfection; treatment with antivirals before HBeAg seroconversion; and first follow-up at >10 years of age.

We recruited another treatment-naïve adult cohort (n = 69), with a mean age of 37.62 years (95% CI 35.05–40.19 years) as a validation cohort for the relationship between *CTLA-4* haplotypes and HBeAg seroconversion in a cross-sectional design.

The HBV genotypes were also determined using serum samples collected during follow-up. Serum samples collected during follow-up were stored at –80 °C within 4 h of collection. The study protocol was approved by the Institutional Review Board of National Taiwan University Hospital (201911047RIND).

Quantification of HBsAg and HBcrAg levels

The HBsAg and HBcrAg levels at 10 and 15 years of age were measured in all serum samples of the study cohort following the methods described in a previous report.[6] The HBsAg levels were determined using the Architect HBSA QT assay (Abbott Laboratories, Abbott Park, IL, USA), and the HBcrAg levels were quantified using the Lumipulse G HBcrAg assay and Lumipulse G1200 Analyzer (Fujirebio, Tokyo, Japan).

CTLA-4 genotyping

Peripheral blood mononuclear cells of all participants were isolated from ethylenediaminetetraacetic acid-anticoagulated blood samples using a density gradient medium (Ficoll-Paque™ Plus; GE Healthcare, Chicago, IL, USA) and cultured at 37 °C in 5% carbon dioxide in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (all purchased from Gibco/Thermo Fisher Scientific, Waltham, MA, USA).

Genomic DNA was isolated from the peripheral blood mononuclear cells of all patients using the Gentra Puregene Blood Kit Plus (QIAGEN, GmbH, Hilden, Germany) according to the manufacturer's protocol. SNPs of the human *CTLA-4* gene rs4553808 (–A1661G) and rs5742909 (–C318T) were genotyped using PCR-restriction fragment length polymorphism.

The fragment for the *CTLA-4* polymorphism was amplified from genomic DNA by PCR using the primer designs in the previous report.[14] Genomic DNA was amplified and digested with the restriction enzyme MseI. All variants identified by restriction fragment length polymorphism were confirmed by direct sequencing.

Statistical analysis

Stata version 17 (StataCorp LLC, College Station, TX, USA) and MedCalc version 22.016 (MedCalc Software, Ostend, Belgium) were used for statistical analyses. Student's *t* test with unequal variance was used to compare continuous variables between the two groups (expressed as mean with 95% CI). Fisher's exact test or the chi-square test was used to compare categorical variables between the two groups and evaluate differences in incidence rates. Cox proportional hazard analysis and Kaplan–Meier survival curves were used for survival analysis. Cox proportional hazard analysis was performed to determine the hazard ratios (HRs) and 95% CIs of binary outcomes in the survival analysis. Logistic regression was also applied to yield the odds ratios (ORs) and 95% CIs of binary outcomes. Statistical significance was set at p <0.05.

Results

Participants' general characteristics

The general characteristics of the study cohort (n = 145) are summarized in Table S1. A total of 112 (77.24%) and 33 (22.76%) patients carried the AA/CC haplotype and GA or GG/CT or TT haplotypes at *CTLA-4* rs4553808 (–A1661G) and rs5742909 (–C318T) promoter SNP sites, respectively. All patients carrying the rs4553808 (–A1661G) AA genotype also carried the CC genotype at the rs5742909 (–C318T) SNP. Thus, we analyzed the difference between patients carrying the AA/CC haplotype and GA or GG/CT or TT haplotypes at the *CTLA-4* rs4553808 (–A1661G) and rs5742909 (–C318T) promoter SNPs.

The HBsAg and HBcrAg levels were significantly lower in patients carrying the AA/CC haplotype than others at the *CTLA-4* rs4553808 and rs5742909 promoter SNP sites (Table 1). The annual rate of decline in the HBsAg level was significantly higher in patients carrying the AA/CC haplotype than in those carrying the GA or GG/CT or TT haplotypes at the *CTLA-4* rs4553808 and rs5742909 promoter SNP sites (0.09 ± 0.20 *vs*. 0.03 ± 0.11 $\log_{10}$ IU/ml/year, 95% CI 0.05–0.13 *vs*. 0–0.07 $\log_{10}$ IU/ml/year, p = 0.02). The annual rate of decline in the HBcrAg level was not significantly different among the different *CTLA-4* haplotypes in this cohort (p = 0.81).

Table 1. Differences in participants with different *CTLA-4* haplotypes in the study cohort (n = 145).

rs4553808/rs5742909	GA or GG/CT or TT (n = 33)	AA/CC (n = 112)	*p* value
Male sex, n (%)	17 (51.52)	65 (58.04)	0.51
Initial visit age, mean ± SD (95% CI), years	6.71 ± 3.10 (5.61-7.80)	7.19 ± 2.66 (6.70-7.69)	0.37
Final follow-up age, mean ± SD (95% CI), years	39.41 ± 6.13 (37.23-41.58)	40.30 ± 4.98 (39.36-41.23)	0.39
Follow-up time, mean ± SD (95% CI), years	32.71 ± 4.49 (31.12-34.30)	33.10 ± 4.35 (32.29-33.92)	0.65
HBV genotype, n (%)			
Genotype B	25 (75.76)	85 (75.89)	
Genotype C	8 (24.24)	23 (20.54)	
Genotypes B+C	0 (0)	4 (3.57)	0.51
HBsAg level, mean ± SD (95% CI), $\log_{10}$ IU/ml			
10 years of age	4.00 ± 1.48 (3.48-4.53)	3.82 ± 1.09 (3.61-4.02)	0.03
15 years of age	3.85 ± 1.45 (3.34-4.36)	3.36 ± 1.46 (3.09-3.64)	0.03
HBcrAg level, mean ± SD (95% CI), $\log_{10}$ U/ml			
10 years of age	6.27 ± 1.38 (5.77-6.76)	5.54 ± 1.75 (5.21-5.87)	0.03
15 years of age	5.61 ± 1.65 (5.03-6.20)	4.98 ± 1.91 (4.62-5.34)	0.09

*p value was obtained using Student's t test for continuous variables and the chi-square test for categorical variables.

Table 2. Predictors of spontaneous HBeAg seroconversion in the study cohort.

	Univariate			Multivariate		
	HR	95% CI	*p* value	HR	95% CI	*p* value
Male *vs.* female	0.92	0.65-1.31	0.66	–	–	–
Genotypes B and B+C *vs.* genotype C	1.72	1.10-2.71	0.02	1.70	1.09-2.66	0.02
CTLA-4 rs455380 rs5742909 AA/CC *vs.* GA or GG/CT or TT	1.58	1.03-2.43	0.03	1.56	1.02-2.39	0.04

*p values were obtained using Cox proportional hazard analysis.
HR, hazard ratio.

We recruited another antiviral treatment-naïve adult cohort (n = 69; Table S2) with a mean age of 37.62 years as a validation cohort. In the validation cohort, 56 (81.16%) patients had achieved HBeAg seroconversion. In the validation cohort, 43 individuals carried the AA/CC haplotype and another 26 carried the GA or GG/CT or TT haplotypes at the *CTLA-4* rs4553808/rs5742909 locus.

Impact of *CTLA-4* haplotypes on spontaneous HBeAg seroconversion

Univariate Cox proportional hazard analyses showed that both HBV genotypes B and B+C and the *CTLA-4* AA/CC haplotype predicted earlier spontaneous HBeAg seroconversion (p = 0.02 and 0.03, respectively) (Table 2). Kaplan–Meier survival curves with the rank-sum test confirmed the beneficial role of the *CTLA-4 AA/CC* haplotype in predicting spontaneous HBeAg seroconversion (Fig. S1). Multivariate Cox proportional hazard analysis further confirmed the significant role of the *CTLA-4* AA/CC haplotype in predicting spontaneous HBeAg seroconversion (HR 1.56; p = 0.04) (Table 2). We were not able to identify an effect of *CTLA-4* rs4553808 and rs5742909 promoter SNPs on HBsAg seroclearance in this cohort (p >0.05).

In the validation cohort (n = 69), Fischer's exact test showed the HBeAg seroconversion rate is significantly higher in participants carrying the AA/CC haplotype than in those carrying the GA or GG/CT or TT haplotypes (90.70% *vs.* 65.38%, p = 0.01, Table S3). The logistic regression also showed a higher prevalence of HBeAg seroconversion in participants carrying the AA/CC haplotype than in those carrying the GA or GG/CT or TT haplotypes (OR 5.16, 95% CI 1.40–19.10, p = 0.01).

Discussion

In this retrospective analysis, the *CTLA-4* promoter SNP (rs4553808 and rs5742909) haplotypes had significant effects on baseline HBsAg and HBcrAg levels in patients with chronic HBV infection. The *CTLA-4* AA/CC haplotype was associated with the occurrence of earlier spontaneous HBeAg seroconversion in this study.

Clearance of HBV is associated with adequate CTL responses to the viral envelope, nucleocapsid, and polymerase proteins.[15,16] HBV-specific CTLs are considered crucial to the control and even clearance of HBV.[15] CTLA-4 is a coinhibitory molecule that attenuates CTL activity and is associated with chronic HBV infection.[17] The expression pattern of the protein encoded by the *CTLA-4* gene can reportedly be altered by the polymorphisms rs4553808 (–A1661G) and rs5742909 (–C318T) located in the promoter region of the *CTLA-4* gene.[13] The role of CTLA-4 in the pathogenesis of HBV infection was demonstrated by a study linking *CTLA-4* gene SNPs to the outcome of acute HBV infection.[12] However, the impact of *CTLA-4* gene SNPs on the natural course of chronic HBV infection has not been discussed in the literature to date. Our study demonstrated that the AA/CC haplotype in the *CTLA-4* rs4553808 and rs5742909 promoter SNPs was associated with lower serum levels of HBsAg and HBcrAg in children with chronic HBV infection. It is possible that chronic HBV infection in combination with the presence of a low *CTLA-4* expression haplotype, indicating higher CTL activity against HBV, may have resulted in lower HBsAg and HBcrAg levels in childhood. We further showed that patients carrying such haplotypes achieved earlier spontaneous HBeAg seroconversion than other patients in survival analysis.

The main limitation of this study was its retrospective design and the fact that only a small number of patients achieved HBsAg seroclearance. This resulted in inadequate statistical power for the analysis of the effect of *CTLA-4* haplotypes on HBsAg seroclearance. Although our data showed a relationship between *CTLA-4* haplotypes and the annual rate of decline in HBsAg, a further large-scale study is needed to confirm the relationship between *CTLA-4* haplotypes and HBsAg seroclearance in patients with chronic HBV infection.

In conclusion, we found a relationship between CTLA-4, HBsAg, and HBcrAg levels in the early phase of chronic HBV infection. The *CTLA-4* AA/CC haplotypes were correlated with the annual decline of HBsAg levels and predicted earlier spontaneous HBeAg seroconversion.

Abbreviations

CTLs, cytotoxic T lymphocyte; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; HBcrAg, hepatitis B core-related antigen; HBeAg, hepatitis B e-antigen; HBsAg, hepatitis B surface antigen; HR, hazard ratio; OR, odds ratio; SNPs, single nucleotide polymorphisms.

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Conflict of interest

The authors declare no potential, perceived, or real conflict of interest.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

JF Wu, the first author of the study, is responsible for the study design, data management, and manuscript writing. CS Tai, KC Chang, TW Chen,

HL Chen, YH Ni, HY Hsu, and MH Chang are responsible for long-term patient follow, recruitment, and critical review of the manuscript. MH Chang, the corresponding author, was responsible for patient recruitment, study design, and critical review of the article, and is the principal investigator of this study. All authors have seen and approved the final version. The corresponding author had full access to all the data in this study and had final responsibility for the decision to submit for publication.

Data availability statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request. and IRB approval of National Taiwan University Hospital, Taiwan.

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Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhepr.2024.101061.

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Author names in bold designate shared co-first authorship

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