The Impact of Hepatitis B Virus Precore/Core Gene Carboxyl Terminal Mutations on Viral Biosynthesis and the Host Immune Response

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Background. We aimed to elucidate the impact of hepatitis B virus (HBV) precore/core gene mutations on spontaneous hepatitis B e antigen (HBeAg) seroconversion, HBV biosynthesis, and the human immune responses in chronic HBV–infected patients.

Methods. We analyzed the HBV precore/core gene sequences by cloning method in 33 chronic HBV–infected patients during the inflammatory phase before spontaneous HBeAg seroconversion. The impact of the most prevalent mutant on HBeAg biosynthesis was assessed by Western blotting and native agarose gel analysis in Huh7 cells, and the human immune responses were assessed by in vitro stimulation of CD3+CD8+ T lymphocytes of chronic HBV–infected subjects.

Results. The P135Q and G1896A were the most prevalent mutants before HBeAg seroconversion, acting as markers of HBeAg seroconversion (hazard ratios = 2.75 and 4.50; \( P = .01 \) and <.001, respectively). The P135Q mutants displayed decreased HBeAg secretion and HBV capsid molecular weight, while showing increased 22 kD HBeAg proprotein accumulation in Huh7 cells. The P135Q mutant peptide induced less interferon-\( \gamma \) expression in CD3+CD8+ T lymphocytes in HBeAg-negative subjects compared to the wild-type peptide (\( P = .03 \)).

Conclusions. The HBV P135Q mutant emergence during the inflammatory phase was associated with HBeAg seroconversion. It was associated with altered HBV capsid assembly, HBeAg biosynthesis, and reduced human immune responses following HBeAg seroconversion.

Keywords. hepatitis B virus; hepatitis B e antigen; hepatitis B e antigen seroconversion; immune escape mutant; precore/core gene.

Persistence of the hepatitis B virus (HBV) e antigen (HBeAg) with high viral load during chronic HBV infection is associated with an increased risk of liver cirrhosis and hepatocellular carcinoma [1–3]. HBeAg is an alternative translation product of the precore/core gene of the HBV genome and is linked with long-term immune tolerance. The 19 amino acids of the N-terminus encoded by the HBV precore gene serve as a signal peptide for the translocation of the 25 kD HBeAg proprotein (p25) to the endoplasmic reticulum of hepatocytes, and these amino acids are cleaved by a signal peptidase to yield a 22-kD HBeAg proprotein (p22) [4]. The cleavage of the arginine-rich carboxyl-terminus (C-terminus) region of the p22 proprotein by furin enhances the secretion of the resulting 17-kD mature HBeAg (p17) into the circulation [4, 5].

The emergence of HBV precore/core gene mutations during has been identified during HBeAg seroconversion in chronic HBV infection [6–8]. Such mutations alter the HBeAg antigenicity, HBV nucleocapsid structure and stability, and even pregenomic RNA (pgRNA) packaging into the nucleocapsid [9]. Mutations in the C-terminus of the HBV precore/core gene have been reported to alter the biosynthesis, transportation, and secretion of HBeAg [10–12]. Accumulation of the HBeAg proprotein (p22) in the cytoplasm has been found to downregulate HBV DNA replication and 21 kD hepatitis B core antigen (HBcAg; p21) capsid polymerization, which results in decreased HBV replication [13].
Host immune pressure may select for HBV strains during chronic HBV infection [8, 14, 15]. However, the inflammatory phase–associated impacts of the naturally occurring HBV precore/core mutants on the biosynthesis, transportation, and secretion of HBeAg, and even on human immune responses, have not been investigated extensively. The current study demonstrates the effects on HBV biosynthesis of HBV mutant strains selected by host immune pressure and the interaction of these strains with the host immune system.

**METHODS**

**Study Subjects**

For this study, 33 patients (24 males) were enrolled based on the following recruitment criteria: (1) follow-up period of >10 years, (2) presence of the inflammatory phase (HBeAg[+]; and anti-HBeAg antibody [anti-HBe] [−]; alanine aminotransferase [ALT] >60 IU/L for >6 months) during the follow-up period, (3) genotype B HBV infection, (4) no antiviral treatment during the inflammatory phase, (5) born to chronic HBV surface antigen (HBsAg)–carrier mothers, and (6) initial serum ALT levels <30 IU/L. Serum samples from the immune-tolerance (HBeAg [+]; anti-HBe[−]; ALT < 30 IU/L) and inflammatory (HBeAg [+]; anti-HBe[−]; ALT >60 IU/L) phases before spontaneous HBeAg seroconversion were analyzed. The study protocol was approved by the Institutional Review Board of the National Taiwan University Hospital; the patients/guardians provided informed consent for blood collection and clinical data analysis.

**HBV Serological Tests, Genotyping, and Viral Load Determination**

Levels of the HBV markers HBsAg, anti-HBsAg antibody (anti-HBs), HBeAg, anti-HBe, and anti-HB core antibody were assessed by enzyme immunoassay (Abbott Laboratories). Spontaneous HBeAg-seroconversion was defined as the clearance of serum HBeAg and the appearance of anti-HBe, persisting for >6 months and without the use of antiviral agents. HBV genotype and viral load in each individual were determined by real-time PCR and melt curve analysis using the LightCycler hybridization probe assay system (Roche) [15].

**HBV Precore/Core Gene Cloning and Sequencing**

Total DNA was extracted from 200-µL serum samples using the QIAamp DNA blood mini kit (Qiagen), according to the manufacturer’s instructions, and eluted in 50 µL of distilled water. The following PCR primers were used for the amplification of the precore/core gene: forward, 5′-AGATTTAGGTTAAGGTCTTTG-3′; reverse, 5′-AGTTTCCACCTTATGAGTC-3′. The DNA amplification was performed using the Extensor Hi-Fidelity PCR Master Mix (Thermo Fisher Scientific) with 5 µL of the serum as the DNA template. PCR reactions were performed under the following conditions: initial activation at 94°C for 1 minute, followed by 34 denaturation cycles at 94°C for 30 second, an annealing step at 55°C for 30 seconds, and extension at 72°C for 1 minute. The last cycle was followed by a final extension at 72°C for 10 minutes. The 700-bp fragment (nt 1769–2469) containing the entire precore/core gene (639 bp) was then amplified, purified using the Gel/PCR DNA Fragments Extraction kit (Geneaid), cloned using the TOPO TA cloning kit (Invitrogen), and transformed into competent ECOS101 *Escherichia coli* (Yeastern Biotech Co). The Mini Plus Plasmid DNA Extraction System (Viogene) was used to purify plasmid DNA. Fifteen positive clones per serum sample were subjected to precore/core gene DNA sequencing using the Big Dye Terminator and sequencer (Applied Biosystems). The genotype Bb HBV precore/core gene sequence was used as the reference sequence [16].

The 1762 + 1764 basal core promoter mutation was shown to play a nonsignificant role in the process of spontaneous HBeAg seroconversion in children infected with genotype B chronic HBV [17]. Hence, we did not assess the basal core promoter mutation in this analysis.

**HBV Precore/Core Gene Expression Constructs**

A wild-type HBV DNA fragment spanning the entire precore/core gene sequence (nt 1785 to 2488) was amplified from DNA extracted from serum samples with the sense (5′-AGATTTAGGTTAAGGTCTTTG-3′) and antisense (5′-AGTTTCCACCTTATGAGTC-3′) primers.

The PCR products were cloned into the TOPO TA cloning vector (Invitrogen). Using EcoRI cutting, PCR fragments were amplified using the sense (5′-GACTATACAAAACTTATGCAA CTTTTTCACC-3′) and antisense (5′-GTCAAGACTCGAGC TAAAGATTCGAC-3′) primers, and then cut with the HindIII and Xhol enzymes. The resulting DNA fragments were cloned into the pcDNA 3.1 myc-His C vector (Invitrogen) and transfected into Huh7 cells.

**Site-Directed Mutagenesis for Generation of Specific Mutant Clones**

The wild-type HBV DNA core/precore gene expression constructs and a 1.5× full-length genotype Bb HBV clone (clone B6) were amplified by PCR. These PCR products were cloned into the pcDNA 3.1-TOPO vector (Invitrogen) as templates for PCR-mediated site-directed mutagenesis. We selected the most prevalent mutants seen during the patients’ inflammatory phases as our candidate targets for point mutagenesis, using the QuickChange Lightning Site-Directed Mutagenesis kit (Agilent Technologies). The primers for point mutagenesis were designed with the QuickChange primer design program (http://www.stratagene.com/sdmdesigner/default.aspx), and PCR was conducted with Pfu Turbo DNA polymerase (Stratagene), followed by a 16-hour digestion with Dpn1 enzyme (New...
England BioLabs) to remove the parental DNA template. The vector DNA was then transformed into competent cells and the resulting single colonies were selected. All DNA constructs were sequenced to confirm the presence of the desired mutations.

**Huh7 Hepatoma Cell Line Culture and Transfection**

Huh7 cells were purchased from the Bioresource Collection and Research Center. Cell culture media and fetal bovine serum (FBS; Invitrogen) were from Thermo Scientific. Transfection into Huh7 cells (wild-type and mutant HBV full-length core/precore gene expression constructs and the B6 clone) was performed using GenJet DNA In Vitro Transfection Reagent (Version II, SignaGen). A control vector was also used for standardization and confirmation of transfection efficiency.

**HBeAg Titer Determination in the In Vitro Cell Line Culture System**

Culture medium samples from Huh7 cells (wild-type and mutant HBV full-length precore/core gene sequences and the B6 clone) were subjected to HBeAg titer determination by enzyme immunoassay (Abbott Laboratories). Each culture condition was performed in triplicate.

**Western Blotting of the Intracellular HBeAg 22 kD Proprotein**

Extracts from Huh7 cells (wild-type and mutant HBV full-length precore/core gene sequences) were prepared following a 48-hour transfection period, and the cells were lysed with urea lysis buffer (10% SDS, 5M urea, β-mercaptoethanol). The proteins were separated by 4%-20% SDS-PAGE and transferred electrophoretically to nitrocellulose membranes. Following transfer, the blots were blocked at room temperature for 1 hour with 3% bovine serum albumin (BSA) dissolved in Tris-base saline —0.05% Tween 20 (TBST) buffer and incubated at 4°C overnight. Subsequent steps were carried out at room temperature. The blots were washed for 30 minutes with TBST buffer and incubated for 2 hours with a 1:500 dilution of a mouse monoclonal antibody specific for the HBV core protein (Santa Cruz Biotechnology). The blots were then washed for 30 minutes with TBST buffer and incubated for 1 hour with horseradish peroxidase (HRP)–conjugated antiserum antibodies (GeneTex) diluted 1:10 000 in 3% BSA-TBST buffer. After another wash for 30 minutes, signals were revealed by enhanced chemiluminescence (Millipore). The difference in intracellular HBeAg protein between the wild-type and mutant precore/core strains was assessed by Western blotting. A commercially sourced 21-kD HBcAg (p21) was used as the positive control.

**Native Agarose Gel Analysis of HBV Nucleocapsid Particles**

Huh7 cells transfected with wild-type and mutant clone B6 HBV sequences were washed with phosphate-buffered saline (PBS, Biological Industries) twice and lysed with NET buffer (50 mM Tris-HCl, pH 8.0; 1 mM ethylenediaminetetraacetic acid [EDTA]; 100 mM NaCl; 0.5% NP-40) at room temperature for 10 minutes. The lysates were then incubated at 4°C with agitation for 1 hour. The lysates were centrifuged at 12 000 rpm at 4°C for 20 minutes to remove the nuclei and cell debris. To eliminate the transfected DNA contamination, the lysates were treated with 300 units of micrococcal nuclease (Thermo Fisher Scientific) and 6 mM CaCl2 at 37°C for 30 minutes, and 25 mM EDTA was added to terminate the reaction. Lysates (25 µg) were separated by electrophoresis on 1% agarose gels with 1× Tris-borate–EDTA buffer at 50 volts for 4–5 hours, followed by electrotransfer onto a PVDF membrane for Western blotting. The PVDF membrane was blocked with 5% nonfat milk in 1× TBST for 1 hour at room temperature, then washed 3 times in TBST. For the antibodies, Hep-B-cAg (1:500, 13A9; sc-23946, Santa Cruz Biotechnology) and HRP-conjugated rabbit antimouse (1:20 000, Jackson ImmunoResearch Lab) were used. Chemiluminescence detection was then performed with the Immobilon Western System (Millipore). To detect HBV capsid-associated HBV DNA, we extracted the DNA from native agarose gel for PCR analysis. The following PCR primers were used for the amplification of the pre-S gene: forward, 5′-GGGTCACCTTATTCTTGGGA-3′; reverse, 5′-CCCCGCTGTAAACAGGACA-3′. The DNA amplification was performed using the Extensor Hi-Fidelity PCR Master Mix (Thermo Fisher Scientific). PCR reactions were performed under the following conditions: initial activation at 95°C for 5 minutes, followed by 35 denaturation cycles at 95°C for 40 seconds, an annealing step at 46°C for 40 seconds, and extension at 72°C for 40 seconds. The last cycle was followed by a final extension at 72°C for 2 minutes [18].

**Peripheral Blood Mononuclear Cell Isolation**

Peripheral blood mononuclear cells (PBMCs) from 10 HBeAg-positive and 10 HBeAg-negative chronic HBV carriers were isolated from 20 cc of whole blood according to established procedures [19]. None of these subjects received antiviral therapy at the time of blood sampling. The PBMCs (10⁶/mL) were cultured in 200 µL of medium with wild-type and mutant fragments of HBV precore/core peptides (amino acids 130–138). Plates were incubated in a humidified incubator with 5% CO₂ at 37°C for 7 days, then assessed for intracellular interferon (IFN-γ) and perforin expression.

**Evaluation of Intracellular Interferon-γ (IFN-γ) and Perforin Expression in T Lymphocytes**

We stained for intracellular IFN-γ and perforin to assess the cytoxicity of HBV-specific cytotoxic T lymphocytes. CD3⁺ CD8⁺ cells were harvested from PBMCs, washed, and then fixed with 2% paraformaldehyde in PBS for 20 minutes at room temperature. Cells were washed and permeabilized by incubation in PBS plus 1% BSA and 0.5% saponin for 10 minutes at room temperature. CD3⁺CD8⁺ cells were stained with
flourescein isothiocyanate (FITC)-antiperforin monoclonal antibody and isotype-matched control FITC–anti-IgG2a to assess perforin expression, and subsequently stained with FITC–anti-IFN-γ monoclonal antibody and isotype-matched control FITC–anti-IgG2a to assess the intracellular IFN-γ expression level in T lymphocytes. After staining, cells were washed twice with PBS plus 1% BSA and 0.5% saponin and once with PBS plus 0.5% BSA and fixed a second time with 2% paraformaldehyde in PBS. Flow cytometry was used to analyze the expression levels of intracellular IFN-γ and perforin in CD3+CD8+ T lymphocytes from HBeAg-positive and -negative chronic HBV–infected patients stimulated with wild-type and mutant fragments of HBV precore/core peptides.

Statistical Analysis
The statistical software package Stata (StataCorp LP) was used for statistical analysis. The Mann–Whitney U test was used to determine the significance of differences in continuous variables, and Fisher exact test for categorical variables. Linear regression was applied to evaluate the associations between HBV viral load and HBV precore/core gene mutations. Survival analysis using the Cox proportional hazard model for the interval censored data was used to calculate the relative hazard ratios (HRs) and hazard ratios (HRs) and P values between the various HBV mutants and viral load during the tolerance phase. P < .05 was considered to indicate statistical significance.

RESULTS

General Characteristics of the Study Population
During the follow-up period, 29 of the 33 subjects (87.9%) developed spontaneous HBeAg seroconversion at the median age of 19.6 years (range, 12.4–33.0 years). Another 4 subjects exhibited persistent HBeAg positivity following the inflammatory phase at a median age of 28.0 years (range, 26.2–30.9 years). The median follow-up period from the time of precore/core gene sequencing during the inflammatory phase to the time of spontaneous HBeAg seroconversion was 0.6 years (range, 0.1–8.2 years) in spontaneous HBeAg seroconverters (n = 29). The median follow-up period from the time of precore/core gene sequencing during the inflammatory phase to the time of the final medical visit in HBeAg nonseroconverters was 9.4 years (range, 2.6–20.0 years). There was no difference in baseline HBV load between HBeAg seroconverters and nonseroconverters (P = .16; Table 1).

Common HBV Precore/Core Gene Mutations and Their Correlation With HBeAg Seroconversion Time
All subjects expressed the wild-type HBV precore/core gene sequence during the immune-tolerance phase. We identified 26 mutation sites located in the HBV precore/core gene sequence during the inflammatory phase in these 33 patients. The HBV precore/core gene mutation profiles are summarized in Table 2.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>HBeAg Seroconverters (n = 29)</th>
<th>HBeAg Nonseroconverters (n = 4)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sex, No. (%)</td>
<td>20 (69.0)</td>
<td>4 (100)</td>
<td>.55</td>
</tr>
<tr>
<td>Age at initial visit, y</td>
<td>6.92 (0.25–9.96)</td>
<td>4.51 (2.56–7.54)</td>
<td>.27</td>
</tr>
<tr>
<td>Follow-up duration, y</td>
<td>24.69 (20.8–28.8)</td>
<td>23.49 (17.18–28.37)</td>
<td>.07</td>
</tr>
<tr>
<td>Age at final visit, y</td>
<td>30.68 (23.99–36.07)</td>
<td>27.99 (24.71–30.93)</td>
<td>.41</td>
</tr>
<tr>
<td>No. of medical visits</td>
<td>45 (31–68)</td>
<td>50.5 (37–68)</td>
<td>.56</td>
</tr>
<tr>
<td>Initial ALT level, IU/L</td>
<td>14 (3–31)</td>
<td>15 (9–29)</td>
<td>.74</td>
</tr>
<tr>
<td>Peak ALT level, IU/L</td>
<td>218 (53–2380)</td>
<td>386.5 (164–888)</td>
<td>.56</td>
</tr>
<tr>
<td>Baseline HBV load, log10 copies/mL</td>
<td>8.51 (6.71–9.39)</td>
<td>7.98 (7.67–8.77)</td>
<td>.33</td>
</tr>
<tr>
<td>HBV-infected mother, No. (%)</td>
<td>29 (100)</td>
<td>4 (100)</td>
<td>–</td>
</tr>
<tr>
<td>Genotype B HBV, No. (%)</td>
<td>29 (100)</td>
<td>4 (100)</td>
<td>–</td>
</tr>
</tbody>
</table>

Data are presented as median (range) unless otherwise specified. Fisher exact test and Mann–Whitney U test were applied to test the difference between categorical and continuous variables. Abbreviations: ALT, alanine aminotransferase; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus.

HBeAg seroconverters displayed more HBV mutant strains than nonseroconverters during the inflammatory phase. The C2304A (62.1%) and G1896A (48.3%) gene mutations were the most prevalent during the inflammatory phase in HBeAg seroconverters. These HBeAg seroconverters displayed a mean of 27% (95% confidence interval [CI], 14.8%–39.2%) C2304A mutant strains per person and 32.1% (95% CI, 18.0%–46.2%) G1896A mutant strains per person (Supplementary Table 1).

The C2304A mutation results in a proline to glutamine substitution at amino acid 135 of HBCAg (P135Q; Figure 1), whereas the G1896A mutation results in a precore stop codon. In the survival analysis, the presence of C2304A (P135Q) and G1896A during the inflammatory phase predicted spontaneous HBeAg seroconversion (HR = 2.8 and 4.5; P = .01 and <.001, respectively; Table 3). This significance persisted in the multivariate model for independent predictors of spontaneous HBeAg seroconversion (Table 3). Among the HBV precore/core gene mutation sites in the HBeAg seroconverters, only the C2304A (P135Q) and G1896A mutant percentages were negatively correlated with HBeAg seroconversion time (correlation coefficient = −0.4 and −0.4; P = .03 and .04, respectively). As C2304A (P135Q) was the most prevalent mutant strain in our study subjects, we further
assessed the impact of C2304A (P135Q) mutation on HBeAg biosynthesis, HBV capsid particle assembly, and immune responses before and after HBeAg seroconversion.

### Impact of C2304A (P135Q) Mutation on HBeAg Biosynthesis and HBV Nucleocapsid Particle Assembly

The HBeAg concentration in the culture medium was significantly lower in Huh7 cells transfected with the C2304A mutant clone compared with those transfected with the wild-type HBV precore/core gene sequence clone (73.6 ± 3.7 vs 181.2 ± 14.3 S/CO; \( P = .005 \)), as well as in Huh7 cells transfected with the C2304A mutant and wild-type B6 clone (1.5× full-length genotype Ba HBV sequence) (75.1 ± 10.7 vs 183.0 ± 24.8 S/CO; \( P = .001 \)).

There was also greater accumulation of the 22-kD HBeAg proprotein (p22) in Huh7 cells transfected with the C2304A (P135Q) mutant than with the wild-type HBV precore/core gene sequence, as demonstrated by Western blotting (Figure 2).

We further assessed the impact of the C2304A (P135Q) mutation on HBV capsid particle assembly by native agarose gel analysis, which showed that the number of low-molecular-weight HBV capsid particles increased in Huh7 cells transfected with the C2304A mutant B6 clone than those with the wild-type B6 clone sequences (Figure 3). From the PCR analysis of pre-S gene sequence in HBV capsid, HBV genome was still identified in low-molecular-weight HBV capsid particles in the C2304A (P135Q) mutants (Supplementary Figure 2).

### Impact of C2304A (P135Q) Mutation on the Host Immune Response

Recombinant C2304A (P135Q) mutant and wild-type peptides (amino acids 130–138) were used for in vitro PBMCs stimulation studies. The wild-type peptide induced higher intracellular IFN-\( \gamma \) expression in CD3+CD8+ T lymphocytes in HBeAg-negative subjects compared to in HBeAg-positive subjects (\( P = .02 \), Figure 4). In HBeAg-negative subjects, the C2304A mutant peptide P135Q induced less intracellular IFN-\( \gamma \) expression in CD3+CD8+ T lymphocytes than did the wild-type peptide (\( P = .03 \), Figure 4).

The effect of the C2304A (P135Q) mutant peptide on intracellular IFN-\( \gamma \) expression levels in CD3+CD8+ T lymphocytes

### Results

#### Table 2. Hepatitis B Virus Precore/Core Gene Mutation Patterns in the Inflammation Phase Between Subjects With and Without Spontaneous Hepatitis B e Antigen Seroconversion

<table>
<thead>
<tr>
<th>Mutation</th>
<th>HBeAg Seroconverters (n = 29)</th>
<th>HBeAg Nonseroconverters (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1896A</td>
<td>14 (48.3%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>C1913A</td>
<td>10 (34.5%)</td>
<td>1 (25%)</td>
</tr>
<tr>
<td>G1937A</td>
<td>1 (3.4%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>T1938C</td>
<td>2 (6.9%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>T1961A</td>
<td>2 (6.9%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>T1976G</td>
<td>2 (6.9%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>A1979G</td>
<td>4 (13.8%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>T1987A</td>
<td>1 (3.4%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>C1991A</td>
<td>1 (3.4%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>T2012C</td>
<td>1 (3.4%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>G2016A</td>
<td>1 (3.4%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>G2018A</td>
<td>0 (0%)</td>
<td>1 (25%)</td>
</tr>
<tr>
<td>A2075G</td>
<td>3 (10.3%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>C2078G</td>
<td>5 (17.2%)</td>
<td>1 (25%)</td>
</tr>
<tr>
<td>G2088T</td>
<td>5 (17.2%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>C2135A</td>
<td>1 (3.4%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>A2149T</td>
<td>1 (3.4%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>G2153A</td>
<td>3 (10.3%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>G2159A</td>
<td>5 (17.2%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>A2187C</td>
<td>1 (3.4%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>A2189C</td>
<td>5 (17.2%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>C2198A</td>
<td>1 (3.4%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>C2288A</td>
<td>2 (6.9%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>C2298A</td>
<td>1 (3.4%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>C2304A</td>
<td>18 (62.1%)</td>
<td>1 (25%)</td>
</tr>
<tr>
<td>A2339G</td>
<td>1 (3.4%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>G2357T</td>
<td>5 (17.2%)</td>
<td>1 (25%)</td>
</tr>
</tbody>
</table>

Data are presented as No. (%); the percentages indicate the percentage of patients carrying the respective mutation.

**Abbreviation:** HBeAg, hepatitis B e antigen.

#### Table 3. Prediction Roles of G1896A and C2304A Mutants During the Inflammatory Phase on the Time Interval to Spontaneous Hepatitis B e Antigen Seroconversion

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Univariate Model</th>
<th>Multivariate Model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>( P ) Value</td>
</tr>
<tr>
<td>G1896A vs others</td>
<td>4.50, 2.04–9.93</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>C2304A vs others</td>
<td>2.75, 1.27–5.98</td>
<td>.01</td>
</tr>
</tbody>
</table>

The Cox proportional hazards model was used to evaluate the likelihood of hepatitis B virus mutation in spontaneous hepatitis B e-antigen seroconversion.

**Abbreviations:** CI, confidence interval; HR, hazard ratio.

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**Impact of C2304A (P135Q) on the Host Immune Response**

The wild-type peptide induced higher intracellular IFN-\( \gamma \) expression in CD3+CD8+ T lymphocytes in HBeAg-negative subjects compared to in HBeAg-positive subjects (\( P = .02 \), Figure 4). In HBeAg-negative subjects, the C2304A mutant peptide P135Q induced less intracellular IFN-\( \gamma \) expression in CD3+CD8+ T lymphocytes than did the wild-type peptide (\( P = .03 \), Figure 4).

The effect of the C2304A (P135Q) mutant peptide on intracellular IFN-\( \gamma \) expression levels in CD3+CD8+ T lymphocytes

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**Figure 1.** Nucleotide and amino acid sequences of the wild-type and C2304A (P135Q) mutant genotype Ba hepatitis B virus gene regions.
DISCUSSION

HBcAg and HBeAg are derived from the HBV precore/core gene, mutations of which can result in changes in the amino acid sequence, protein structure, antigenicity, and even the biological functions of HBeAg and HBcAg [11, 20, 21]. HBcAg is the key component of HBV capsid particles, whereas HBeAg may be responsible for long-term immune tolerance in chronic HBV-infected patients. Changes in the amino acid sequence of HBcAg can alter the structure, capsid stability, pgRNA packaging, and replication efficacy of HBV [9, 22]. Changes in the HBeAg amino acid sequence may cause changes in its tertiary structure and immunogenicity, which may be linked to the breakthrough of long-term immune tolerance [23].

In this study, we demonstrated that both G1896A and C2304A mutations are predictors of spontaneous HBeAg seroconversion following long-term immune-tolerance development within genotype B chronic HBV–infected subjects. The G1896A results in a precore stop codon, which abolishes HBeAg proprotein biosynthesis. The C2304A mutation, which substitutes a glutamine for the proline at amino acid 135 of HBcAg (P135Q), was found to be the most prevalent mutation during the inflammatory phase before spontaneous HBeAg seroconversion (62.1%).

We further demonstrated that the C2304A (P135Q) mutation was significantly associated with increased cytoplasmic HBeAg 22 kD proprotein (p22) accumulation, decreased 17-kD...
mature HBeAg (p17) secretion, and decreased HBV capsid particle molecular-weight in HuH7 hepatoma cells. These findings suggest that this mutation may be associated with reduced efficacy in the cleavage of the 22-kD HBeAg proprotein (p22) C-terminus, resulting in decreased secretion of the 17-kD mature HBeAg (p17) [4, 9, 22, 23]. The C2304A (P135Q) mutation lies within the proline-rich loop (amino acids 128–136) of the HBcAg C-terminus. The crystallographic structure of HBcAg suggests that this proline-rich loop serves important roles within HBV capsid pores, in terms of pgRNA packaging and stabilization of the HBV capsid polymer structure [24–26]. The substitution of proline to glutamine at amino acid 135 may alter the biological function of the proline-rich domain of HBcAg and perhaps even the biosynthesis of HBV. Cytoplasmic accumulation of the HBeAg proprotein (p22) has been found to down-regulate HBcAg (p21) capsid polymerization previously [13]. Our data showed the increased percentage of HBV DNA containing low-molecular-weight HBV capsid in the C2304A (P135Q) mutant. The decrease in the molecular weight of HBV capsid particles seen in the C2304A (P135Q) mutants may be due to immature capsids because of alterations in the capsid pore structure.

The C2304A (P135Q) mutant peptide was also shown to induce less intracellular IFN-γ expression than the wild-type peptide in CD3+CD8+ T lymphocytes of HBeAg-negative subjects. Furthermore, the wild-type peptide induced higher IFN-γ expression in CD3+CD8+ T lymphocytes within HBeAg-negative subjects compared to HBeAg-positive subjects. Hence, the emergence of the C2304A (P135Q) mutation during the inflammatory phase under immune selection pressure might lead to the development of an immune-escape mutant after HBeAg seroconversion; moreover, this mutation appears to play an important role in the HBV life cycle. None of our 33 study subjects displayed any signs of HBeAg-negative hepatitis, liver cirrhosis, or hepatocellular carcinoma at the end of the follow-up period. Further studies are necessary to clarify the long-term consequences of C2304A (P135Q) mutation on the clinical course of chronic HBV infection.

The various HBV genotypes are associated with differences in the clinical outcomes of HBV infection [27, 28]. We investigated only the mutation patterns in subjects with chronic genotype B HBV infection, as genotype B is predominant in Taiwan; thus, future studies in subjects chronically infected by other HBV genotypes are required. In addition, the route of HBV transmission and the preceding elevation in serum ALT levels may also alter the course of HBV infection and the accumulation of different HBV mutants. Hence, only subjects infected with genotype B HBV, born to HBsAg carrier mothers, and displaying normal ALT levels at the first medical appointment were enrolled in this study to minimize any possible confounding effects.

In conclusion, the naturally occurring C2304A (P135Q) HBV precore/core gene immune escape mutant, arising during the inflammatory phase, was associated with spontaneous HBeAg-seroconversion in chronic genotype B HBV–infected patients. The C2304A (P135Q) mutation altered the biosynthesis of HBeAg in human hepatocytes and HBV capsid particle assembly, and suppressed the immune response following HBeAg seroconversion.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyrighted. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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