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Prevalence of Basal Core Promoter and Precore Mutations in Chinese Chronic Hepatitis B Patients and Correlation With Serum HBeAg Titers

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Abstract

The A1762T and G1764A mutations in the basal core promoter (BCP) region and the G1896A mutation in the precore (PC) region of hepatitis B virus (HBV) genome are found commonly in HBeAg-negative patients. Experiments in vitro suggest that BCP and PC mutation reduce and abolish HBeAg expression, respectively. In the present study, the prevalence of the BCP and PC mutations were determined in 207 patients with HBeAg positive chronic hepatitis B from China and correlated with the titers of serum HBeAg. None of the patients received antiviral therapy. The HBV genotype was determined by direct sequencing of the HBsAg gene. The BCP and PC mutations were detected by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and confirmed by DNA sequencing. The HBeAg titer was measured by the microparticle enzyme immunoassay. Fifty-one of the 207 patients (24.6%) were infected with genotype B and the remainder with genotype C. The BCP mutations were detected in 103 patients (50%) while the PC mutation was present in 43 (20.8%). Thirteen patients (6.3%) harbored both BCP and PC mutations. No significant difference in the titers of HBeAg was found between patients infected with the two HBV genotypes, but the presence of either the BCP or PC mutation was associated with reduced HBeAg titer ($P < 0.05$). The presence of both the BCP and PC mutations was accompanied by even lower HBeAg titer ($P < 0.05$). These findings confirm that in patients with HBeAg, the BCP and PC mutations reduced the expression of HBeAg.

Keywords

chronic hepatitis B; core promoter mutations; genotypes; HBeAg titer; precore mutation; restriction fragment length polymorphism

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INTRODUCTION

Over 350 million people worldwide are infected chronically with hepatitis B virus (HBV), of whom 250 millions reside in Asia [Magnius and Norder, 1995; Kao and Chen, 2002]. HBV is classified into eight genotypes designated as A–H [Magnius and Norder, 1995; Chu et al., 2002a]. The most prevalent genotypes in Asia are hepatitis B virus genotype B and C. Infection with genotype C is associated with HBeAg seroconversion (loss of HBeAg and presence of the corresponding antibody, anti-HBe) a decade later than genotype B infection [Chu et al., 2002b; Yuen et al., 2003]. Such seroconversion leads frequently to the selection of mutations in the precore (PC) region [Carman et al., 1989; Okamoto et al., 1990; Tong et al., 1990] and/or basal core promoter (BCP) region of the HBV genome [Okamoto et al., 1994]. The most common PC mutation is a G to A transition at nucleotide (nt) 1896 (A1896) that creates a premature stop codon and abolishes HBeAg translation [Carman et al., 1989; Blum et al., 1991; Tong et al., 1991]. The most common BCP mutations are A to T at nt 1762 and G to A at nt 1764 (T1762/A1764) [Okamoto et al., 1994; Kidd-Ljunggren et al., 1997; Chan et al., 1999; Honda et al., 1999; Lindh et al., 1999]. The double mutation decreases the transcription of pre-C mRNA, the mRNA for HBeAg, and consequently reduces HBeAg expression [Buckwold et al., 1996; Moriyama et al., 1996; Parekh et al., 2003]. Previous studies in Japanese and South African patients revealed an association of BCP mutations in HBV genome with reduced titers of HBeAg [Takahashi et al., 1995; Kurosaki et al., 1996; Baptista et al., 1999]. In the present study, prevalence of the PC and BCP mutations was determined among HBeAg positive Chinese chronic hepatitis B patients and sought for their possible correlation with HBeAg titers.

PATIENTS AND METHODS

Patients

Between July and October 2003, 207 Chinese patients with HBeAg-positive chronic hepatitis B were recruited from six hospitals in Shanghai, Beijing, Guangzhou, and Changchun. All the patients met the following criteria: (1) 18–70 years of age; (2) HBsAg positive for at least 6 months before entry; (3) HBeAg positive and HBV DNA titer > 5 $\log_{10}$ copies/ml; (4) ALT levels within 2–10 times the upper limit of normal (ULN); (5) lack of evidence for auto-immune hepatitis or markers of infection with hepatitis C virus, hepatitis D virus, or HIV. Patients who had received any type of antiviral therapy were also excluded. The earliest available serum samples before Peg-interferon clinical trial were used for this study. The serum samples were stored at −20°C until use. The study was conducted in accordance with the ethics principles of the Declaration of Helsinki. Written informed consent was obtained from each participant.

Serological Assays

The presence of HBsAg, HBeAg, anti-HBe, anti-HCV, and anti-HDV antibodies was determined using commercial AxSYM MEI kits (Abbott Laboratories, North Chicago, IL). The HBeAg values were measured using the microparticle enzyme immunoassay and expressed as signal/cutoff. The HBV DNA level was quantified using a real-time fluorescence quantitative commercial kit (Shenzhen PG Biotech, Shenzhen, China), which

has a detection limit of 500 HBV copies/ml. Assay calibration using the standard HBV DNA was supplied by the National Institute for the Control of Pharmaceutical and Biological Products, China.

HBV DNA Extraction, Amplification, and Genotype Detection

One hundred microliters of serum were mixed with equal volume of lysis buffer [25 mM Tris–HCl (pH 8.3), 10 mM EDTA, 1%SDS] and 500 μg/ml fresh proteinase K (TaKaRa Dalian, Dalian, China) and incubated at 65°C for 3 hr. DNA was extracted with phenol/chloroform, precipitated with ethanol, and resuspended in 20 μl distilled water. Genotyping was performed using a 1.3-kb fragment of the HBV genome covering the entire HBsAg gene (nt 2,816–886), which was amplified by the polymerase chain reaction (PCR). Primers S1 and AS (Table I), which occupied conserved regions, were designed according to Naito et al. [2001] and Kirschberg et al. [2004]. The PCR was performed in a volume of 50 ml containing 1 PCR buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM each of dNTPs (TaKaRa Bio, Inc.), 1 μM of primers, 4.0 U of Taq DNA polymerase (TaKaRa Dalian) and 5 μl of template DNA. Thermal cycling conditions consisted of 95°C for 10 min followed by 30 cycles of 94°C for 30 sec, 55°C for 45 sec, and 72°C for 1 min, and a final extension step at 72°C for 5 min. Precautions were taken to avoid contamination. Serum samples from individuals without HBV infection were used as negative controls during PCR. The PCR products were subjected to direct sequencing. HBV genotype was determined according to phylogenetic analysis [Simmonds and Midgley, 2005] using the Vector NTI 9.0 TreeView software [Zhou et al., 2004]. Since sequences from each genotype were monophyletic, only the most recent common ancestor is shown for non-gibbon genotypes. The tree was constructed by neighbor-joining using Jukes-Cantor corrected distances in the MEGA2 package [Tamura et al., 2001], using 1000 bootstrap replicates.

Detection of Precore and Core Promoter Mutations by RFLP and DNA Sequencing

Nested and semi-nested PCR were used to detect the A1896 mutation in the precore region and T1762/A1764 double mutation in the core promoter region, respectively. First, a fragment spanning nt 1,643–2,477 was amplified using primers B1 and C1 (Table I) under the following conditions: 94°C for 5 min; 30 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min; and finally 72°C for 10 min. Next, two parallel PCR reactions were carried out to detect the BCP and PC mutations using restriction fragment length polymorphism (RFLP). Although the T1762/A1764 double mutation does not create a novel restriction site, introduction of artificial A1767T mutation into the antisense primer B-Bc1 produces a *Bcl*I cleavage site (TGATCA) in conjunction with the double mutation [Amini-Bavil-Olyaee et al., 2005]. Similarly, due to the artificial G1888C/T1889C/G1890T mutations in the sense primer C-Xag, an *Xag*I cleavage site ($CCTN_5AGG$) was created in the presence of the A1896 [Xing et al., 2000]. The first-round PCR product (0.1 μl) was reamplified using B1/B-Bc1 primer pair (for BCP mutations) or C-Xag/C2 primer pair (for PC mutation) under the following conditions: 94°C for 5 min; 32 cycles of 94°C for 50 sec, 56°C for 1 min, and 72°C for 55 sec; a final 72°C for 10 min. The sensitivity of this nested PCR method was 100 copies/ml. All necessary precautions were observed to prevent cross-contamination, and negative controls were included in each assay. All the tests were performed in duplicate to confirm the results.

PCR product of the B1/B-Bc1 primer pair was digested with *Bcl*I and separated on 3% agarose gel. The 144-bp (uncleaved) and 120-bp (cleaved) bands correspond to the wild-type sequence and T1762/A1764 double mutant, respectively. The presence of both 144-bp and 120-bp fragments suggests a mixture of wild-type virus and the BCP mutant. PCR product of the C-Xag/C2 primer pair was digested with *Xag*I and also analyzed on 3% agarose gel. The 198-bp (uncleaved) and 176-bp (cleaved) bands correspond to wild-type virus and the A1896 mutant, respectively.

To confirm the presence of the BCP and PC mutations and the ratio between wild-type and mutant virus when a mixture was present, the first round PCR product of six samples was sequenced directly. The sequences were analyzed with software Vector NTI 9.0 and Chromas.

Statistical Analysis

Data were analyzed by: the Mann–Whitney rank sum test, the chi-square, Spearman correlation, Linear correlation and regression. A *P* value below 0.05 was considered statistically significant.

RESULTS

Prevalence of Genotypes B and C

A total of 207 patients were studied. Viral genotype was established by PCR and sequencing of the HBsAg gene (GenBank accession numbers: EU915485; EU 921790–EU921799; EU926420–EU926473; EU927150–EU927286), followed by phylogenetic analysis. Fifty-one isolates were classified into genotype B while the remaining 156 belonged to genotype C (Table II).

Prevalence of PC and BCP Mutations

The presence of PC mutation was determined by digestion of the nested PCR product with *Xag*I, which cleaves the A1896 mutant but not wild-type virus. As shown in the upper panel of Figure 1, samples contained a 198-bp band (wild-type), a 176-bp band (A1896 mutant), or both bands at various ratios. The wild-type was defined as dominant if the 198-bp band is stronger than the 176-bp band, and the A1896 mutant as dominant if the 176-bp band is stronger. In six samples, the presence of the wild-type, mutant, or mixed sequence was confirmed by direct sequencing of the first round PCR product (Fig. 1, lower panel and Fig. 3). Presence of the BCP mutation was established by digestion of the 144-bp semi-nested PCR product with *Bcl*I, which converts it into 120 bp if the T1762/A1764 double mutation is present. Again patterns of wild-type, mutant, and mixture at different ratios were observed (Fig. 2, top panel). A sample is considered as wild-type dominant if the 144-bp band is stronger than the 120-bp band. The result was confirmed by direct sequencing of the PCR product (Fig. 2, lower panel and Fig. 3).

The PC mutant was detectable in 43 samples (20.8%), with 19 of them showing dominance of the mutant species (Table III). In the remaining 24 samples, the wild-type was dominant. The BCP mutant was present in 103 samples (50%), of which 77 showed dominance of the

mutant (Table III). When both the PC and BCP mutations were considered (Table IV), the samples fall into four groups: wild-type sequence at both regions (74), presence of PC but no BCP mutant (30), presence of BCP but no PC mutant (90), presence of both PC and BCP mutants (13).

Comparison of HBeAg Titers and Mutation Patterns Between Genotypes B and C

Table II compares patients infected with genotypes B and C. There were no significant differences in serum ALT level and HBV DNA titer between the two genotypes. The mean HBeAg titer was slightly higher in genotype C than genotype B (197 vs. 189) but the difference was not statistically significant. Patients with genotype C isolates were older than those with genotype B isolates (mean age: 33.43 vs. 28.08; $P = 0.0002$) and had higher percentage of males (86%) than patients with genotype B (75%), although the difference did not reach statistical significance ($P = 0.083$). The genotype C isolates had a higher prevalence of BCP mutations but a lower prevalence of the PC mutation than genotype B ($P = 0.001$).

Correlation of HBeAg Titers With Patient Gender, Age, and Mutations in the BCP and PC Regions

Table III compares impact of factors other than viral genotype on HBeAg titer. The HBeAg titers did not differ between male and female patients, but were higher in younger patients (<35 years of age) in comparison to older patients (≥35 years) (206.9 ± 87.5 vs. 173.7 ± 101.9; $P = 0.015$). Since the prevalence of BCP and PC mutations differed in the younger and older patients, HBeAg titers were compared among patients infected with wild-type PC and BCP sequence using linear regression analysis. The regression coefficient between age and HBeAg titers was 1.651 (Fig. 4). Although the value did not reach statistical significance ($P = 0.078$), this is consistent with a negative correlation of age with HBeAg titers. No correlation was found for patients infected with the PC mutant/BCP WT sequence (Fig. 4). The HBeAg titers were lower in patients harboring the BCP mutant (whether alone or together with wild-type virus) than those harboring the pure wild-type sequence at this position (170.6 ± 96.1 vs. 219.9 ± 85.2; P = 0.001); of the former group, it was lower in those with dominance of the mutant than those with wild-type dominance (159.6 ± 95.2 vs. 209.1 ± 87; $P = 0.017$). The HBeAg titer was also lower in patients harboring the PC mutant than those with pure wild-type sequence at this position (170.6 ± 96.1 vs. 219.9 ± 85.2; $P = 0.001$). In those samples harboring the PC mutant, the mutant dominant group had lower HBeAg titer than the wild-type dominant group (126.2 ± 108.8 vs. 183 ± 101.3), although the difference did not reach statistical significance ($P = 0.084$).

The impact of dual mutations in both the BCP and PC regions versus single region mutation on HBeAg titers were compared. As shown in Table IV, the HBeAg titer was 192.2 ± 96.7 in those harboring the PC mutation alone, 185.6 ± 88.7 in those harboring the BCP muta-tions alone ($P = 0.552$), but only 78.92 ± 89.2 in those with mutations in both regions ($P < 0.001$). Therefore, the presence of mutant populations at both the precore and core promoter regions led to lower HBeAg titer than the presence of mutation in either region alone.

Correlation of HBeAg Titers With HBV DNA

Figure 5 shows the relationship between HBeAg titers and viral load in patients infected with the PC WT/BCP WT sequence ($P = 0.288$), the PC mutant/BCP WT ($P = 0.454$), and the BCP mutant/PC WT sequence ($P = 0.057$). There was no statistically significant correlation although the value was nearly significant for the BCP mutant/PC WT group.

DISCUSSION

Genotypes B and C are the dominant HBV genotypes in East Asia. Their distribution shows a North-to-South Gradient, with genotype C predominating in the north and B in the south [Orito et al., 2001a; Zeng et al., 2005]. In the present study of HBeAg positive patients from six hospitals in China, it was found that the prevalence of genotype C (75.4%) was higher than genotype B (24.6%). Consistent with the earlier reports that genotype C patients clear HBeAg about a decade later than genotype B patients [Chu et al., 2002b; Yuen et al., 2003], this study also showed that patients with genotype C were 5 years older than patients with genotype B. These HBeAg positive patients of genotype C infection also had a higher prevalence of the A1762T/G1764A BCP mutations but lower prevalence of the G1896A PC mutation, as have been reported by many other investigators [Orito et al., 2001b; Chu et al., 2003; Sumi et al., 2003]. At present, the preferential development of the BCP mutations in genotype C as opposed to PC mutation in genotype B remains unknown. C1858, which is found in majority of genotype A isolates and some isolates of genotypes C and F, precludes the emergence of 1896 mutation because of the base pairing restrictions of the encapsidation signal [Li et al., 1993; Lok et al., 1994; Arauz-Ruiz et al., 1997; Alestig et al., 2001]. Since most samples in this study were analyzed by restriction fragment length polymorphism, the prevalence of C1858 variant in our genotype C isolates is unknown. On the other hand, the serum HBV DNA titer did not differ between patients infected with these two genotypes.

Despite the importance of the presence of HBeAg/anti-HBe in the pathogenesis, few studies have attempted to measure HBeAg quantitatively. In the present quantitative assay of HBeAg, no difference in HBeAg titers was found between patients infected with genotype B and C. However, the titers were higher in patients <35 years of age than those >35 years, which is consistent with age-dependent decline and loss of HBeAg expression. Importantly, presence of either precore or core promoter mutations was associated with reduced HBeAg titer. It is possible that this reduction of HBeAg titer is caused by immune clearance that reduces viral load and also provides a selection force for the precore and core promoter mutants. If this is true, HBV DNA replication should also decline in patients harboring such mutants. However, no difference was found in the titers of HBV DNA between patients with the wild-type or mutated precore or core promoter sequence. Thus, the reduced HBeAg titer is more likely a direct consequence of precore and core promoter mutations, which are known to reduce and abolish HBeAg expression, respectively [Carman et al., 1989; Blum et al., 1991; Tong et al., 1991; Buckwold et al., 1996; Moriyama et al., 1996; Parekh et al., 2003]. The reason why patients harboring the G1896A precore mutation continued to express HBeAg was the co-existence of wild-type virus (the mutant was never detected as a pure population in any of our patients). In fact patients with mutant precore sequence as the dominant species tended to have lower HBeAg titer than those with predominantly wild-type

sequence, although the difference was not statistically significant probably due to small sample size (Table III). The coexistence of mutant and wild-type sequence also explains why presence of both precore and core promoter mutations led to a further reduction in HBeAg titer than presence of either mutation alone: these mutations probably were present in separate molecules and had additive effects. This issue could not be addressed in the present study because the second round of PCR did not amplify the two regions as a single DNA fragment.

In conclusion, the HBeAg titer correlated with the age of the patients and precore/core promoter mutations, but not with the viral genotype. Follow-up studies will establish whether patients with a lower HBeAg titer will seroconvert to anti-HBe sooner than those with a higher HBeAg titer. Indeed, a recent study demonstrated that patients with a lower HBeAg titer were more likely to lose HBeAg during interferon therapy [Fried et al., 2008].

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Abbreviations used:

ALT	alanine aminotransferase
HBeAg	hepatitis B e antigen
HBsAg	hepatitis B surface antigen
BCP	basal core promoter
HBV	hepatitis B virus
PCR	polymerase chain reaction
PC	precore
RFLP	restriction fragment length polymorphism
ULN	upper limit of normal

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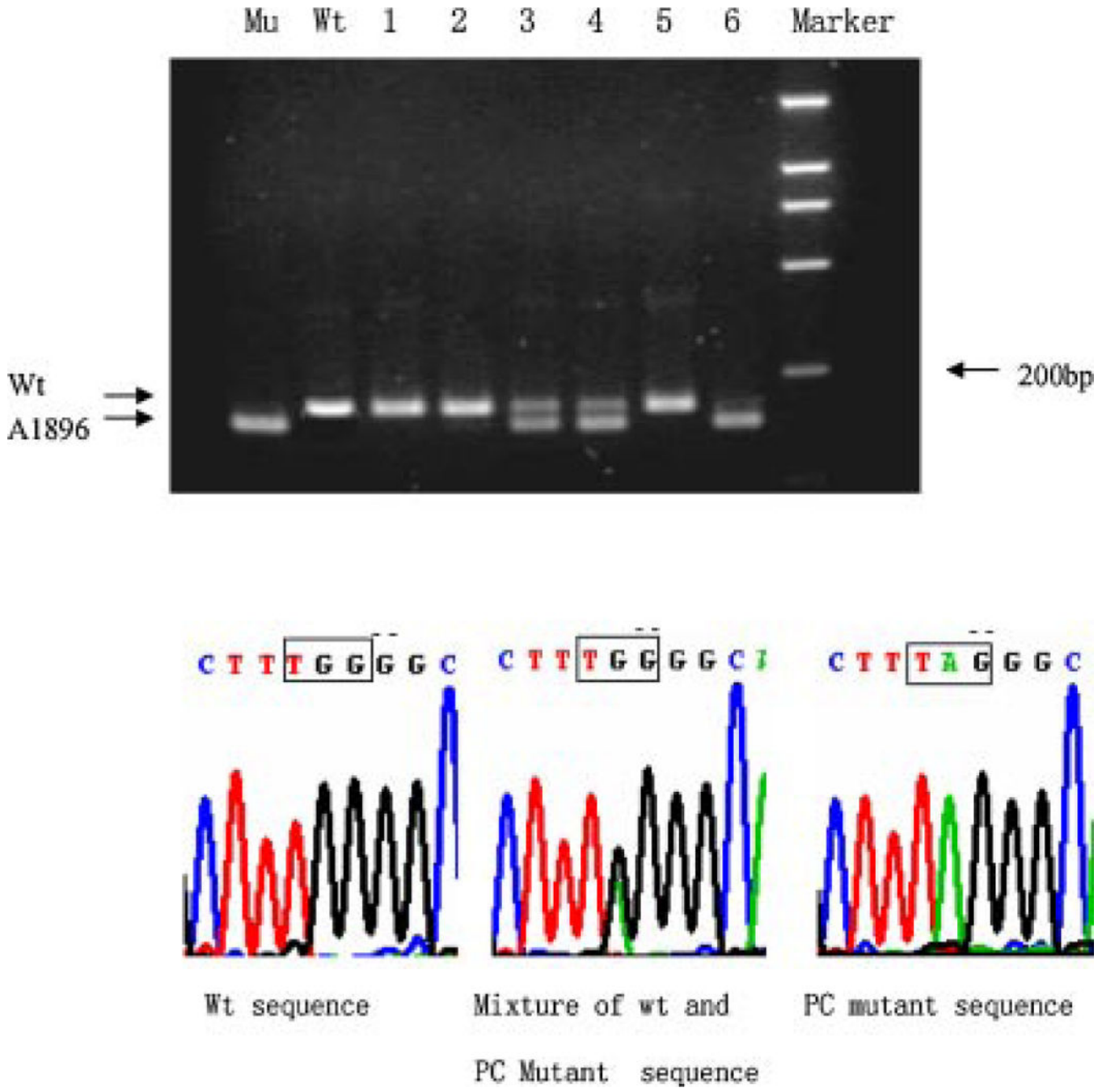

Fig. 1.

Detection of G1896A precore mutation by RFLP assay and direct sequencing. For the RFLP assay (**top panel**), the second round PCR product was digested with XagI and separated in 3% agarose gel. Positions of the wild-type band and A1896 band are indicated. Marker, 2,000-bp molecular size markers; Mu, control of PC mutant; Wt, control of PC wild-type; 1–6, digested DNA from six samples. For direct sequencing (**lower panels**), the first round PCR product was used. The three panels correspond to samples number 1 (**left panel**), 3 (**middle panel**), and 6 (**right panel**).

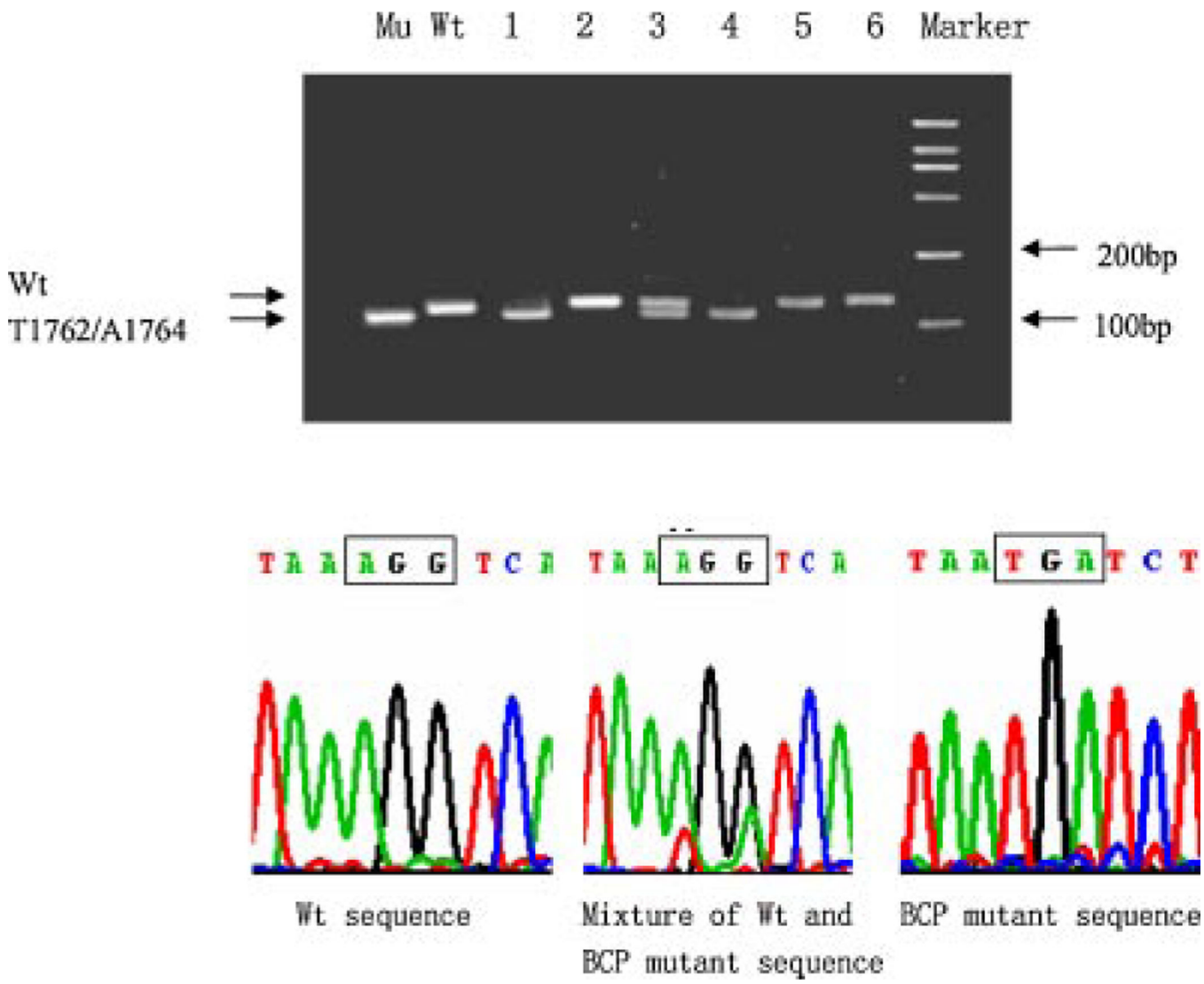

Fig. 2.

Detection of T1762/G1764 BCP mutations by RFLP assay and direct sequencing. The six samples analyzed here are identical to those shown in Figure 1. For the RFLP assay (**top panel**), the second round PCR product was digested with BclI and separated in 3% agarose gel. Positions of the wild-type band and mutant band are indicated. Marker, 2,000-bp molecular size markers; Mu, control of BCP mutant; Wt, control of BCP wild-type; 1–6, digested DNA from the six samples. For direct sequencing (lower panels), the first round PCR product was used. The three panels correspond to samples number 2 (**left panel**),3 (**middle panel**), and 4 (**right panel**).

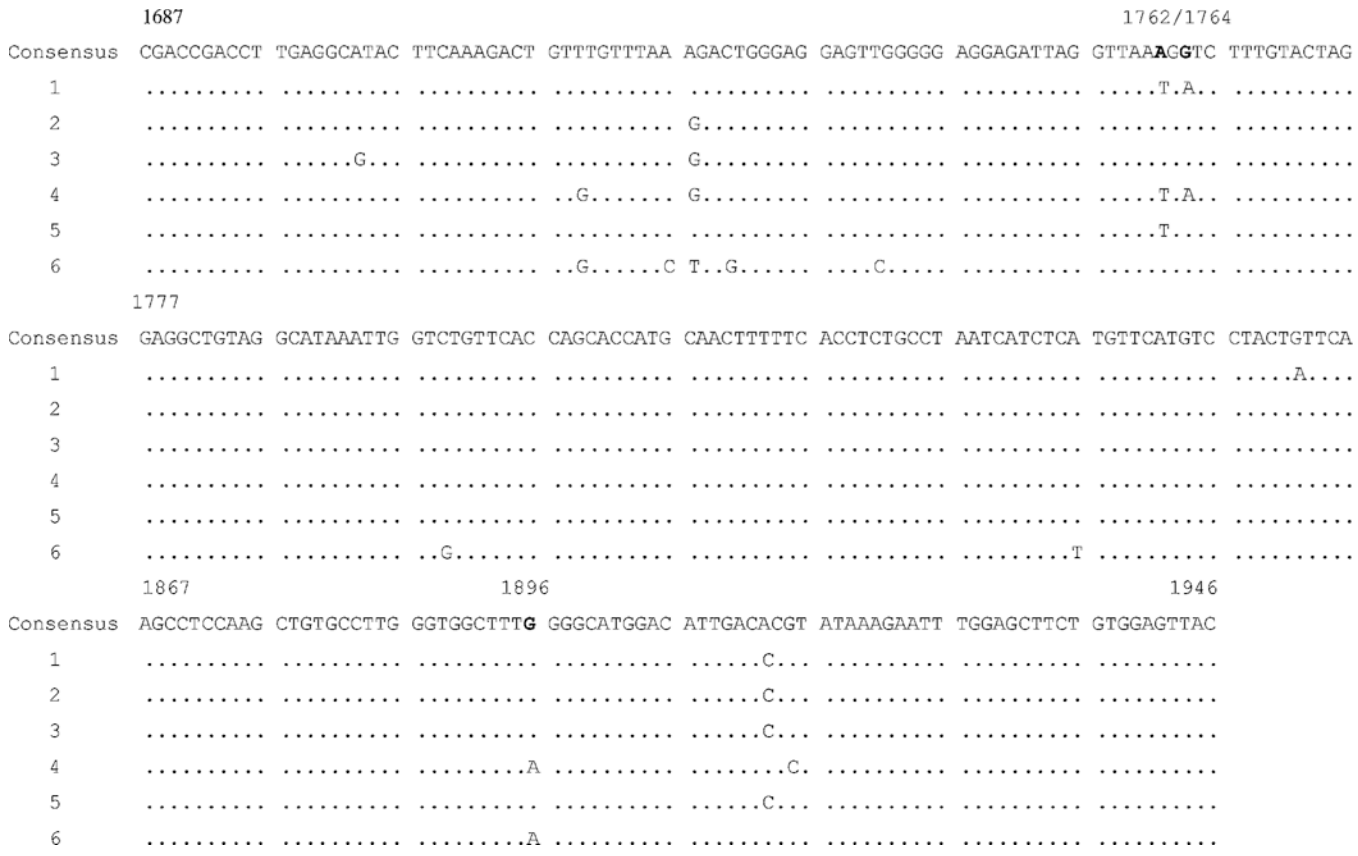

Fig. 3.

Sequence alignment of the six samples shown in Figures 1 and 2. The first round PCR product was sequenced directly. Shown here are nucleotides 1,687–1,946 covering both basal core promoter and precore regions.

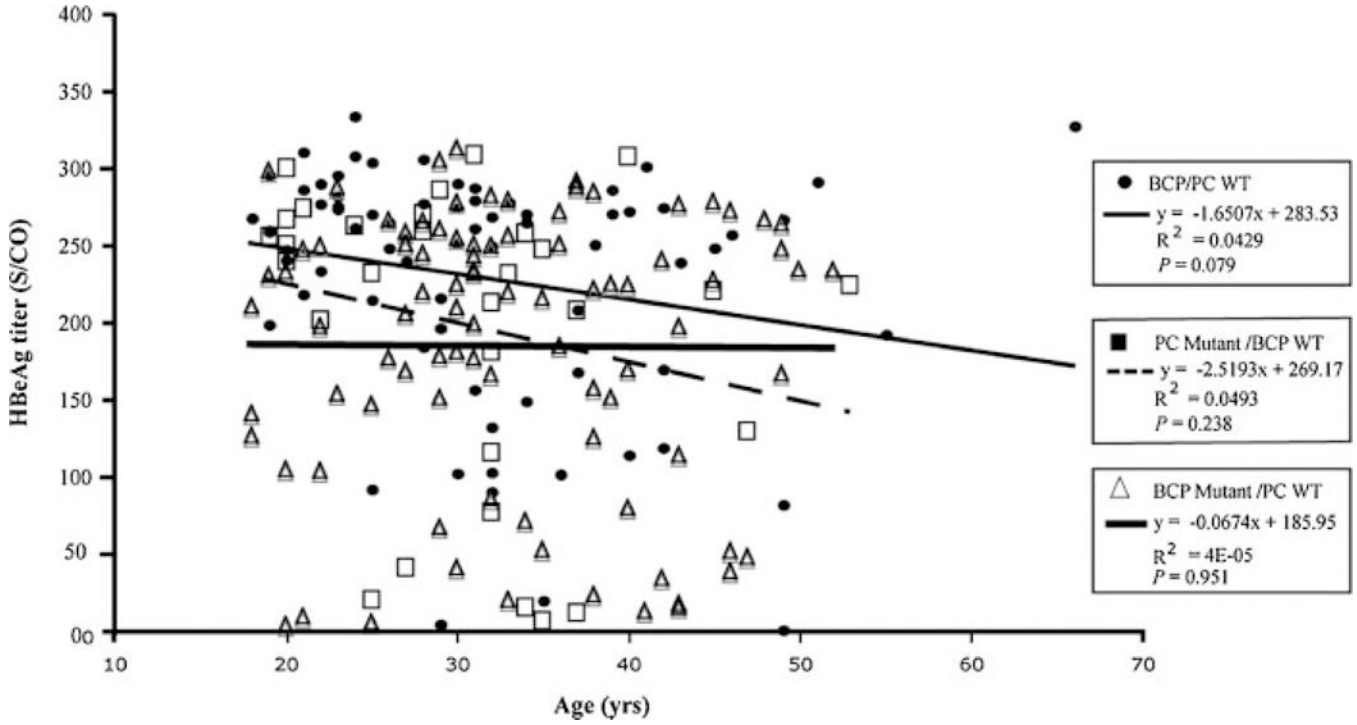

Fig. 4.

Correlation of HBeAg titers with age in patients infected with BCP/PC WT sequence (closed circle), PC mutant/BCP WT (solid square), and BCP mutant/PC WT (open triangle).

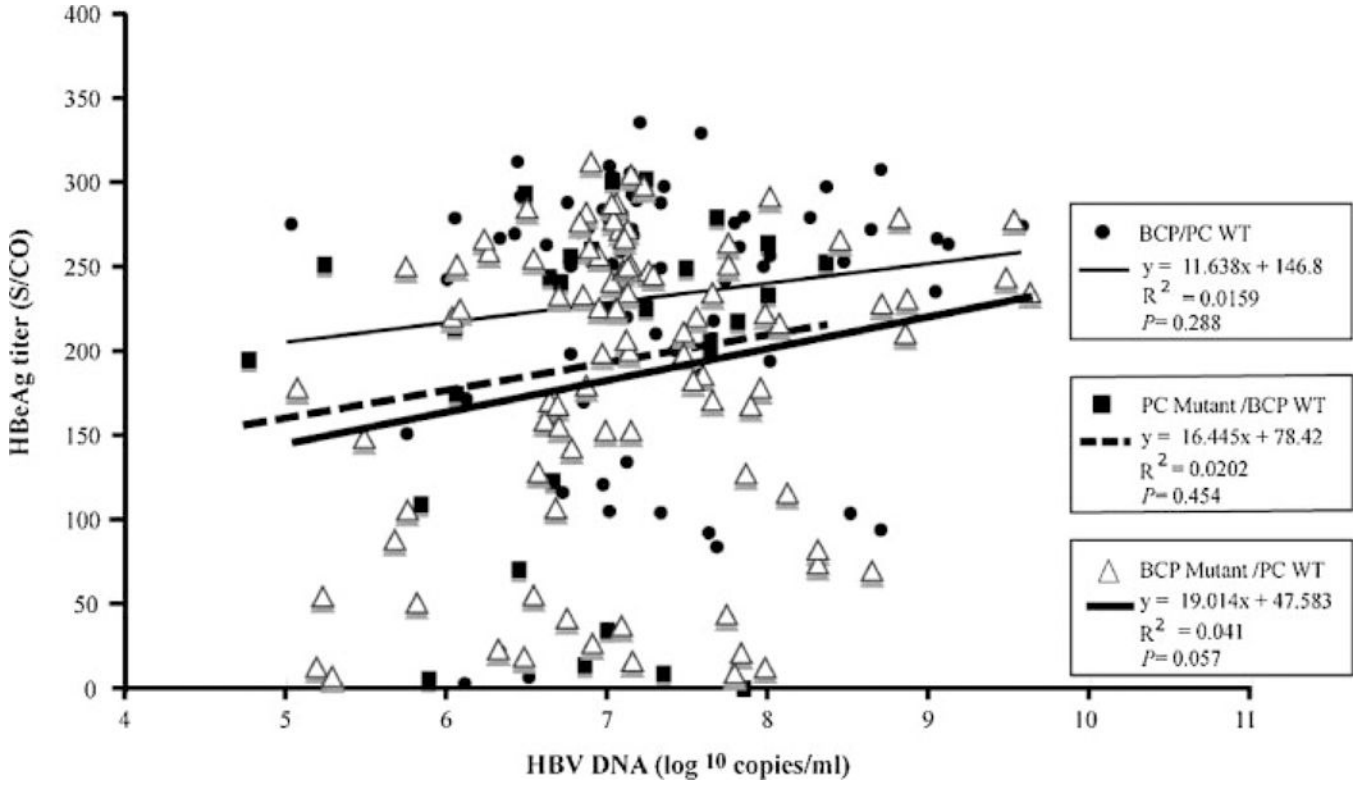

Fig. 5.

Correlation of the HBeAg titers with HBV DNA levels in patients infected with BCP/PC WT sequence (closed circle), PC mutant/BCP WT (solid square), and BCP mutant/PC WT (open triangle).

TABLE I.

Primers Used in This Study and Their Locations on the HBV Genome

Primer	Sequence (5′–3′)	Location	Polarity	Purpose
S1	GTC ACC ATA TTC TTG GGA AC	2,816 –2,835	Forward	S gene PCR for genotyping
AS	CAT ATC CCA TGA AGT TAA GG	886 –867	Reverse	S gene PCR for genotyping
B1	CAA GGT CTT GCA TAA GA GGA CT	1,643 –1,664	Forward	1st PCR for BCP and PC mutations; 2nd PCR for BCP mutations
C1	CCC CAC CTT ATG AGT CCA AG	2,477 –2,458	Reverse	1st PCR for BCP and PC mutations
C-Xag	CCT CCT AGC TGT GCC TTG GCC TGC TTT	1,869 –1,895	Forward	2nd PCR for PC mutation
C2	TGA GAG CAG TAT GGT GAG GTG AAC AAT G	2,066 –2,039	Reverse	2nd PCR for PC mutation
B-Bcl	CTA CAG CCT CCT AGT ACA ATG A	1,786 –1,765	Reverse	2nd PCR for BCP mutations

Boldface: mismatches in the C-Xag and B-Bcl primers to introduce the XagI and BclI sites for PC and BCP mutants, respectively.

TABLE II.

Comparison of Clinical and Virological Features Between Genotype B and Genotype C Patients

Factor	Total (n = 207)	Genotype B (n = 51)	Genotype C (n = 156)	*P*-value
Gender (M/F)	173/34 ± (1/0.2)	38/13 (1/0.34)	135/21 (1/0.16)	0.083
Age (years)[a]	32.2 ± 9.2	28.08 ± 7.77	33.43 ± 9.16	0.0002
ALT (U/L)[a]	176.6 ± 86.5	177 ± 100	177 ± 82	0.987
HBeAg titer (S/CO)[a]	195.2 ± 98.9	189 ± 99	197 ± 92	0.591
HBV DNA titer[b]	7.15 ± 0.89	7.08 ±1.15	7.17 ± 0.81	0.532
T1762/A1764	103	14/51 (27.5%)	89/135 (65.9%)	0.001
A1896	43	19/51 (37.2%)	24/135 (17.8%)	0.001
T1762/A1764/A1896	13	2/51 (3.9%)	11/135 (8.1%)	0.527

[a]Expressed as X ± SD.

[b]$\log_{10}$ copies/ml.

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TABLE III.

HBeAg Titers in Relationship to Gender, Age, as Well as BCP and PC Mutations

Factor	Subgroup	Number	HBeAg titer (S/CO)[a]	*P*-value	HBV DNA[b]	*P*-value
Sex	Male	173	194.2 ± 94.5		7.20 ± 0.90	
	Female	34	200.1 ± 92.2	0.743	6.93 ± 0.89	0.125
Age (years)	<35	134	206.9 ± 87.5		7.14 ± 0.93	
	≥35	73	173.7 ± 101.9	0.015	7.18 ± 0.86	0.736
BCP mutations	Pure wild-type	104	219.9 ± 85.2		7.16 ± 0.84	
	With A1762T/G1764A mutants	103	170.6 ± 96.1	0.0001	7.13 ± 0.94	0.85
	Wild-type dominant	26	209.1 ± 87		7.03 ± 0.87	
	BCP mutant dominant	77	159.6 ± 95.2	0.017	7.17 ± 0.97	0.64
PC mutation	Pure wild-type	164	204.9 ± 87.9		7.20 ± 0.88	
	With G1896A mutants	43	157.9 ± 104.3	0.003	6.99 ± 0.89	0.095
	Wild-type dominant	24	183.0 ± 101.3		7.15 ± 0.84	
	PC mutants dominant	19	126.2 ± 108.8	0.084	6.70 ± 0.91	0.098

[a] Expressed as X ± SD.

[b] $\log_{10}$ copies/ml.

TABLE IV.

Impact of Combined BCP and PC Mutations on HBeAg Titers

Group	Sequence	Number	HBeAg titer (S/CO)[a]	HBV DNA[b]
A	Wild-type	74	228.5 ± 81.4	7.27 ± 0.84
B	PC mutation alone	30	192.2 ± 96.7	6.91 ± 0.83
C	BCP mutations alone	90	185.6 ± 88.7	7.16 ± 0.95
D	BCP + PC mutations	13	78.9 ± 89.2	7.03 ± 1.04

P values (HBeAg titers): A/B, 0.019; A/C, 0.0001; A/D, 0.0000; B/C, 0.552; B/D, 0.0008; C/D, 0.0003.

P values (HBV DNA): A/B, 0.132; A/C, 0.446; A/D, 0.363; B/C, 0.326; B/D, 0.915; C/D, 0.627.

[a] Expressed as X ± SD.

[b] $\log_{10}$ copies/ml