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Precore/core promoter mutations and hepatitis B virus genotype in hepatitis B and C dually infected patients treated with interferon-based therapy

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ABSTRACT

We studied the prevalence and distribution of precore/basal core promoter (BCP) mutations and hepatitis B virus (HBV) genotypes in HBV/hepatitis C virus (HCV) dually-infected patients, and evaluated their impact on long-term HBV response of interferon (IFN)-based therapy. The HBV genotypes and sequences of the precore/BCP regions were determined in 180 HBV/HCV dually-infected patients and were compared with 90 age, sex and hepatitis B e antigen-matched chronic hepatitis B controls. Serum HBV DNA and hepatitis B surface antigen (HBsAg) were assessed every 3–6 months after therapy with IFN or pegylated-IFN plus ribavirin in 135 dually-infected patients with active hepatitis C. Dually-infected patients had a higher prevalence of genotype C HBV ($P = 0.022$) and a lower frequency of G1896A mutation ($P = 0.004$) as compared with controls. Among dually-infected patients, genotype C was associated with a higher frequency of A1762T/G1764A mutation ($P < 0.001$), but with lower HBV DNA ($P < 0.001$) and a lower frequency of A1752T/G ($P = 0.008$), C1799G ($P < 0.001$) and G1896A mutation ($P < 0.001$) than genotype B. Based on Cox proportional hazards model, young age (hazard ratio (HR) = 0.952, $P = 0.001$), sustained virological response to HCV (HR = 4.638, $P = 0.044$), C1766T mutation (HR = 5.216, $P = 0.003$) and A1846T mutation (HR = 2.332, $P = 0.031$) correlated with HBV DNA reactivation ($\geqslant$2000 IU/ml) after therapy. Age (HR = 1.068, $P = 0.020$), G1896A mutation (HR = 0.140, $P = 0.01$) and A1846T mutation (HR = 0.086, $P = 0.018$) were associated with HBsAg seroclearance independently. In conclusion, specific mutations in the precore/BCP regions could be useful in predicting long-term HBV response in HBV/HCV dually-infected patients treated with IFN-based therapy.

1. Introduction

Hepatitis B virus (HBV) and hepatitis C virus (HCV) infections account for the major causes of chronic liver disease worldwide (Lee, 1997; World Health Organization, 2002). Both viruses are transmitted by exposure to infected blood, thus it is not unusual to find patients dually infected with HBV and HCV, especially in areas endemic for both viruses (Liaw, 1995; Lee et al., 1999; Zarski et al., 1998). Previous studies have shown that patients with dual infection carry a significantly higher risk of developing cirrhosis or hepatocellular carcinoma (HCC) than those with either infection alone (Chu et al., 1994; Donato et al., 1998; Hung et al., 2010; Zarski et al., 1998).

Although there is no standard of care for HBV/HCV dually-infected patients, several studies have confirmed that co-existing HBV infection does not influence the clearance of HCV in dually-infected patients with active hepatitis C receiving interferon (IFN) or pegylated-IFN plus ribavirin therapy (Chuang et al., 2005; Hung et al., 2005, 2011; Liu et al., 2003; Liu et al., 2009; Raimondo et al., 2006). Recently, we have demonstrated that sustained HCV clearance by IFN-based therapy may significantly reduce HCC in HBV/HCV dually-infected patients (Hung et al., 2011). However, the treatment might alter the dominant hepatitis virus with subsequent HBV reactivation (Chuang et al., 2005; Hung et al., 2011), which could decrease the benefit of sustained virological response (SVR) to HCV (Hung et al., 2011). By contrast, pegylated-IFN plus ribavirin therapy could promote hepatitis B surface antigen (HBsAg) seroclearance in HBV/HCV dually-infected patients (Hung et al., 2011; Liu et al., 2009; Yu et al., 2010), which could reach 11.2% 24 weeks after the end of treatment (Liu et al., 2009). HBsAg

Abbreviations: AST, aspartate aminotransferase; ALT, alanine aminotransferase; BCP, basal core promoter; CI, confidence interval; HR, hazard ratio; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCV, hepatitis C virus; HCC, hepatocellular carcinoma; IFN, interferon; IU, international unit; PCR, polymerase chain reaction; SVR, sustained virological response.

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seroclearance usually confers a favorable outcome and is the optimal treatment goal for chronic HBV infection. To date, the factors associated with long-term HBV response such as HBV reactivation and HBsAg seroclearance in HBV/HCV dually-infected patients treated with IFN-based therapy have not been investigated.

Patients dually infected with HBV and HCV usually present with a lower level of serum HBV DNA and negative hepatitis B e antigen (HBeAg) compared to those with HBV infection alone (Chuang et al., 2005; Hung et al., 2005, 2011; Liaw, 1995; Liu et al., 2003, 2009; Raimondo et al., 2006; Yu et al., 2010). Previous studies have shown that mutations in the precore and basal core promoter (BCP) regions of the HBV genome are frequently observed in patients with HBeAg-negative chronic hepatitis B. The most common precore variant has a G to A substitution at nucleotide 1896 (G1896A), which prevents the production of HBeAg by introducing a premature stop codon into the open reading frame of the precore region (Carman et al., 1989; Okamoto et al., 1990). Longitudinal studies have shown that G1896A emerges or is selected around the time of HBeAg seroconversion, and high precore mutant ratios have been associated with persistent hepatitis after HBe seroconversion (Brunetto et al., 1991; Hamasaki et al., 1994). The most frequent BCP mutation is a double mutation involving an A to T substitution at nucleotide 1762 and a G to A substitution at nucleotide 1764 (A1762T/G1764A), which results in a substantial decrease in HBeAg expression but enhances viral genome replication in vitro (Buckwold et al., 1996; Kurosaki et al., 1996; Okamoto et al., 1994). So far, the prevalence and the influence of these virological mutants in HBV/HCV dual infection have remained unclear.

The impact of HBV genotypes on the clinical outcome of chronic HBV infection has been clarified (Chu et al., 2002a; Kao et al., 2000; Lee et al., 2003). HBV has been classified into eight genotypic groups (A–H) based on genome sequence divergence with distinct geographical distribution (Norder et al., 1992; Okamoto et al., 1998; Stuyver et al., 2000). Genotypes B and C HBV are the most predominant variants in Asia (Kao et al., 2000; Lee et al., 2003). It has been reported that genotype C HBV is associated with higher levels of HBV DNA replication, more advanced liver disease, and a decreased rate of response to IFN therapy compared with genotype B (Kao et al., 2000; Lee et al., 2003; Lin and Kao, 2011).

In this study, we attempted to investigate the prevalence and implications of precore/BCP mutations and HBV genotypes in dual HBV/HCV infection. This longitudinal study also evaluated the role of precore/BCP mutations and HBV genotypes in HBV reactivation and HBsAg seroclearance among dually-infected patients after IFN-based therapy.

2. Patients and methods

2.1. Patients

From January 1999 to December 2007, 180 patients with chronic HBV/HCV dual infection in a single medical center were enrolled in this study. Dual infection was defined as seropositivity for both antibody to HCV (anti-HCV) and HBsAg for more than 6 months. Patients with positive antibody to hepatitis D virus (HDV) or human immunodeficiency virus were excluded. Pathologic diagnoses were performed by percutaneous liver biopsies ($n = 136$), according to a modified Knodell histology index (Desmet et al., 1994). Fibrosis score 4 was defined as cirrhosis (Desmet et al., 1994). Clinical diagnosis of cirrhosis was based on repeated ultrasound findings suggestive of cirrhosis at least twice 3 months apart (Hung et al., 2003), supplemented with clinical criteria or other signs of portal hypertension. During the same period, 90 age, sex and HBeAg-matched controls with chronic hepatitis B were selected randomly from our database (Chen et al., 2005, 2007a). This study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institutional human research committee.

Of the 180 dually-infected patients, 135 subjects received IFN-based therapy. The exclusion criteria for antiviral therapy included decompensated liver disease, alcohol abuse, and major contraindications to IFN or ribavirin therapy. Patients were treated with pegylated-IFN alfa-2a (Pegasys, F. Hoffmann-La Roche, Basel, Switzerland; 180 μg/week subcutaneously) ($n = 54$), pegylated-IFN alfa-2b (Peg-Intron, Schering-Plough Corporation, Kenilworth, NJ; 1–1.5 μg/kg/week subcutaneously) ($n = 19$), or IFN alfa-2b (Intron-A, Schering-Plough Corporation, Kenilworth, NJ; 3 or 5 million units subcutaneously thrice weekly) ($n = 62$) and oral ribavirin daily for 12 to 48 weeks. Ribavirin was given at a total daily dose of 1000 mg for patients who weighed 75 kg or less and 1200 mg for patients who weighed more than 75 kg.

Serum HCV RNA was assessed at the end of treatment and 24 weeks after discontinuation of therapy. SVR was defined as undetectable serum HCV RNA at week 24 posttreatment. Serum HBV DNA and HBsAg status were assessed every 3 to 6 months after therapy. Reactivation of HBV DNA was defined as $\geqslant$2000 international units (IU)/ml since the week 24 posttreatment. In addition, other end points of HBV DNA reactivation including $\geqslant$60 IU/ml, $\geqslant$20,000 IU/ml and $\geqslant$2000 IU/ml twice or more were also examined. For each patient, study entry began at the treatment initiation.

2.2. Serological and virological assays

HBsAg, HBeAg, antibody to HBeAg (anti-HBe) and anti-HDV were assayed using commercially available enzyme immunoassay kits (Abbott Laboratories, North Chicago, IL). Serum HBV DNA was quantified with a sensitive polymerase chain reaction (PCR) assay (COBAS Amplicor HBV Monitor, Roche Diagnostics), with a detection limit of 200 copies/ml (60 IU/ml). Dilution was performed if HBV DNA levels were more than 10^6 copies/ml.

Anti-HCV was assessed using third generation ELISA (Ax SYM HCV 3.0, Abbott Laboratories, Chicago, IL). Qualitative detection of HCV RNA was performed by a standardized qualitative reverse transcription-polymerase chain reaction (RT-PCR) assay (Amplicor™, Roche Diagnostics, Branchburg, NJ), using biotinylated primers for the 5′ noncoding region. The lowest detection limit of this assay was 100 copies/ml (50 IU/ml). Serum HCV RNA levels were determined by a branched-DNA (b-DNA) signal amplification assay (VERSANT HCV RNA 3.0. Assay, Bayer Diagnostics, Emeryville, CA). This assay was a sandwich nucleic-acid hybridization procedure with a detectable limit of 3400 copies/ml. Genotyping of HCV was performed by reverse hybridization assay (Inno-LiPA™ HCV II; Innogenetics N.V., Gent, Belgium) using the HCV-Amplicor products.

2.3. HBV genotyping and sequencing of the precore and BCP regions

HBV genotypes were determined using restriction fragment length polymorphism on the surface-gene sequence, amplified by PCR with nested primers, as described previously (Mizokami et al., 1999). The precore and BCP sequences in sera were determined using nested PCR and direct sequencing, as described previously (Chen et al., 2007a). DNA was extracted from 100 μL serum using the QIAamp DNA Mini kit (Qiagen Inc, Hilden, Germany) according to the Manufacturer's recommendations. First-round PCR was performed on 5 μL of DNA extract in a 50 μL reaction mix containing 10× buffer (100 mM Tris–HCl, pH 9.0, 500 mM KCl, 15 mM $MgCl_2$, and 1% Triton X-100), 2.5 mM dNTP, 1U Taq polymerase and 20 μM of external primers. PCR was performed

as follows: 96 °C for 2 min; 94 °C for 1 min, 54 °C for 1 min, and 72 °C for 2 min, for 36 cycles; and finally 72 °C for 10 min. For the second-round PCR, 1 μL of first-round PCR product was re-amplified under the same condition of the first-round reaction, except that internal primers were used. The sensitivity of this method was 100 copies/ml. All necessary precautions to prevent cross-contamination were taken, and negative controls were included in each assay. The nucleotide sequences of the amplified products were directly determined by using fluorescent-labeled primers with an ABIPRISM™ 377 Genetic Analyzer (Applied Biosystems, Foster City, CA).

2.4. Statistical analysis

Continuous data are expressed as median (interquartile range), and the categorical data are expressed as number (percentage). Comparisons of differences in categorical data between groups were performed using the chi-square test. Distributions of continuous variables were analyzed by the Mann–Whitney *U* test for the two groups. Kaplan–Meier curves were generated for the cumulative incidences of HBsAg seroclearance and HBV DNA reactivation. The differences between groups were determined with the log-rank test. Cox proportional hazards regression analysis was performed to examine the independent factors for HBsAg seroclearance and HBV DNA reactivation. All analyses were carried out using SPSS software version 15.0 (SPSS, Inc., Chicago, IL). A *P* value below 0.05 was considered to be statistically significant.

3. Results

3.1. Baseline characteristics

The clinical and virological features of the 180 HBV/HCV dually-infected patients are shown in Table 1. There were 107 men and 73 women, with a median age of 54.6 years (range: 20–80 years). Sixty-four patients had cirrhosis diagnosed by biopsy histology ($n = 45$) or clinical criteria ($n = 19$). Five patients (2.8%) were found to be positive for HBeAg and negative for anti-HBe. The genotype distribution of HBV was B in 70 (39%), C in 73 (41%) and non-classified in 37 (20%) patients due to undetectable viral genomes or too weak signals of PCR products for further genotyping. Of these patients, precore and BCP regions could be successfully sequenced in 134 (74%) patients.

There were no significant differences in age, gender, body mass index, rate of cirrhosis, platelet count, HCV load and HCV genotype between HBV genotype B and C dually-infected patients. However, genotype C HBV patients had higher serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels, but had a lower rate of positive HBV DNA (>60 IU/ml) ($P < 0.001$) than genotype B subjects. As regards to precore and BCP mutation, genotype C HBV had a higher frequency of A1762T/G1764A mutation ($P < 0.001$), but had a lower frequency of A1752TG ($P = 0.008$), C1799G ($P < 0.001$) and G1896A mutation ($P < 0.001$) than genotype B (Fig. 1).

A higher serum HBV DNA level ($\geqslant$2000 IU/ml) was found to be correlated with C1766T ($P < 0.001$), T1768A ($P = 0.006$), G1896A ($P = 0.019$) and G1899A mutation ($P = 0.019$), whereas there was no significant association between serum HBV DNA level and A1726C ($P = 0.446$), A1752TG ($P = 0.382$), A1762T/G1764A ($P = 0.589$), C1799G ($P = 0.554$) and A1846T mutation ($P = 0.287$).

Comparison of HBV genotypes and precore/BCP mutations between HBV/HCV dually-infected patients and age, sex and HBeAg-matched chronic hepatitis B controls.

As shown in Table 2, there was no significant difference with respect to age, sex and HBeAg status between these two groups. Dually-infected patients had higher serum AST and ALT levels, but had lower mean HBV DNA levels than those with chronic hepatitis B ($P < 0.001$). Dually-infected patients had a higher prevalence of genotype C HBV ($P = 0.022$) and a lower frequency of G1896A mutation ($P = 0.004$) as compared with chronic hepatitis B controls.

Fig. 1 shows the comparison of precore and BCP mutation rates between HBV genotype B and C in dual HBV/HCV infection and single HBV infection. Among patients with single HBV infection, genotype C HBV had a higher frequency of A1762T/G1764A mutation ($P = 0.008$), but had a lower frequency of A1752TG ($P = 0.030$), C1766T ($P = 0.002$) and C1799G mutation ($P < 0.001$) than genotype B. In subgroup analysis for HBV genotype B patients, there were no differences in precore and BCP mutation rates between dual infection and single HBV infection. While in HBV genotype C patients, dually-infected patients had a lower frequency of A1762T/G1764A ($P = 0.005$) and G1896A mutation ($P = 0.009$) than those with single HBV infection.

Table 1
Clinical and virological characteristics of chronic HBV and HCV dually-infected patients between different HBV genotypes.

	Total	HBV genotypes		
	($n = 180$)	B ($n = 70$)	C ($n = 73$)	NC ($n = 37$)
Age (years)	54.6 (46.6–61.7)	55.9 (49.9–62.2)	55.1 (45.5–62.6)	51.0 (44.4–59.5)
Male gender (%)	107 (59%)	42 (60%)	41 (57%)	24 (65%)
BMI (kg/m^2)	24.0 (21.9–26.5)	24.9 (21.8–27.0)	23.6 (21.6–26.2)	23.4 (22.6–25.9)
Cirrhosis	64 (36%)	25 (36%)	31 (43%)[c]	8 (22%)[c]
AST (U/L)	78 (46–116)	72 (39–105)	93 (43–130)	78 (47–122)
ALT (U/L)	109 (63–182)	87 (52–140)[a,b]	129 (99–190)[a]	138 (90–220)[b]
Platelet (10^4/μL)	15.3 (10.9–19.1)	15.9 (11.0–19.2)	13.8 (10.1–18.0)[c]	17.3 (12.7–19.8)[c]
HBeAg+ve (%)	5 (3%)	2 (3%)	3 (4%)	0 (0%)
HBV DNA (IU/ml)				
$\geqslant$2000 (%)	29 (16%)	16 (23%)	10 (14%)	3 (8%)
60–2000 (%)	50 (28%)	32 (46%)[b]	15 (21%)	3 (8%)[b]
<60 (%)	101 (56%)	22 (31%)[a,b]	48 (66%)[a]	31 (84%)[b]
Precore/BCP sequence available (%)	134 (74%)	60 (86%)[a,b]	71 (97%)[a,c]	3 (8%)[b,c]
HCV RNA+ (%)	171 (95%)	64 (91%)	70 (96%)	37 (100%)
Log HCV RNA (copies/ml)	5.7 (4.5–6.4)	5.8 (4.3–6.5)	5.5 (4.5–6.3)	5.9 (4.8–6.4)
HCV genotype (1/non-1)	82/78	28/32	34/31	20/15

Data were presented as median (interquartile range); *P*-value by Mann–Whitney *U* test or x^2 test.
Abbreviations: HBV, hepatitis B virus; NC, non-classified; BMI, body mass index; AST, aspartate transaminase; ALT, alanine transaminase; HBeAg, hepatitis B e antigen; BCP, basal core promoter; HCV, hepatitis C virus.
[a] $P < 0.05$ between genotype B and C.
[b] $P < 0.05$ between genotype B and NC.
[c] $P < 0.05$ between genotype C and NC.

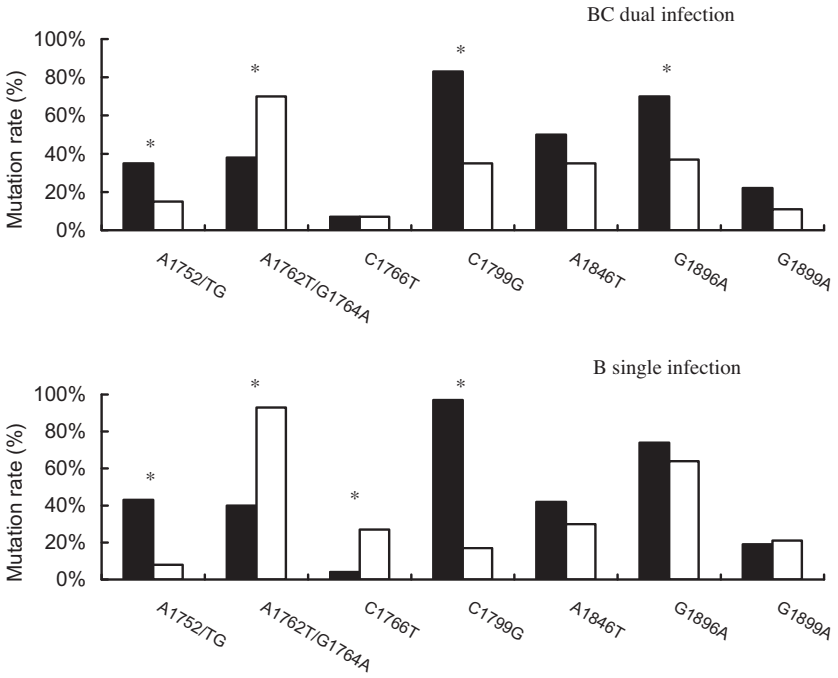

Fig. 1. Comparison of prevalence of precore and basal core promoter mutations between HBV genotype B and C in dual HBV/HCV infection and single HBV infection, respectively (black column: genotype B; white column: genotype C; $^{*}P < 0.05$ between genotype B and C).

Table 2
Comparison of clinical features, HBV genotypes and precore/BCP mutations between HBV/HCV dually-infected patients and age-, sex- and HBeAg-matched chronic hepatitis B controls.

	Dual infection ($n = 180$)	Chronic hepatitis B ($n = 90$)	P-value
Age (years)	54.6 (46.6–61.7)	52.0 (48.0–56.0)	0.176
Male gender (%)	107 (59%)	54 (60%)	0.519
AST (U/L)	78 (46–116)	41 (29–94)	0.001
ALT (U/L)	109 (63–182)	54 (26–119)	<0.001
HBeAg + ve (%)	5 (3%)	2 (2%)	0.562
Log HBV DNA (copies/ml)	2.3 (2.3–3.6)	5.6 (4.8–6.4)	<0.001
HBV genotype (B/C)	70/73	57/33	0.022
A1752T/G	32 (24%)	30 (33%)	0.155
A1762T/G1764A	75 (56%)	54 (60%)	0.323
C1766T and/or T1768A	13 (10%)	11 (12%)	0.350
A1846T	55 (41%)	34 (38%)	0.364
G1896A	69 (51%)	63 (70%)	0.004
G1899A	21 (16%)	18 (20%)	0.254

Data were presented as median (interquartile range); P-value by Mann–Whitney U test or x^2 test.
Abbreviations: HBV, hepatitis B virus; BCP, basal core promoter; HBeAg, hepatitis B e antigen; AST, aspartate transaminase; ALT, alanine transaminase.

3.2. Risk factors for HBV DNA reactivation in dually-infected patients receiving IFN or pegylated-IFN plus ribavirin therapy

Of the 135 dually-infected patients receiving IFN-based therapy, 96 (71%) subjects achieved HCV-SVR, which was durable until the end of follow-up in each individual. Two of the five patients with baseline HBeAg positivity had achieved HBeAg seroconversion at the end of follow-up.

As shown in Fig. 1A, 37 (27%) patients had HBV DNA reactivation ($\geqslant$2000 IU/ml) after a median follow-up of 3.3 years (interquartile range, 1.7–5.7). Of them, 4 HBeAg negative patients (all HCV-SVR) with peak ALT level of 115 IU/ml (interquartile range, 59–240) had received nucleoside analogs (lamivudine, $n = 1$; entecavir, $n = 3$). Kaplan–Meier method showed that young age (<50 years) at entry ($P = 0.001$), baseline non-cirrhosis ($P = 0.009$), baseline HBV DNA $\geqslant$2000 IU/ml ($P < 0.001$), HBV genotype B ($P = 0.024$), HCV-SVR ($P = 0.008$) and C1766T mutation ($P = 0.001$) were associated with HBV DNA reactivation $\geqslant$2000 IU/ml (Fig. 2).

Univariate analyses of risk factors for HBV DNA reactivation $\geqslant$2000 IU/ml in dually-infected patients receiving IFN-based therapy are shown in Supplement Table 1. Based on stepwise multivariate Cox regression analysis, age (hazard ratio (HR) = 0.961; 95% confidence interval (CI), 0.938–0.986; $P = 0.002$), HCV-SVR (HR = 6.456; 95% CI, 1.522–27.39; $P = 0.011$) and baseline HBV DNA $\geqslant$2000 IU/ml (HR = 2.396; 95% CI, 1.093–5.254; $P = 0.029$) were independent factors associated with HBV DNA reactivation. With regard to those with precore and BCP sequence available, age (HR = 0.952; 95% CI, 0.925–0.979; $P = 0.001$), HCV-SVR (HR = 4.638; 95% CI, 1.040–20.69; $P = 0.044$), C1766T mutation (HR = 5.216; 95% CI, 1.745–15.59; $P = 0.003$) and A1846T mutation (HR = 2.332; 95% CI, 1.081–5.032; $P = 0.031$) correlated with HBV DNA reactivation independently (Table 3).

When the end point of HBV DNA reactivation was $\geqslant$60 IU/ml, stepwise Cox regression analysis showed that age (HR = 0.960; 95% CI, 0.938–0.982; $P = 0.001$), baseline HBV DNA $\geqslant$60 IU/ml (HR = 6.428; 95% CI, 3.260–12.67; $P < 0.001$), C1766T mutation (HR = 2.897; 95% CI, 1.106–7.585; $P = 0.030$) and baseline cirrhosis (HR = 0.480; 95% CI, 0.239–0.966; $P = 0.040$) were independent factors (Table 3). In addition, age and C1766T mutation were significant variables associated with HBV DNA reactivation $\geqslant$20,000 IU/ml (Supplement Table 2). Age, male gender, baseline cirrhosis and pretreatment HBV DNA $\geqslant$2000 IU/ml were independent factors of HBV DNA reactivation $\geqslant$2000 IU/ml twice or more (Supplement Table 3).

Among patients who achieved HCV-SVR, stepwise Cox regression analysis showed that age (HR = 0.945; 95% CI, 0.914–0.977; $P = 0.001$), C1766T mutation (HR = 4.694; 95% CI, 1.177–18.73; $P = 0.028$) and A1846T mutation (HR = 3.379; 95% CI, 1.419–8.048; $P = 0.006$) were independent factors of HBV DNA reactivation $\geqslant$2000 IU/ml (Table 4).

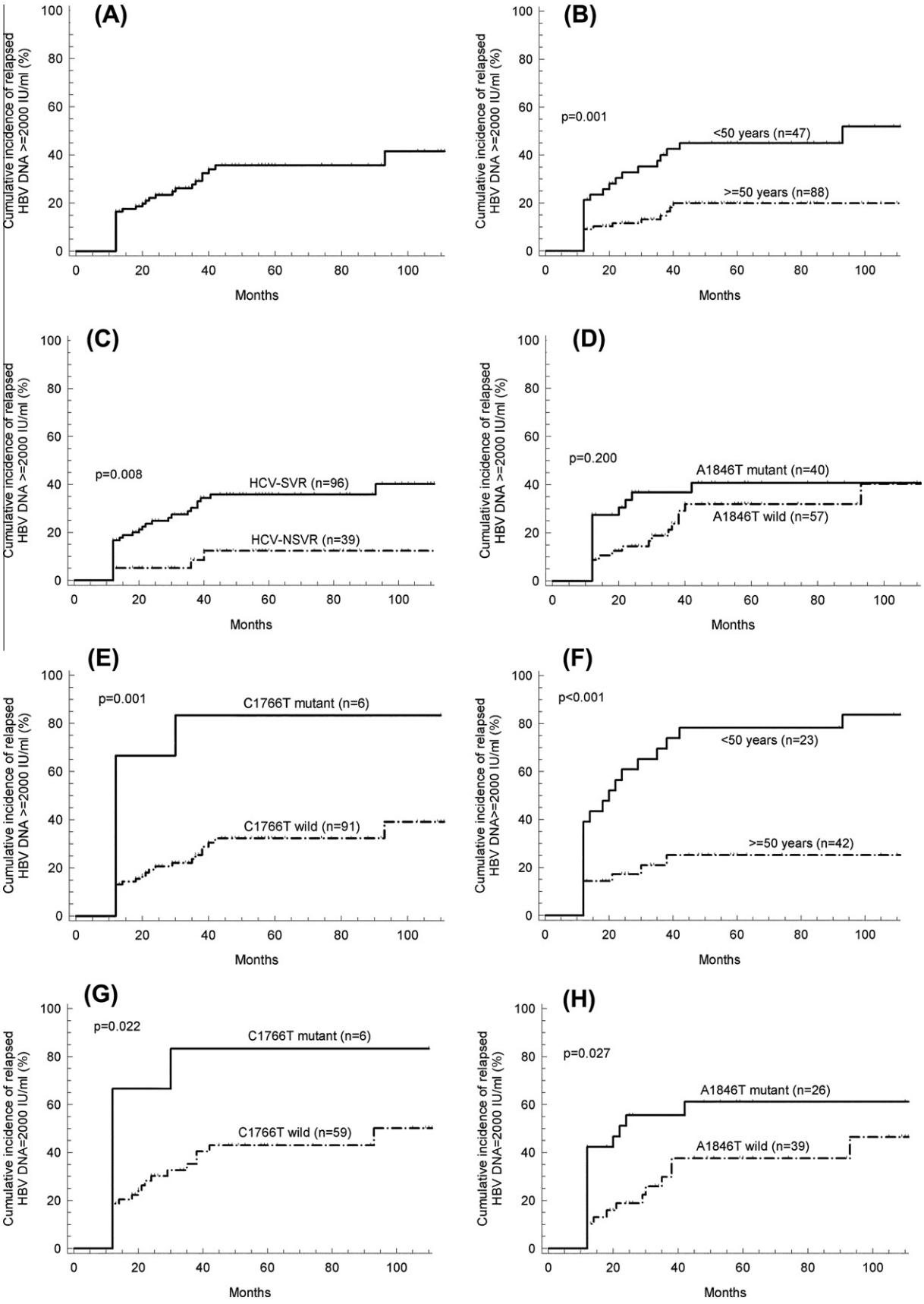

Fig. 2. Cumulative incidence of HBV DNA reactivation ($\geqslant$2000 IU/ml) in HBV/HCV dually-infected patients treated with interferon-based therapy. (A) All patients (n = 135); (B) <50 years vs. $\geqslant$50 years, P = 0.001; (C) HCV-SVR vs. NSVR, P = 0.008; (D) A1846T mutated vs. wild type, P = 0.200; (E) C1766T mutated vs. wild type, P = 0.001; (F) <50 years vs. $\geqslant$50 years (HCV-SVR patients), P < 0.001; (G) C1766T mutated vs. wild type (HCV-SVR patients), P = 0.022 and (H) A1846T mutated vs. wild type (HCV-SVR patients), P = 0.027 (compared by the log-rank test).

Table 3
Stepwise multivariate analyses of factors associated with HBV DNA reactivation by Cox proportional hazards model in HBV/HCV dually-infected patients after IFN-based therapy.[a]

	Hazard ratio	95% CI	*P*-value
HBV DNA reactivation ⩾ 2000 IU/ml			
Age (per 1 year increase)	0.952	0.925–0.979	0.001
HCV-SVR achievement	4.638	1.040–20.69	0.044
C1766T	5.216	1.745–15.59	0.003
A1846T	2.332	1.081–5.032	0.031
HBV DNA reactivation ⩾ 60 IU/ml			
Age (per 1 year increase)	0.960	0.938–0.982	0.001
Pretreatment HBV DNA ⩾ 60 IU/ml	6.428	3.260–12.67	<0.001
C1766T	2.897	1.106–7.585	0.030
Baseline cirrhosis	0.480	0.239–0.966	0.040

Abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus; CI, confidence interval; SVR, sustained virological response.
[a] Data were analyzed in 97 patients with precore/basal core promoter sequence available.

Table 4
Stepwise multivariate analyses of factors associated with HBV DNA reactivation by Cox proportional hazards model in HBV/HCV dually-infected patients with HCV-SVR achievement.[a]

	Hazard ratio	95% CI	*P*-value
HBV DNA reactivation ⩾ 2000 IU/ml			
Age (per 1 year increase)	0.945	0.914–0.977	0.001
C1766T	4.694	1.177–18.73	0.028
A1846T	3.379	1.419–8.048	0.006
HBV DNA reactivation ⩾ 60 IU/ml			
Age (per 1 year increase)	0.960	0.938–0.986	0.002
Pretreatment HBV DNA ⩾ 60 IU/ml	5.406	2.471–11.83	<0.001

Abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus; CI, confidence interval; SVR, sustained virological response.
[a] Data were analyzed in 65 patients with precore/basal core promoter sequence available.

3.3. Factors of HBsAg seroclearance in dually-infected patients receiving IFN or pegylated-IFN plus ribavirin therapy

As shown in Fig. 3A, the 5-year cumulative rate of HBsAg seroclearance was 27%. Kaplan–Meier method showed that old age (⩾50 years) at entry ($P = 0.011$), baseline HBV DNA < 60 IU/ml ($P < 0.001$), G1896A mutation ($P = 0.002$) and A1846T mutation ($P = 0.002$) were associated with HBsAg seroclearance (Fig. 3B–E).

As shown in Table 5, old age (HR = 1.053; 95% CI, 1.006–1.102; $P = 0.027$) and baseline HBV DNA < 60 IU/ml (HR = 3.077; 95% CI, 1.018–9.346; $P = 0.046$) were independent predictors of HBsAg seroclearance by stepwise multiple logistic regression analysis. As regards to those with precore and BCP sequence available, age (HR = 1.068; 95% CI, 1.010–1.129; $P = 0.020$), G1896A mutation (HR = 0.140; 95% CI, 0.031–0.626; $P = 0.010$) and A1846T mutation (HR = 0.086; 95% CI, 0.011–0.661; $P = 0.018$) were independently associated with HBsAg seroclearance.

4. Discussion

A wide spectrum of HBV and HCV virological patterns may occur in cases of dual infection. Longitudinal studies have shown complex and dynamic profiles that change over time in HBV/HCV dual infection and therefore characterization of disease requires repeated monitoring of both viral levels (Raimondo et al., 2006). In regions endemic for HBV such as Asian-Pacific countries, the most common scenario of HBV/HCV dual infection is HCV superinfection in chronic hepatitis B, which is often acquired perinatally or at early infancy (Chuang et al., 2005; Hung et al., 2005, 2011; Liaw, 1995; Liu et al., 2003, 2009; Yu et al., 2010). In our study, the lower levels of HBV-DNA observed in HBV/HCV dual infection as compared with those in HBV mono-infection agree with the well-recognized observation that HCV superinfection inhibits HBV replication (Chuang et al., 2005; Hung et al., 2005, 2011; Jardi et al., 2001; Liaw, 1995; Liu et al., 2003, 2009; Sagnelli et al., 2000; Yu et al., 2010).

There has been limited data regarding the prevalence of HBV genotypes and precore/BCP mutations and their impact on outcomes in patients with HBV-HCV dual infection (Jardi et al., 2001; Yeh et al., 1994, 1998). Jardi et al. have reported that the distribution of HBV genotypes is similar in HBV/HCV dually-infected and in HBV mono-infected patients (Jardi et al., 2001). By contrast, our data revealed that dually-infected patients had a higher prevalence of genotype C HBV than age-, sex- and HBeAg-matched HBV controls. This discrepant result might be associated with the fact that the studies were conducted in patient populations from different geographic areas (Europe and East Asia), where the viral strains differed. In Europe, HBV genotype A and D are the major strains (Norder et al., 1992; Stuyver et al., 2000), whereas genotype B and C are predominant in East Asia (Kao et al., 2000; Lee et al., 2003). However, the higher prevalence of HBV genotype C in dually-infected patients remains to be further investigated in large-scale studies. In particular, it is worth noting that genotype C dually-infected patients had lower levels of HBV DNA replication (>60 IU/ml) compared with genotype B subjects, which is significantly different from that observed in HBV mono-infected patients.

Previous studies have shown that precore/BCP mutations are found less frequently in patients with HBV/HCV dual infection than in patients with HBV infection alone (Jardi et al., 2001; Yeh et al., 1994, 1998). The authors suggested that the inhibitory effect of HCV on HBV might limit the emergence of precore/BCP variants because the lesser the viral genomes replicated, the fewer mutant genomes would be created (Jardi et al., 2001; Yeh et al., 1994, 1998). In our study, we have found a lower frequency of G1896A mutation in HBV/HCV dually-infected patients compared to HBV mono-infected patients. In particular, a subgroup analysis for HBV genotype C patients showed that dually-infected patients had a lower frequency of A1762T/G1764A ($P = 0.005$) and G1896A mutation ($P = 0.009$) than those with HBV monoinfection. This can partially explain our observation that genotype C dually-infected patients had lower levels of HBV DNA compared with genotype B subjects because of less G1896A mutation, which has been reported to be associated with lower serum viral loads and ALT levels among HBV genotype C patients (Kawabe et al., 2009). In addition, among dually-infected patients, genotype C HBV had a higher frequency of A1762T/G1764A mutation, but had a lower frequency of A1752T/G, C1799G and G1896A mutation than genotype B. These results concur with the findings observed in HBV mono-infected patients, suggesting that different HBV genotypes are associated with various mutations in the precore/BCP regions (Chan et al., 1999, 2007b).

Little is known about the HBV reactivation and HBsAg seroclearance following therapy with IFN or pegylated-IFN plus ribavirin among HBV/HCV dually-infected patients. In this study, we found that young age, HCV-SVR and baseline HBV DNA ⩾2000 IU/ml were independently associated with HBV DNA reactivation. These data in keeping with the previous observation that mutual interference between HCV and HBV may develop after IFN-based therapy indicate that closely monitoring serum HBV DNA level is necessary in dually-infected patients with HCV-SVR (Chuang et al., 2005; Hung et al., 2011). In addition, baseline HBV DNA level correlated with HBV DNA reactivation after IFN-based therapy, implying that combination with or sequential other antiviral therapy for HBV such as nucleoside/nucleotide analogs might be considered. However, further controlled trials are needed to elucidate this issue.

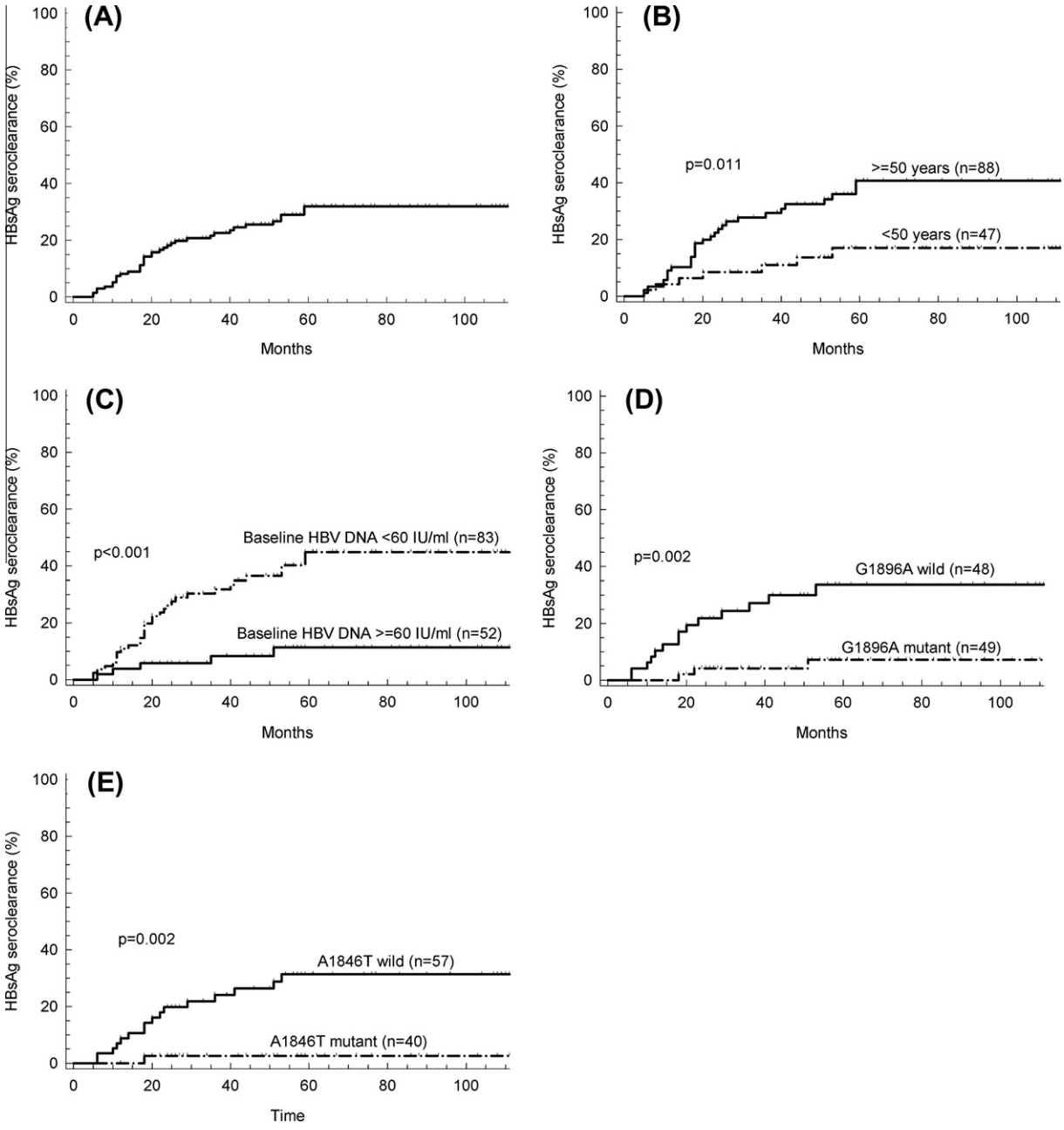

Fig. 3. Cumulative incidence of HBsAg seroclearance in HBV/HCV dually-infected patients treated with interferon-based therapy (A) All patients ($n = 135$); (B) $\geqslant$50 years vs. <50 years, $P = 0.011$; (C) baseline HBV DNA < 60 IU/ml vs. $\geqslant$60 IU/ml, $P < 0.001$; (D) G1896A wild vs. mutated type, $P = 0.002$ and (E) A1846T wild vs. mutated type, $P = 0.002$ (compared by the log-rank test).

Table 5
Stepwise multivariate analyses of factors associated with HBsAg seroclearance by Cox proportional hazards model.

	Hazard ratio	95% CI	*P*-value
All treated patients (n = 135)			
Age (per 1 year increase)	1.053	1.006–1.102	0.027
Pretreatment HBV DNA < 60 IU/ml	3.077	1.018–9.346	0.046
Sequence data available[a] *(n = 97)*			
Age (per 1 year increase)	1.068	1.010–1.129	0.020
G1896A	0.140	0.031–0.626	0.010
A1846T	0.086	0.011–0.661	0.018

Abbreviations: HBsAg, hepatitis B surface antigen; CI, confidence interval; HBV, hepatitis B virus.

[a] Sequence: precore/basal core promoter regions of HBV.

In regard of patients with precore/BCP sequencing available, genotype B was a significant factor for HBV reactivation by univariate analysis, but its impact was masked in the presence of other stronger risk factors, such as high serum HBV DNA level. C1766T mutation and A1846T mutation were two other independent factors associated with HBV DNA reactivation. Ren et al. have recently reported that C1766T mutation is one of the mutations that are more frequently detected in patients with HBV-related acute-on-chronic liver failure than in those with chronic hepatitis B (Ren et al., 2010). Also, we have previously demonstrated that C1766T and/or T1768A mutations correlate with the reactivation of hepatitis after HBeAg seroconversion (Chen et al., 2007b). On the other hand, the real significance of the A1846T mutant remains to be clarified. Our previous study has indicated that A1846T mutant is an inde-

pendent factor associated with HBeAg seroconversion during the natural course of chronic hepatitis B (Chen et al., 2007b). Moreover, a recent study has shown that A1846T is a novel factor independently associated with the risk of liver cirrhosis compared with asymptomatic HBV carriers and chronic hepatitis B patients (Yin et al., 2011). By contrast, the most frequent BCP mutation, A1762T/G1764A that has frequently been reported to be associated with advanced liver disease and enhanced viral genome replication in vitro (Chen et al., 2007a; Okamoto et al., 1994), appears to be unrelated to the HBV DNA reactivation in dually-infected patients after IFN-based therapy.

Seroclearance of HBsAg is a rare event in patients with chronic hepatitis B. Nevertheless, in accordance with previous reports and our cross-sectional study (Hung et al., 2011; Liu et al., 2009; Yu et al., 2010), we showed that IFN-based therapy had long-term effect on HBsAg seroclearance with a 5-year cumulative rate of 27% among HBV/HCV dually-infected patients. This incidence was substantially higher than that in HBV mono-infected patients receiving current antiviral agents (Chu and Liaw, 2007; Liu et al., 2010; Marcellin et al., 2004). Based on Cox proportional hazards regression analysis, old age and baseline HBV DNA < 60 IU/ml were independent predictors of HBsAg seroclearance. These results were very similar to those recently reported in a large community-based follow-up study for chronic HBV carriers (Liu et al., 2010). Alternatively, wild type C1766 and A1846 were other significant factors associated with HBsAg seroclearance in those with precore and BCP sequence available. Previous studies have shown that many patients with spontaneous HBeAg seroconversion have detectable precore mutants, but high precore mutant ratios are associated with persistent hepatitis after anti-HBe seroconversion and increased risk of cirrhosis (Chu et al., 2002b). In particular, G1896A is also predominantly found in patients with fulminant hepatitis, suggesting that G1896A mutant may be more pathogenic than wild type (Brunetto et al., 1991; Carman et al., 1989; Okamoto et al., 1990). Taken together, these data support the notion that HBsAg seroclearance among HBV/HCV dually-infected patients receiving IFN-based therapy is associated with sustained low HBV DNA possibly due to less G1896A and A1846T mutations. However, this cannot explain why C1766 is not a significant factor for HBsAg seroclearance. Further studies are necessary to explore the detailed mechanism.

This present study is limited by the absence of analysis in pre-S/S gene sequences, in which some mutations have been identified in patients with spontaneous or nucleotide/nucleoside analog-treated HBsAg seroclearance (Yeh, 2010). However, this can be almost ignored by the undetectable HBV DNA levels in our patients who had HBsAg seroclearance (Yeh, 2010). Another limitation is the lack of long-term follow-up study on the evolution of precore/BCP mutations. Our previous studies have shown that the frequency of A1762T/G1764A and G1896A mutations may increase after HBe seroconversion (Chen et al., 2007b) and pegylated-IFN therapy seems to suppress the G1896A mutant during treatment (Chen et al., 2011). Further work is necessary to evaluate the evolution of precore/BCP mutations and the association with IFN-based therapy in HBV/HCV dual infection.

In conclusion, this study showed that HBV/HCV dually-infected patients had a higher prevalence of genotype C HBV and a lower frequency of G1896A mutation as compared with those with single HBV infection. Specific mutations in precore/BCP regions such as C1766T, G1896A and A1846T mutations could be useful in predicting long-term HBV response in HBV/HCV dually-infected patients treated with IFN-based therapy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2011.10.018.

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