Various approaches have been used for the treatment of osteomalacia associated with Fanconi's syndrome. Clarke et al. [38] reported successful treatment of osteomalacia associated with acquired Fanconi's syndrome with calcium, phosphate and vitamin D, regardless of the underlying cause of the disease. Eight cases of Fanconi's syndrome with ADV-related hypophosphatemic osteomalacia were reported in the past 5 years [14, 16, 17, 39–41]. Three of the 8 patients were treated with oral phosphate only; while 3 other patients received oral phosphate and vitamin D, and one patient was treated with the combination of oral phosphate, vitamin D and calcium. In all cases, treatment increased serum phosphate level and improved musculoskeletal symptoms. Similar to the eight cases reported in the literature, our 2 patients showed normalization of phosphate level and symptomatic improvement after treatment. Treatment with oral phosphate for ADV-related hypophosphatemic osteomalacia is considered effective.

We also examined the clinical characteristics of the 14 patients who developed persistent hypophosphatemia. Three of the 14 patients developed ostealgia during the treatment. Patients 1 and 10 were diagnosed with acquired Fanconi's syndrome with subsequent hypophosphatemic osteomalacia. Although we could not confirm the diagnosis of Fanconi's syndrome in patient 2 because she was transferred to another hospital, she was considered to have developed Fanconi's syndrome based on the clinical course. Despite persistent hypophosphatemia, serum creatinine remained within the normal range. In addition, 6 of the 14 patients also had low eGFR ($\geq$30 % decrease relative to baseline), and two patients with Fanconi's syndrome showed $\geq$40 % decrease in eGFR, relative to baseline. Based on the above features, patients can develop marked hypophosphatemia and serious complications, such as Fanconi's syndrome, following significant fall in eGFR, irrespective of the level of serum creatinine. In the three patients who developed Fanconi's syndrome, a gradual increase in serum ALP level and simultaneous fall in serum uric acid were noted more than one year before the appearance of ostealgia. Based on the above findings, we recommend reducing the dose or changing medications to other nucleotide analogues in patients who develop hypouricemia, hyper-ALPemia, hypophosphatemia, and low eGFR, to avoid the development of ADV-induced Fanconi's syndrome.

In our study, the dosing interval of ADV was modified by the attending physician following increase in serum creatinine level. Seventeen (5.8 %) patients required such modification, their eGFR and serum phosphate showed significant improvement at 6 and 12 months after the modification, in agreement with previous reports [13, 42]. However, the modification in ADV dosing interval from 10 mg every day to every other day neither affected HBV-DNA level nor the antiviral effect. Therefore, the ADV dose should be modified in patients who show decrease in eGFR and/or serum phosphate.

In conclusion, our results showed that even at low dose of 10 mg/day, long-term combination therapy of ADV and LAM can cause renal impairment and hypophosphatemia, and lead to Fanconi's syndrome in a subgroup of patients. ADV-based treatment tends to reduce eGFR and serum phosphate especially in elderly male patients and those with HCC. We recommend regular monitoring of serum phosphate and evaluation of eGFR, in addition to serum creatinine, in patients treated with ADV. Suspicion of Fanconi's syndrome requires early reduction of ADV dose or switching to other antiviral agents.

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Conflict of interest None.

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ORIGINAL ARTICLE—LIVER, PANCREAS, and BILIARY TRACT

Long-term efficacy and emergence of multidrug resistance in patients with lamivudine-refractory chronic hepatitis B treated by combination therapy with adefovir plus lamivudine

Fumitaka Suzuki · Tetsuya Hosaka · Yoshiyuki Suzuki · Norio Akuta · Hitomi Sezaki · Tasuku Hara · Yusuke Kawamura · Masahiro Kobayashi · Satoshi Saitoh · Yasuji Arase · Kenji Ikeda · Mariko Kobayashi · Sachiyo Watahiki · Rie Mineta · Hiromitsu Kumada

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Abstract
Background Few studies have investigated the emergence of multidrug resistance to adefovir dipivoxil (ADV) plus lamivudine (LAM) combination therapy for patients with LAM-refractory chronic hepatitis B (CHB). In this retrospective study, we investigated the long-term clinical course of these patients with or without multidrug resistance mutations.
Methods We analyzed 406 Japanese patients with LAM-refractory CHB treated with combination therapy with follow-up for a median of 5.4 (0.5–9.5) years. Multidrug resistance of hepatitis B virus (HBV) DNA was analyzed using direct sequencing or cloning methods at baseline and viral breakthrough or insufficient decline during combination therapy.
Results Ratio of patients with undetectable serum HBV DNA levels (<2.6 log copies/mL) during combination therapy was 63, 72, 75, 79, 82, 80 and 85 % at years 1 through 7, respectively. Substitutions associated with multidrug resistance were identified in 11 patients (2.7 %) at baseline, and in 12 patients (3 %) during therapy. HBV DNA levels of patients with rtA181S mutation at baseline and emergence of rtA181T + rtN236T double mutation or a wide variety of mutations during combination therapy could not be suppressed. Moreover, using ultra-deep sequencing, rtA181T/V mutations were detected at baseline in 7 of 10 patients with emergent multidrug resistance during combination therapy, although 6 of these 7 patients had very low frequency (<1 %) variants.
Conclusion Long-term ADV plus LAM combination therapy is effective in LAM-refractory patients. However, HBV DNA levels of the patients with multidrug resistance at baseline or during combination therapy sometimes could not achieve complete suppression or were re-elevated after a decrease.

Keywords Adefovir dipivoxil · Lamivudine · Hepatitis B virus · Ultra-deep sequence · Multidrug resistance

Abbreviations

HBV	Hepatitis B virus
IFN	Interferon
NA	Nucleoside/nucleotide analogues
LAM	Lamivudine
ADV	Adefovir dipivoxil
ETV	Entecavir
TDF	Tenofovir disoproxil fumarate
CHB	Chronic hepatitis B
HBeAg	Hepatitis B e antigen
ALT	Alanine aminotransferase
HBsAg	Hepatitis B surface antigen
PCR	Polymerase chain reaction
CLEIA	Chemiluminescent enzyme immunoassay
rt	Reverse transcriptase

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F. Suzuki (✉) · T. Hosaka · Y. Suzuki · N. Akuta · H. Sezaki · T. Hara · Y. Kawamura · M. Kobayashi · S. Saitoh · Y. Arase · K. Ikeda · H. Kumada
Department of Hepatology, Toranomon Hospital, 2-2-2 Toranomon, Minato-ku, Tokyo 105-8470, Japan
e-mail: fumitakas@toranomon.gr.jp

F. Suzuki
Okinaka Memorial Institute for Medical Research, Tokyo, Japan

M. Kobayashi · S. Watahiki · R. Mineta
Research Institute for Hepatology, Toranomon Hospital, Tokyo, Japan

VBT Viral breakthrough
AST Aspartate aminotransferase
CI Confidence interval
Pt Patient

Introduction

Hepatitis B virus (HBV) infection is a common disease that can induce a chronic carrier state, and is associated with the risk of developing progressive disease and hepatocellular carcinoma [1]. Interferon (IFN) and several nucleoside/nucleotide analogues (NA) such as lamivudine (LAM), adefovir dipivoxil (ADV), entecavir (ETV), and tenofovir disoproxil fumarate (TDF) are currently approved for treatment of chronic hepatitis B (CHB) in most countries [2–8]. Successful treatment of CHB with clearance of hepatitis B e antigen (HBeAg), reduction in serum HBV DNA levels, and normalization of alanine aminotransferase (ALT) levels are associated with favorable long-term outcomes, independent of the antiviral drug used [9–11].

LAM is effective in suppressing HBV replication, improving transaminase levels and liver histology, and enhancing the rate of loss of HBeAg. A major problem with the long-term use of lamivudine, however, is its potential to induce viral resistance, with associated increases in HBV DNA and serum transaminases [3, 12, 13]. ADV is reportedly effective in suppressing HBV replication and is approved as a standard therapy in LAM-resistant patients in Japan [14, 15]. However, data concerning the long-term efficacy of ADV treatment in LAM-resistant CHB patients remain limited.

Although both experimental and clinical studies have shown that ADV suppresses not only wild-type but also LAM-resistant strains, the potential for ADV-resistance mutation has emerged. Selection of the rtA181V/T or rtN236T mutant was associated with ADV [13, 16]. Moreover, we previously reported that the emergence of ADV-resistant mutations before and during combination therapy for a period of 2 years was rare [17]. However, ADV-resistant mutations emerging before and during combination therapy might be caused by a poor response to therapy. Moreover, long-term clinical and virological data concerning ADV- or ETV-resistant mutations in LAM-resistant CHB patients receiving long-term ADV plus LAM combination therapy are limited.

The aims of this study were to evaluate the long-term efficiency of ADV plus LAM combination therapy based on virological response (VR), HBeAg clearance, and Hepatitis B surface antigen (HBsAg) clearance, and to investigate the emergence of ADV-, ETV-, or TDF-resistant (or multidrug resistant) mutations before and during combination therapy, and the clinical course of these patients.

Patients and methods

Patients

A total of 406 consecutive adult Japanese patients with chronic HBV infection were treated with ADV in addition to ongoing LAM treatment from 2002 at Toranomon Hospital (Table 1). Several of these patients were included in previous reports [14, 15, 17, 18]. Enrollment in this study and the start of ADV treatment were determined by the following criteria. First, an increase in serum HBV DNA levels of $\geq$1 log copies/mL during LAM treatment compared with the nadir of initial antiviral efficacy on at least two consecutive occasions, or a serum HBV DNA level of $\geq$5 log copies/mL after 1 year of LAM monotherapy; and second, no history of treatment with other NAs such as ETV or TDF. Exclusion criteria were a serum creatinine level $\geq$1.2 mg/dL; coinfection with hepatitis C virus or HIV; and history of other liver diseases, such as autoimmune hepatitis, alcoholic liver disease, or metabolic liver disease. The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and was approved by the Toranomon Hospital Ethical

Table 1 Characteristics of patients at the commencement of adefovir dipivoxil plus lamivudine combination therapy

Demographic data	
Total number	406
Sex (female/male)	86/320
Age, years (range)	48 (25–78)
Duration of treatment, years (range)	5.4 (0.5–9.5)
History of IFN therapy (+/−)	157/249
Laboratory data	
Aspartate aminotransferase, IU/L (range)	54 (12–1413)
Alanine aminotransferase, IU/L (range)	76 (9–1563)
Bilirubin, mg/dL (range)	0.7 (0.2–15.5)
Albumin, g/dL (range)	3.9 (1.9–4.7)
Platelets, $\times 10^3/\mu$L (range)	160 (28–452)
Staging of liver histology (CH/LC)	325/81
Serum HBV DNA, log copies/mL (range)	6.7 (<2.6 to >7.6)
HBeAg, positive/negative/unknown	208/193/5
HBV genotype (A/B/C/D/F)	14/25/364/2/1
rtM204 mutant (%)	365 (90 %)

Values are expressed as the median and range in parentheses, or number and percentage in parentheses

IFN interferon, *HBV* hepatitis B virus, *CH* chronic hepatitis, *LC* liver cirrhosis, *HBeAg* hepatitis B e antigen

Committee (approval no. 714). Informed consent was obtained from all patients.

Patients received a single daily oral administration of ADV 10 mg, in addition to ongoing LAM treatment (100 mg/day). The dosing interval of ADV was modified by the attending physician when serum creatinine level increased to >1.2 mg/dl. Liver cirrhosis was defined by the presence of stage 4 fibrosis on histopathological examination and/or clinical evidence of portal hypertension.

Blood tests and serum viral markers

Routine biochemical tests were performed using standard procedures before and during therapy at least once every 3 months. Levels of HBsAg, HBeAg, and anti-HBe were determined using radioimmunoassay kits (Abbot Diagnostics, Chicago, IL, USA) or Chemiluminescent enzyme immunoassay (CLEIA; Lumipulse System, Fujirebio, Inc. Tokyo, Japan). Serum HBV DNA was quantified using the polymerase chain reaction (PCR)-based Amplicor HBV Monitor assay (Roche Diagnostics, Indianapolis, IN; lower limit of detection, 2.6 log copies/mL).

Determination of nucleotide sequences of HBV DNA

DNA was extracted from 100 μL of serum. PCR reactions for detection of the reverse transcriptase (rt) region (nt 130–1161) of HBV DNA were performed in two parts. The first and second PCR reactions for detection of the first part of the rt region were performed using primers BGF1 (sense; 5′-CTGTGGAAGGCTGGCATTCT-3′) and BGR2 (antisense; 5′-GGCAGGATAGCCGCATTGTG-3′), and PreSBamH1 (sense; 5′-CTTGGGATCCAGAGCTAC AGCATGG-3′) and BR112 (antisense; 5′-TTCCGTCG ACATATCCCATGAAGTTAAGGGA-3′), respectively, under conditions of initial denaturation for 4 min, 35 cycles of amplification with 94 °C for 1 min, 55 °C for 2 min, 72 °C for 3 min, and a final extension at 72 °C for 7 min. The first and second PCR reactions for detection of the second part of the same region were performed using primer pairs B11F (sense; 5′-GGCCAAGTCTGTACAA CATC-3′) and B12R (antisense; 5′-TGCAGAGGTG AAGCGAAGTG-3′), and B11F and B14R (antisense; 5′-GATCCAGTTGGCAGCACACC-3′), respectively, under the same conditions. The amplified PCR products were used for direct sequencing or cloning methods as previously described [19, 20]. When mutations as a mixed viral population with the wild type sequence for direct sequencing were present, PCR was performed using a cloning method. Sequences of 9–26 independent clones from the sample were determined and analyzed. Measurement of sequences in the rt region was performed at the start of ADV treatment, and on viral breakthrough (VBT) during ADV plus LAM combination therapy. VBT was defined as any increase in serum HBV-DNA by >1 log copies/mL from the nadir or redetection of serum HBV-DNA at levels tenfold the lower limit of detection of the HBV-DNA assay after having an undetectable result. Moreover, sequences for serum HBV DNA level of ≥4 log copies/mL after 1 or 2 years of ADV plus LAM combination therapy were also measured.

Measurement of LAM-, ADV-, ETV- and TDF-resistant variants using ultra-deep sequencing

Ultra-deep sequencing was performed using the Ion Personal Genome Machine (PGM) Sequencer (Life Technologies), as described previously [21]. An Ion Torrent adapter-ligated library was prepared using an Ion Xpress Plus Fragment Library Kit (Life Technologies). Briefly, 100 ng of fragmented genomic DNA was ligated to the Ion Torrent adapters P1 and A. The adapter-ligated products were nick-translated and PCR-amplified for a total of eight cycles. Subsequently, the library was purified using AM-Pure beads (Beckman Coulter, Brea, CA) and the concentration was determined using the StepOne Plus Real Time PCR (Life Technologies) and Ion Library Quantitation Kit in accordance with the manufacturer's instructions. Emulsion PCR was performed using Ion OneTouch (Life Technologies) in conjunction with an Ion OneTouch 200 Template Kit v2 (Life Technologies). Enrichment for templated ion spheres particles (ISPs) was performed using the Ion OneTouch Enrichment System (Life Technologies) in accordance with the manufacturer's instructions. Templated ISPs were loaded onto an Ion 314 chip and subsequently sequenced using 130 sequencing cycles in accordance with the Ion PGM 200 Sequencing Kit user guide. Total output read length per run is over 10 M base (0.5 M-tag, 200 base read). The results were analyzed with the CLC Genomics Workbench software (CLCbio, Aarhus, Denmark). A control experiment was included to validate the error rates in ultra-deep sequencing of the viral genome. In this study, amplification products of the second-round PCR were ligated with plasmid and transformed in Escherichia coli in a cloning kit (TA Cloning; Invitrogen, Carlsbad, CA). A plasmid-derived rt sequence was determined as the template by the control experiment. Coverage per position for aa180, aa181, aa184, aa194, aa202, aa204, aa233, aa236 and aa250 in the rt region was 63320, 63890, 67737, 49273, 57410, 57211, 40155, 34801 and 42914, respectively. Thus, using the control experiment based on the plasmid encoding rt sequence, amino acid mutations were defined as amino acid substitutions at a ratio of more than 0.25 % of total coverage. This frequency ruled out putative errors caused by the deep sequence platform used in this study.

HBV genotype

The major genotypes of HBV were determined using the enzyme-linked immunosorbent assay (ELISA, Institute of Immunology, Tokyo, Japan) or the PCR-invader assay (BML, Inc, Tokyo, Japan) according to the method described by Usuda et al. [22] or Tadokoro et al. [23].

Statistical analysis

Differences between groups were examined for statistical significance using the χ^2 or Fisher's exact test where appropriate. Independent risk factors predicting the achievement of HBeAg seroclearance were studied using stepwise Cox regression analysis. The following 14 potential predictors of HBeAg seroclearance were assessed in this study: age, sex, pretreatment with IFN, severity of liver disease (CH or liver cirrhosis), duration from LAM to ADV, substitution of rtM204, HBV genotype, and levels of aspartate aminotransferase (AST), ALT, bilirubin, albumin, γ-glutamyl transpeptidase, platelets, and HBV DNA. Each was transformed into categorical data consisting of two simple ordinal numbers for univariate and multivariate analyses. All factors found to be at least marginally associated with HBeAg seroclearance ($P < 0.10$) were tested in the multivariate Cox proportional hazards model, and hazard ratios and 95 % confidence intervals (CIs) were calculated to assess the relative risk confidence. The above calculations were performed using the Windows IBM SPSS version 19.0.0 software (IBM Corp., Armonk, NY, USA). A Kaplan–Meier estimate was also performed using the SPSS software.

Results

Study population

Clinical and virological profiles of the 406 patients at the start of ADV plus LAM combination therapy are shown in Table 1. At the start of combination therapy, 81 patients (20 %) had cirrhosis and 208 (51 %) were positive for HBeAg. Fourteen (3 %), 25 (6 %), 364 (90 %), 2 (0.5 %), and 1 (0.2 %) patients were infected with HBV genotypes A, B, C, D, and F, respectively. During the clinical course, 48 of 406 patients (12 %) showed an elevation in serum creatinine >1.2 mg/dL, and their ADV dose was accordingly reduced to 10 mg every second day.

Response to ADV plus LAM combination therapy

The ratio of patients with undetectable serum HBV DNA levels (<2.6 log copies/mL) was 63 % (231/367), 72 % (254/352), 75 % (249/331), 79 % (235/297), 82 % (210/256), 80 % (137/171), and 85 % (94/110) at years 1 through 7, respectively (Fig. 1a). Among HBeAg-positive patients at baseline, undetectable rates of serum HBV DNA levels gradually increased from 1 to 7 years (42, 57, 65, 70, 76, 75, 83 % at years 1 through 7, respectively; $n = 208$). In contrast, ratios in HBeAg-negative patients at baseline were >80 % at all points (86, 89, 88, 90, 91, 87, 89 % at years 1 through 7, respectively; $n = 193$). The undetectable rates of serum HBV DNA in HBeAg-negative patients

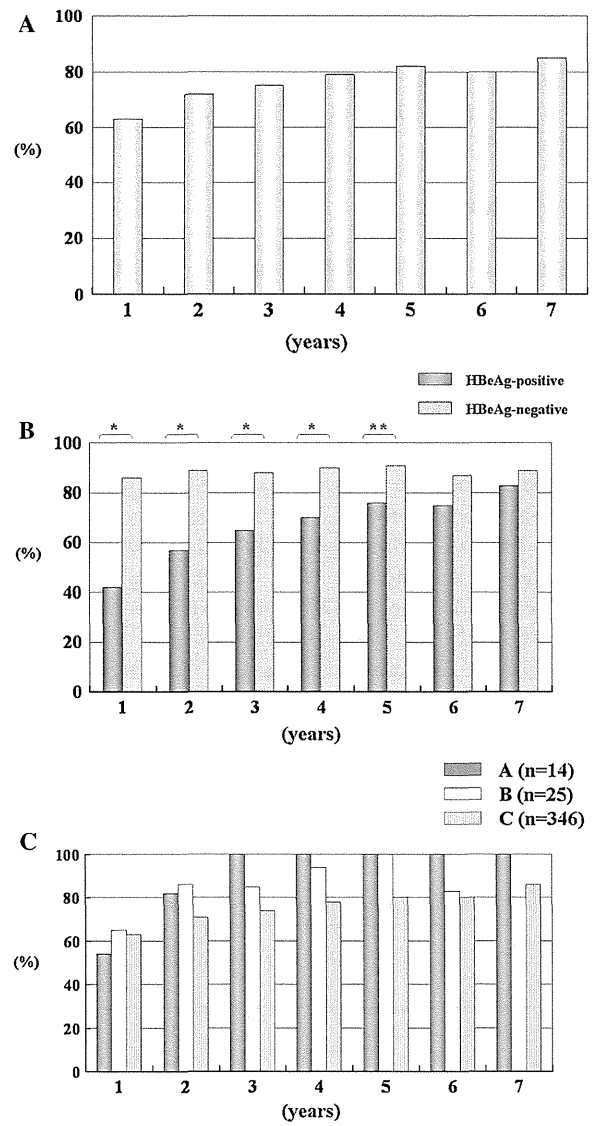

Fig. 1 Undetectable serum HBV DNA levels (<2.6 log copies/mL) in years 1 through 7, respectively. **a** All patients. **b** HBeAg status. A *single asterisk* indicates a statistical significance of $P < 0.0001$ and a *double asterisk* indicates $P = 0.0044$, as determined at the χ^2 test. **c** Genotypes A, B and C

were significantly higher than those in HBeAg-positive patients at years 1 through 5 ($P < 0.0001$ at years 1 through 4, and $P = 0.0044$ at year 5) (Fig. 1b).

By genotype, serum HBV DNA levels were undetectable after 3 years in 100 % of those with genotype A (54, 82, 100, 100, 100, 100, 100 % at years 1 through 7, respectively; $n = 14$), and in >80 % after 2 years in those with genotype B (65, 86, 85, 94, 100, 83, 80 %, at years 1 through 7, respectively; $n = 25$). In contrast, ratios in patients with genotype C gradually increased from 1 to 7 years (63, 71, 74, 78, 80, 80, 86 %, at years 1 through 7, respectively; $n = 364$) (Fig. 1c).

Moreover, the ratio of patients with ALT normalization (≤30 IU/L) was 66 % (250/380), 73 % (262/358), 78 % (255/327), 77 % (226/292), 77 % (194/251), 76 % (125/165), and 77 % (81/105) at years 1 through 7, respectively.

HBeAg clearance

Eighty-four of 208 HBeAg-positive patients (40 %) achieved seroclearance of HBeAg. Cumulative HBeAg seroclearance rates from the commencement date of ADV plus LAM combination therapy were 13 % at 1 year, 24 % at 3 years, 35 % at 5 years, and 52 % at 7 years (Kaplan–Meier method; Supplementary Figure). No patients experienced the reappearance of HBeAg after seroclearance. Six factors found to be associated with the achievement of HBeAg seroclearance in univariate analysis were: AST upper limit of normal (ULN: 30 IU/L)×2<($P = 0.017$), bilirubin 1.1 < mg/dL ($P = 0.020$), ALT ULN×3 <($P = 0.040$), history of IFN therapy ($P = 0.068$), platelets 150<×10^3 μL ($P = 0.074$), and non C genotype ($P = 0.081$). In multivariate analysis, independent factors predicting the achievement of HBeAg seroclearance were history of IFN therapy ($P = 0.009$), AST ($P = 0.016$), bilirubin ($P = 0.030$), and genotype ($P = 0.042$) (Table 2).

HBsAg clearance

Eight of 406 patients (1.9 %) achieved seroclearance of HBsAg (Supplementary Table). All patients were older than 40 years, and all but one was male. Three, two, and three patients were infected with HBV genotypes A, B, C, respectively; two patients were HBeAg-positive at baseline of combination therapy; and five patients had a history of IFN therapy. The duration of HBsAg seroclearance was 2.1–6.8 years.

Genotypic analysis of ADV- and ETV-resistant mutants at baseline of combination therapy and clinical course

Genotypic resistance to LAM, ADV, ETV or TDF was analyzed in baseline samples before the start of ADV plus LAM combination therapy. Substitutions were assessed by direct sequencing or cloning, namely those at rtL180 or rtM204 associated with LAM resistance; rtA181, rtI233, or rtN236 associated with ADV resistance; rtT184, rtS202, or rtM250 associated with ETV resistance; and rtA194 associated TDF resistance. At baseline, substitutions associated with resistance to ADV or ETV were identified in 11 patients (2.7 %) (Table 3). RtA181S/T mutations without substitution at rtM204 were identified in four patients, whereas rtA181T mutation with substitution at rtM204 on the same clones was identified in three patients. RtA181T mutation and rtM204V/I mutation, which existed together on other clones, was identified in two patients. Substitutions related with ETV resistance were identified in the remaining two patients. All but one (Pt. 11) patient was HBeAg-positive and most were younger (<40 years old) and had a high viral load at baseline of LAM therapy. In the remaining 395 patients, rtM204 mutations without substitutions associated with resistance to ADV, ETV or TDF were identified in 358 patients, whereas 37 patients had no substitutions associated with resistance to LAM, ADV, ETV or TDF.

Table 2 Factors associated with HBeAg seroclearance due to ADV plus LAM combination therapy on univariate and multivariate analyses

Parameter	Univariate analysis		Multivariate analysis	
	Hazard ratio (95 % CI)	*P*	Hazard ratio (95 % CI)	*P*
AST (≤UNL×2/UNL×2<)	1.717 (1.102–2.676)	0.017	1.750 (1.112–2.754)	0.016
Bilirubin (≤1.1/1.1<)	1.783 (1.095–2.903)	0.020	1.743 (1.056–2.876)	0.030
ALT (≤UNL×3/UNL×3<)	1.577 (1.008–2.468)	0.040		
History of IFN therapy (−/+)		0.068	1.824 (1.164–2.857)	0.009
Platelets (≤150 × 10^3/150 × 10^3<)		0.074		
Genotype (C/non C)		0.081	2.096 (1.025–4.274)	0.042

HBeAg hepatitis B e antigen, *ADV* adefovir dipivoxil, *LAM* lamivudine, *CI* confidence interval, *AST* aspartate aminotransferase, *UNL* upper limit of normal: 30 IU/L, *ALT* alanine aminotransferase, *IFN* interferon

Table 3 Characteristics of patients with resistance to ADV, ETV or TDF at baseline of ADV plus LAM combination therapy

No.	Baseline of LAM therapy					Baseline of ADV plus LAM combination therapy	
	Age	Sex	Genotype	HBeAg	HBV DNA level	Mutation type (rt region)	Duration from start of LAM to emergence of mutation (years)
1	29	M	C	+	7.6<	A181S	3.3
2	32	M	C	+	7.6<	A181T	1.3
3	23	M	C	+	7.6	A181T	2
4	34	M	C	+	nd	A181T	5
5	35	M	C	+	7.6<	A181T (17/19), L180M + M204V (2/19)	1
6	37	M	C	+	6.5	A181T (7/24), M204I (15/24), L180M + M204V (2/24)	1.3
7	51	M	C	+	7.4	A181T + M204I	1.3
8	38	F	C	+	nd	A181T + M204I (7/13), M204I (6/13)	4
9	33	M	C	+	nd	A181T + M204I (10/21), A181T + M204V(1/21), M204I (10/21)	1.3
10	25	F	D	+	nd	L180M + S202G + M204V	5
11	31	F	C	−	7.6<	L180M + M204V + M250L	6

No. of clones with combined mutations in rt region/total clones are shown in parentheses

ADV adefovir dipivoxil, *ETV* entecavir, *TDF* tenofovir disoproxil fumarate, *LAM* lamivudine, *HBV* hepatitis B virus, *HBeAg* hepatitis B e antigen, *nd* not done, *rt* reverse transcriptase, *M* male, *F* female

Following ADV plus LAM combination therapy, HBV DNA levels of four patients (Pt. 5, 6, 8, 10) were undetectable (<2.6 log copies/mL) (Fig. 2a), while those of the remaining seven were ≥2.6 log copies/mL. One patient (Pt. 7) achieved HBeAg clearance at 2 weeks, while HBeAg reappeared in a second patient (Pt. 11) at 40 weeks. Ratios of patients with undetectable levels of HBV DNA were 9 % (1/11) at 1 year, 22 % (2/9) at 2 years and 50 % (4/8) at 3 years. Three patients (Pt. 1, 2, 9) received TDF plus LAM or TDF plus ETV therapy after ADV plus LAM combination therapy due to insufficient virological response. Mutations of rtA181T + rtM204I, rtA181T + rtM204V and rtM204I in Pt. 9 changed to rtA181T + rtN236T and rtL180V + rtM204V after 3 years of combination therapy, and HBV DNA level was again thereafter elevated.

Genotypic analysis of ADV- and ETV-resistant mutants during combination therapy and clinical course

Genotypic resistance to ADV, ETV or TDF was analyzed during ADV plus LAM combination therapy in 395 patients without ADV- or ETV-resistant mutants at baseline. During combination therapy, substitutions associated with resistance to ADV or ETV were identified in 12 patients (3 %) (Table 4). All patients were genotype C and had a high viral load (>5.0 log copies/ml) at baseline of combination therapy. Substitutions of rtM204 were identified in all but one patient (Pt. 19) at baseline. RtA181V/S/

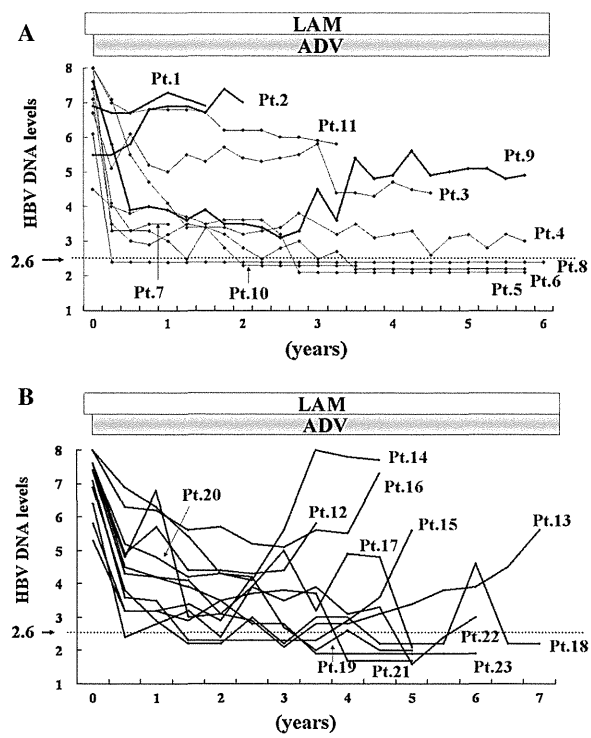

Fig. 2 Clinical course (HBV DNA load) of patients. **a** Patients with resistance associated with ADV or ETV at baseline of ADV plus LAM combination therapy. **b** Patients with resistance associated with ADV or ETV during ADV plus LAM combination therapy

Table 4 Characteristics of patients with emergence of resistance to ADV, ETV or TDF during ADV plus LAM combination therapy

No.	Baseline of ADV plus LAM combination therapy						During ADV plus LAM combination therapy	
	Age	Sex	Genotype	HBeAg	HBV DNA level	rtM204 mutant type	Mutation type(rt)	Duration from start of ADV + LAM to emergence of mutations (years)
12	32	M	C	+	7.6	M204I/V	A181T + N236T (12/16), L180M + N236T (1/16), A181T (1/16), L180M + A181T + M204V (1/16), L180M + M204V + N236T (1/16)	1
13	29	M	C	+	7.6	M204I/V	A181T + M204I + M250L (13/18), L180M + M204V + M250L (2/18), L180M + T184I + M204I + M250L(1/18), L180M + M204I + M250L(1/18), A181T + M204I (1/18)	5
14	58	M	C	+	7.6 <	M204I	L180M + T184I + M204I + M250L(16/26), L180M + T184I + M204I(6/26), A181T(4/26),	3.5
15	49	M	C	+	5.1	M204I/V	A181V + M250L	5
16	46	M	C	+	7.6	M204V	A181T + N236T	3
17	30	F	C	+	7.4	M204I	A181T	0.2
18	40	M	C	+	6.9	M204I	A181S	4
19	40	M	C	+	5.3	M204	A181S	2.3
20	49	M	C	+	7.6	M204V	A181V	0.1
21	63	M	C	−	5.8	M204I	A181T(10/11), A181T + M204I(1/11)	2
22	56	M	C	−	6.4	M204V	A181S	0.6
23	36	M	C	+	7.4	M204I	M180M + A181T(5/9), L180M + A181T + M204I + M250I (3/9), L180M + M204I + M250I(1/9)	1

No. of clones with combined mutations in the rt region/total clones are shown in parentheses

ADV adefovir dipivoxil, *ETV* entecavir, *TDF* tenofovir disoproxil fumarate, *LAM* lamivudine, *HBV* hepatitis B virus, *HBeAg* hepatitis B e antigen, *rt* reverse transcriptase, *M* male, *F* female

T mutation with or without substitution at rtM204 was identified in all patients, whereas rtT184I or rtM250I/L mutation with or without substitution at rtM204 was identified in 4 patients. Moreover, rtA181T + N236T double mutation related with ADV resistance was identified in two patients (Pt. 12 and 16). Interestingly, substitutions of rtM204 were not detected in five patients (Pt 15, 17, 18, 19, 22) when these ADV- or ETV-related mutations emerged.

Following ADV plus LAM combination therapy, the ratio of patients with undetectable levels of HBV DNA was 0 % (0/12) at 1 year, 25 % (3/12) at 2 years, 27 % (3/11) at 3 years, and 20 % (2/10) at 4 years (Fig. 2b). The HBV DNA levels of five patients (Pt. 12–16) were re-elevated after a decrease, and these patients were then switched to a different treatment (TDF plus LAM or TDF plus ETV in four patients and ETV plus ADV in one). Two of these five patients (Pt. 12 and 16) had rtA181T + rtN236T double mutation-related ADV resistance, while three (Pt. 12–14) had a wide variety of mutations. In contrast, HBV DNA levels of patients who had HBeAg clearance (Pt. 17–19, 23) during ADV plus LAM combination therapy were sustained at ≤5 Log copies/mL after 1 year, and only three patients (Pt. 19, 21, 22) showed sustained levels of ≤2.6 Log copies/mL after 4 years.

Evolution of LAM-, ADV-, ETV- and TDF-resistant variants using ultra-deep sequencing

In 10 of 12 patients with emergent substitutions associated with resistance to ADV or ETV during combination therapy, LAM-, ADV-, ETV- and TDF-resistant variants were analyzed by ultra-deep sequencing at baseline (Table 5). Patients 13 and 20 could not be analyzed due to insufficient stored serum. RtA181T/V mutations were detected in all 7 patients by ultra-deep sequencing at baseline, although 6 of these 7 patients had very low frequency (<1 %) variants. Interestingly, rtA181S mutation in 3 patients could not be

Table 5 Detection of resistance to ADV, ETV or TDF by ultra-deep sequencing at baseline in patients with emergence of resistance during ADV plus LAM combination therapy

No.	Baseline of ADV plus LAM combination therapy (ultra-deep sequencing)									During therapy
	rtL180	rtA181	rtT184	rtA194	rtS202	rtM204	rtI233	rtN236	rtM250	Mutation type(rt)
12	L (50.7 %)	A (96.4 %)	T (99.9 %)	A (99.9 %)	S (99.9 %)	I (59.1 %)	I (99.8 %)	N (99.9 %)	M (99.8 %)	A181T,
	M (49 %)	**T (3.5 %)**				V (34.5 %)				N236T
14	L (81.2 %)	A (99.4 %)	T (99.9 %)	A (99.7 %)	S (99.8 %)	I (99.6 %)	I (99.7 %)	N (99.8 %)	M (99.5 %)	A181T, T184I,
	M (15.6 %)	**T (0.56 %)**							I (0.38 %)	M250L
15	L (75.3 %)	A (97.5 %)	T (99.7 %)	A (99.7 %)	S (99.7 %)	I (70.6 %)	I (99.7 %)	N (99.8 %)	M (99.6 %)	A181V,
	M (24.4 %)	S (1.5 %)				V (27.2 %)				M250L
		V (0.75 %)								
16	M (99.3 %)	A (99.7 %)	T (99.9 %)	A (99.7 %)	S (99.8 %)	V (99.5 %)	I (99.7 %)	N (99.8 %)	M (99.4 %) I (0.51 %)	A181T
	L (0.26 %)	**T (0.27 %)**		T (0.27 %)						
17	L (99.8 %)	A (99.7 %)	T (99.9 %)	A (99.9 %)	S (99.9 %)	I (80.3 %)	I (99.7 %)	N (99.8 %)	M (99.7 %)	A181T
		T (0.25 %)				M (19.5 %)				
18	L (87.9 %)	A (98.7 %)	T (99.9 %)	A (99.4 %)	S (99.5 %)	I (98.2 %)	I (99.7 %)	N (99.8 %)	M (98.9 %)	A181S
	M (11.9 %)	T (1.3 %)		T (0.55 %)		V (1.7 %)			I (0.97 %)	
19	L (99.8 %)	A (98.8 %)	T (99.9 %)	A (99.8 %)	S (99.8 %)	M (99.5 %)	I (99.6 %)	N (99.7 %)	M (99.6 %)	A181S
		T (0.89 %)								
21	L (98.8 %)	A (98.2 %)	T (99.9 %)	A (99.8 %)	S (99.8 %)	I (72.3 %)	I (99.6 %)	N (99.7 %)	M (99.6 %)	A181T
	M (0.96 %)	V (0.99 %)				M (27.0 %)				
		S (0.48 %)				V (0.49 %)				
		T (0.35 %)								
22	M (99.4 %)	A (99.8 %)	T (99.8 %)	A (99.8 %)	S (99.8 %)	V (99.8 %)	I (99.6 %)	N (99.8 %)	M (99.6 %)	A181S
23	L (87.5 %)	A (99.1 %)	T (99.9 %)	A (99.9 %)	S (99.8 %)	I (99.4 %)	I (99.8 %)	N (99.8 %)	M (99.6 %)	A181T, M250I
	M (12.3 %)	**T (0.81 %)**				M (0.48 %)			**I (0.31 %)**	

Bold values indicate emergent substitutions during combination therapy

ADV adefovir dipivoxil, *ETV* entecavir, *TDF* tenofovir disoproxil fumarate, *LAM* lamivudine, *rt* reverse transcriptase

detected at baseline. In contrast, rtT184I, rtN236T or M250I/L mutations were detected in 1 of 4 patients with emergent mutations during combination therapy.

Discussion

Although ADV plus LAM combination therapy is a standard rescue treatment for patients with LAM-refractory HBV, the virological benefits of long-term therapy have not yet been fully assessed. Here, we evaluated the long-term efficacy of ADV plus LAM combination therapy in 406 LAM-refractory patients over a median follow-up period of 5.4 years. We also investigated baseline factors associated with HBeAg clearance and HBsAg clearance. We found long-term combination therapy produced a gradual virological improvement. In particular, virological response was higher in patients who were HBeAg-negative at baseline, and genotype A and B. Toyama et al. [24] recently evaluated the long-term (median 41 months, 158 patients) efficacy of add-on ADV treatment for patients with LAM-resistant HBV and reported a rate of virological response of 90.8 % at 4 years. Inoue et al. [25] reported that HBV-DNA levels were undetectable (<2.6 log copies/mL) on long-term ADV plus LAM combination therapy (median 47 months; 28 patients, including 7 genotype B) in 56, 80, 86, and 92 % of patients at 12, 24, 36, and 48 months, respectively, whereas Aizawa et al. [26] reported undetectable levels on the same long-term regimen (median 46 months, 72 patients) in 61, 74, 81, 84, and 85 % at 12, 24, 36, 48, and 60 months, respectively, a pattern of response that was similar to our present findings. These differences in virologic response among these Japanese studies might have been due to treatment duration, genotype, or number of patients. Nevertheless, all these long-term studies in Japanese showed a gradual increase in virological response rate for 7 years, and that combination therapy with ADV plus LAM was effective for LAM-refractory patients without multidrug-resistant HBV.

The rate of HBeAg clearance at the end of follow-up in our study of 40 % was compatible with previous reports [13, 24]. The strongest predictor of HBeAg clearance on multivariate analysis was IFN history, as in a previous report [24]. Moreover, we recently reported that HBsAg clearance during NA therapy in patients with HBeAg was influenced by previous IFN therapy and HBV genotype [27]. These results suggest that previous IFN therapy might have an immunomodulatory effect on NA therapy. In addition, baseline levels of AST and bilirubin were also significantly associated with HBeAg clearance in this study. Our results agree with those of many clinical studies that have shown baseline transaminase levels to be the strongest predictor of HBeAg seroconversion in response to both IFN [11] and NA therapy [6, 28]. On the other hand, the rate of HBsAg clearance at the end of follow-up in the present study was only 1.9 %. As mentioned above, we reported that HBsAg clearance during NA therapy was influenced by previous IFN therapy and HBV genotype as well as HBsAg level at baseline or by a decrease in HBsAg level within 6 months [27]. That study [27] included patients originally treated with LAM monotherapy or ETV therapy who switched to LAM monotherapy along with ADV plus LAM combination therapy. In this regard, further study to evaluate factors affecting HBsAg clearance in ADV plus LAM combination therapy is necessary.

We previously reported the emergence of ADV-resistant mutations (rtA181T, rtA181S and rtA181T + rtN236T) in 3 of 132 patients at baseline and in 2 during subsequent combination therapy for a period of 2 years [17]. Moriconi et al. [29] reported that rtA181S and rtT184S mutations, either alone or with rtM204 mutation, at baseline in combination therapy in patients with viral breakthrough during LAM monotherapy correlated negatively with virologic response. Moreover, Heo et al. [30] reported that the presence of the rtA181V/T mutation at baseline was associated with a decreased rate of virologic response at 12 months of combination therapy. In the present study, we analyzed more patients with multidrug resistance during combination therapy over a longer clinical course. Substitutions associated with resistance to ADV or ETV were identified at baseline in 11 of 406 patients (2.7 %), most of whom were HBeAg-positive, of younger age, and had a high viral load. Moreover, a virological response during combination therapy was obtained in only four patients. On this basis, substitution of rtA181 without rtM204 mutation might correlate with a poor virological response in combination therapy. In contrast, virological response rate in patients with mutations associated with ETV (Pt. 10 and 11) was 50 %. Inoue et al. [25] detected ETV-resistant mutations of rtT184S and rtS202C during ADV plus LAM combination therapy, and noted that these patients also showed an ADV resistance profile on in vitro analysis. Moreover, a previous report showed that A181S, A181S + M204I, and L180M + T184S + M204V/I mutations were associated with a poor response to ADV plus LAM combination therapy [29]. In light of these results, A181S mutation and A181T without rtM204I/V mutation at baseline might be associated with multidrug resistance.

On the other hand, substitutions associated with resistance to ADV or ETV were identified in 12 of 395 patients (3 %) during combination therapy. Two patients (Pt. 12 and 16) in this group and a patient (Pt. 9) with rtA181T + M204V/I mutations at baseline developed rtA181T + rtN236T double mutation-related ADV resistance. Considering our clinical study, rtA181T + rtN236T

double mutation correlated with a poor virological response. Moreover, a wide variety of mutations (Pt. 12–14) might be correlated with a poor virological response. Inoue et al. reported that 1 of 28 patients developed virologic breakthrough after combination therapy and sequence analysis identified a wide variety of mutations, including L180M + A200V + M204V + N236T, L180M + A200V + M204V, L180M + M204V, L180M + T184S + M204V and L180M + S202C + M204V [25]. The replication capacity of each clone differed [25], and accordingly a wide variety of mutations might be associated with the development of multidrug resistance. Although rtA181S mutation emerged in three patients (Pt. 18, 19, 22), their HBV DNA level was sustained below 5 log copies/mL. This might be explained by the fact that two of these patients (Pt. 18, 19) had HBeAg clearance during combination therapy while the third (Pt. 22) was HBeAg-negative at baseline. In contrast, Lampertico et al. [31] reported that 9 of 145 (6 %) LAM-resistant patients developed rtA181T/V mutation before and during combination therapy for 4 years, but that HBV DNA levels progressively declined to become undetectable in 7 (78 %). In that report, however, rtA181T and rtA181V mutations were detected as a mixed population together with the wild-type sequence rtA181 in all serum samples. In our study, in contrast, rtA181S/T/V mutations were the major population and may accordingly have influenced the poor virologic response. In any case, response to combination therapy may be influenced by amino acid substitutions other than the well-known mutations associated with LAM, ADV, or ETV resistance, and further in vivo and in vitro studies are required.

Moreover, rtA181T/V mutations were detected by ultra-deep sequencing at baseline in 7 of 10 patients with emergent substitutions associated with resistance to ADV or ETV during combination therapy. It was possible that these mutant viruses increased during combination therapy. However, rtA181S, rtT184I or rtN236T or M250L were not detected at baseline. These data indicate that resistant variants of a minor population increased in some cases, whereas de novo resistant variants emerged during combination therapy in others. However, the number of patients analyzed by ultra-deep sequencing in this study was small; and we did not obtain data from patients without emergent substitutions associated with resistance during combination therapy. Further studies should be performed to interpret the significance of the presence of low frequency variants detected by ultra-deep sequencing.

In conclusion, this study shows that long-term ADV plus LAM combination therapy is effective for LAM-refractory patients. A history of IFN therapy, AST, bilirubin, and genotype were important factors in predicting HBeAg seroclearance. However, some patients did not achieve complete viral suppression of HBV DNA level (<2.6 Log copies/mL). We speculate that incomplete suppression might favor further selection of drug-resistant mutants, albeit that the frequency of multidrug resistance in the present study (5.7 %, 23/406) was low. Moreover, the presence of rtA181S mutation at baseline and emergence of rtA181T + rtN236T double mutation or a wide variety of mutations during combination therapy might be associated with a poor virological response. Several recent reports have indicated the effectiveness of TDF for ADV- or ETV-refractory patients [32–34]. Where indicated, HBV DNA and virological analysis should be carefully monitored.

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Conflict of interest The authors declare that they have no conflict of interest.

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Correlation Between Hepatitis B Virus Surface Antigen Level and Alpha-Fetoprotein in Patients Free of Hepatocellular Carcinoma or Severe Hepatitis

Norio Akuta,[1]* Fumitaka Suzuki,[1] Mariko Kobayashi,[2] Tasuku Hara,[1] Hitomi Sezaki,[1] Yoshiyuki Suzuki,[1] Tetsuya Hosaka,[1] Masahiro Kobayashi,[1] Satoshi Saitoh,[1] Kenji Ikeda,[1] and Hiromitsu Kumada[1]

[1]*Department of Hepatology and Okinaka Memorial Institute for Medical Research, Toranomon Hospital, Tokyo, Japan*
[2]*Liver Research Laboratory, Toranomon Hospital, Tokyo, Japan*

Alfa-fetoprotein (AFP) is used as a marker of early hepatocarcinogenesis. However, the impact of hepatitis B virus surface antigen (HBsAg) on this relationship in patients with HBV infection is not clear. The present study evaluated the relation between HBsAg and AFP levels at the initial visit in 1,610 untreated HBV patients, free of hepatocellular carcinoma (HCC) or severe hepatitis. The cumulative rate of HCC was significantly lower in patients with a low AFP level (≤10 μg/L; below the upper limit of normal) than in those with a high AFP level (≥11 μg/L) at the initial visit. In patients with HBsAg levels more than 500 IU/ml, HBsAg levels correlated significantly and negatively with AFP levels, and significantly with platelet count. Multivariate analysis of data of patients with HBsAg more than 500 IU/ml identified HBsAg (<7,000 IU/ml), albumin (<3.9 g/dl), platelet count ($<20.0 \times 10^4/mm^3$), gamma-glutamyl transpeptidase (≥50 IU/L), aspartate aminotransferase (≥34 IU/L), HBeAg (positive), and HBV core-related antigen (≥3.0 log U/ml) as determinants of a high AFP. Especially, in patients with HBsAg more than 500 IU/ml and low transaminase levels (below the upper limit of normal), HBsAg was identified as significant determinant of a high AFP. On the other hand, in patients with HBsAg less than 500 IU/ml, multivariate analysis identified albumin, gamma-glutamyl transpeptidase, and HBV core-related antigen as determinants of a high AFP. The results indicated that HBsAg level seems to affect, at least in part, the AFP levels, and that it can be used as a surrogate marker of early hepatocarcinogenesis. ***J. Med. Virol. 86:131–138, 2014.*** © 2013 Wiley Periodicals, Inc.

KEY WORDS: HBV; AFP; HBsAg; HBcrAg; genotype; hepatocellular carcinoma

INTRODUCTION

Hepatitis B virus (HBV) is a small, enveloped DNA virus known to cause chronic hepatitis and often leads to liver cirrhosis and hepatocellular carcinoma (HCC) [Viola et al., 1981; Kobayashi et al., 2002; Yao, 2003]. Evidence suggests that the use of elevated alpha-fetoprotein (AFP) for the prediction of early hepatocarcinogenesis in non-HCC patients could be clinically useful. AFP is a fetal glycoprotein produced by the yolk sac and fetal liver [Bergstrand and Czar, 1956] and has been widely used as a serum marker for the diagnosis of HCC [Sato et al., 1993; Johnson, 2001]. Furthermore, high serum AFP levels are also associated with various chronic liver diseases and hepatic regeneration [Kew et al., 1973; Silver et al., 1974; Elftherious et al., 1977; Alpert and Feller, 1978]. Many patients with chronic hepatitis B who are free of HCC have high AFP levels [Chen and Sung, 1979; Di Bisceglie and Hoofnagle, 1989], and some patients with cirrhosis and concomitant high

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*Correspondence to: Norio Akuta, M.D., Department of Hepatology, Toranomon Hospital, 2-2-2 Toranomon, Minato-ku, Tokyo 105-0001, Japan.
E-mail: akuta-gi@umin.ac.jp

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inflammatory activity have very high AFP levels [Yao, 2003; Cheema et al., 2004]. On the other hand, some patients with small HCC lesions have only moderately elevated levels of AFP [Shinagawa et al., 1984; Ebara et al., 1986; Bruix and Sherman, 2005]. At present, however, there are no cutoff levels for serum AFP used to predict HCC in patients with HBV infection.

There is growing interest in the use of hepatitis B surface antigen (HBsAg) level as a prognostic marker in chronic hepatitis B patients [Chan et al., 2010]. The HBsAg levels are useful for identifying the stage of disease [Jaroszewicz et al., 2010; Nguyen et al., 2010], to distinguish true inactive carriers from patients with HBe antigen-negative disease [Brunetto et al., 2010; Martinot-Peignoux et al., 2010; Chan et al., 2011; Liaw, 2011], and to predict the response to interferon therapy [Brunetto et al., 2009; Moucari et al., 2009]. Recent studies has also demonstrated that the HBsAg levels are associated with the risk of progression to HCC, especially in patients with low HBV DNA levels [Chan, 2012; Tseng et al., 2012], and that there is a potential correlation between the HBsAg levels and the stage of liver fibrosis [Seto et al., 2012; Martinot-Peignoux et al., 2013]. However, the impact of viral factors, such as the HBsAg level, on serum AFP level as a predictor of early HCC is not clear at present.

The present study included 1,610 untreated patients with HBV infection, free of HCC or severe hepatitis. The present study was designed to provide answers to the following questions: (1) what is the relation between a high serum AFP level at the initial outpatient visit and subsequent development of hepatocarcinogenesis in antiviral-therapy-naive patients with hepatitis B viral infection? (2) What is the impact of viral factors, such as the HBsAg level, on serum AFP level in such patients, and (3) What is a good surrogate marker for a high serum AFP at the initial visit.

PATIENTS AND METHODS

Patients

Among 6,466 consecutive patients who were diagnosed with HBV infection between March 1972 and December 2012 at Toranomon Hospital, 1,610 were selected in the present study based on the following criteria: (1) They were positive for HBsAg (radioimmunoassay, Dainabot, Tokyo, Japan) and negative for anti-HCV (third-generation enzyme immunoassay, Chiron, CA). (2) They were free of HCC at the initial visit. (3) HBV hepatitis was assessed as less than severe at the initial visit, in order to minimize the potential effects of high inflammatory activity. Severe hepatitis was defined as serum transaminase level of $\geq$300 IU/L, and/or total bilirubin level of $\geq$3.0 mg/dl. (4) They had not received antiviral therapy in the past (e.g., interferon and/or nucleot(s)ide analogs) at the initial visit. (5) They underwent examination of the AFP level (upper limit of normal, 10 μg/L) at the initial visit. Furthermore, the HBsAg level, HBV core-related antigen (HBcrAg) level, and HBV DNA were also assayed using stored frozen sera obtained at the initial visit. (6) They were free of coinfection with human immunodeficiency virus. (7) They were free of other types of chronic liver disease, including hemochromatosis, Wilson disease, primary biliary cirrhosis, alcoholic liver disease, autoimmune liver disease, inherited liver disease including alpha-1 antitrypsin deficiency, and hepatic venous outflow block. (8) They consented to the study.

Table I summarizes the profile and laboratory data at the initial visit of the 1,610 patients included in the present study. They included 1,047 males and 563 females, with a median age of 40 years (range: 18–83 years). The median AFP level was 4 μg/L (range, 1–1,770 μg/L) and the median follow-up time (from the initial visit until the last visit) was 6.0 years (range, 0.0–34.6 years). The study protocol was approved by the Human Ethics Review Committee of Toranomon Hospital.

Laboratory Tests

HBsAg, HBcrAg, and HBV DNA levels were assayed using stored frozen sera obtained at the initial visit. Blood samples were frozen at −80°C within 4 hr of collection and were not thawed until used for testing. Serum HBsAg level was measured using Architect HBsAg QT assay kit (Abbott Laboratories, Tokyo, Japan), which has a lower limit of detection of

TABLE I. Profiles and Laboratory Data at the Initial Visit of 1,610 Patients Infected With HBV

Demographic data	
Number of patients	1,610
Sex (male/female)	1,047/563
Age (years)*	40 (18–83)
Family history of liver disease[a]	836 (51.9%)
Lifetime cumulative alcohol intake (≥500 kg)	112 (7.0%)
Laboratory data*	
Total bilirubin (mg/dl)	0.6 (0.1–2.9)
Aspartate aminotransferase (IU/L)	37 (5–220)
Alanine aminotransferase (IU/L)	48 (5–297)
Albumin (g/dl)	4.2 (1.0–5.6)
Gamma-glutamyl transpeptidase (IU/L)	37 (2–2,370)
Hemoglobin (g/dl)	14.5 (6.9–18.2)
Platelet count (×104/mm^3)	19.1 (2.7–44.7)
Alpha-fetoprotein (μg/L)	4 (1–1,770)
Virological data	
HBeAg (No. of positive)	690 (42.9%)
HBsAg (IU/ml)*	2,845 (0.09 to >125,000)
HBcrAg (log U/ml)*	4.9 (<3.0 to >6.8)
HBV DNA (log copies/ml)*	5.7 (<2.1 to >9.1)
HBV genotype (A/B/C/others/ND)	65/218/1,119/6/202

Data are number and percentages of patients, except those denoted by *, which represent the median (range) values.
[a]Family history of positivity for hepatitis B surface antigen including third-degree relatives.

0.05 IU/ml and upper limit of detection of 250 IU/ml. To expand the upper range from 250 to 125,000 IU/ml, serum samples with the HBsAg levels above the upper range were diluted in a stepwise fashion to 1:20 and 1:500 with Architect diluents using the information supplied by the manufacturer. HBeAg was determined by enzyme-linked immunosorbent assay kit (HBeAg EIA; Institute of Immunology, Tokyo, Japan). Serum HBcrAg level was measured using a Cleia HBcrAg assay kit (Fujirebio, Tokyo, Japan) using a fully automated analyzer system (Lumipulse System; Fujirebio). The cut-off value of HBcrAg was 3.0 log U/ml. HBV DNA was quantified using the Cobas TaqMan HBV v.2.0 (Roche Diagnostics, Tokyo, Japan), which has a dynamic range of 2.1–9.0 log copies/ml.

A commercial kit (HBV Genotype EIA; Institute of Immunology) was used to determine serologically the HBV genotypes using the combination of epitopes expressed on the pre-S2 region product, which is specific to each of the major genotypes.

Follow-Up and Diagnosis of Future Hepatocellular Carcinoma

After the initial visit, patients were followed-up once or three times a month. Imaging studies (ultrasonography, computed tomography, or magnetic resonance imaging) were conducted once or more per year.

Statistical Analysis

Non-parametric tests (Mann–Whitney *U*-test, chi-squared test and Fisher's exact probability test) were used to compare differences between two groups. Correlation analysis was evaluated by the Spearman rank correlation test. The cumulative rate of hepatocarcinogenesis was calculated using the Kaplan–Meier technique; differences between cumulative carcinogenesis curves between groups were tested using the log-rank test. Statistical analyses of the rate of hepatocarcinogenesis according to groups were calculated using the period from the initial visit. Univariate and multivariate logistic regression analyses were used to determine the independent surrogate markers of elevated AFP at the initial visit. The odds ratios (OR) and 95% confidence intervals (95% CI) were also calculated. A two-tailed *P*-value less than 0.05 was considered significant. Variables that achieved statistical significance ($P < 0.05$) on univariate analysis were entered into multiple logistic regression analysis to identify significant independent factors for elevated AFP. Potential surrogate markers of elevated AFP at the initial visit included the following pretreatment variables: age, sex, family history of liver disease, lifetime cumulative alcohol intake, total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, gamma-glutamyl transpeptidase (GGT), hemoglobin, platelet count, HBV genotype, HBeAg, HBsAg levels, HBcrAg levels, and HBV DNA levels. Statistical analyses were performed using the Statistical Package for Social Sciences software (SPSS, Inc., Chicago, IL).

RESULTS

Cumulative Rate of Hepatocarcinogenesis According to the AFP Level at the Initial Visit

A total of 1,061 patients naïve to antiviral therapy from the initial visit until the last visit were evaluated for the rate of development of HCC based on the AFP levels at the initial visit. During the follow-up period, HCC was diagnosed in 31 of 905 patients (3.4%) with a low AFP level (≤10 μg/L; below the upper limit of normal) and 37 of 156 patients (23.7%) with a high AFP level (≥11 μg/L) at the initial visit. The cumulative hepatocarcinogenesis rates for patients with low and high AFP levels at the initial visit were 4.7% and 30.2% at the end of 10-year follow-up; 9.1% and 36.5% at the end of 20-year follow-up; and 13.2% and 42.9% at the end of 30-year follow-up, respectively. These results indicate that the rate of hepatocarcinogenesis is significantly higher in patients with HBV infection and high AFP levels than their counterparts with low AFP levels ($P < 0.001$; Log-rank test) (Fig. 1).

HBsAg and AFP Levels at the Initial Visit

Blood samples from all patients were analyzed to determine the relationship between the HBsAg and the AFP levels at the initial visit. The proportions of patients with high AFP levels among those with the HBsAg levels below 500 IU/ml, from 500 to 1,999 IU/ml, from 2,000 to 6,999 IU/ml, from 7,000 to 24,999 IU/ml, and above 25,000 IU/ml were 12.6% (42 of 333 patients), 26.7% (89 of 333), 22.6% (94 of 416), 10.4% (29 of 278), and 6.4% (16 of 250), respectively (Fig. 2A). The relationship between the HBsAg and

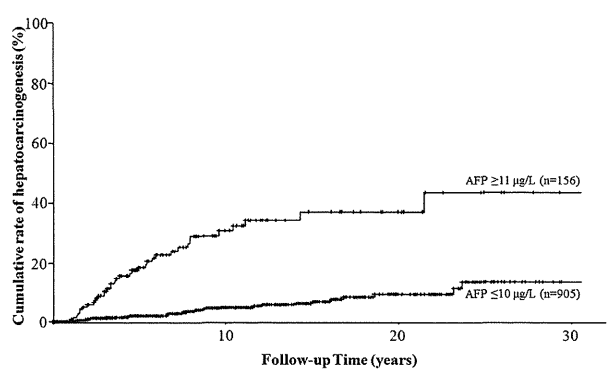

Fig. 1. Cumulative rate of hepatocarcinogenesis according to the AFP level at the initial visit in patients naïve to antiviral therapy from the initial visit until the last visit. The rate of hepatocarcinogenesis was significantly higher in patients with high AFP levels (≥11 μg/L) than in those with low levels (≤10 μg/L) at the initial visit ($P < 0.001$; Log-rank test).

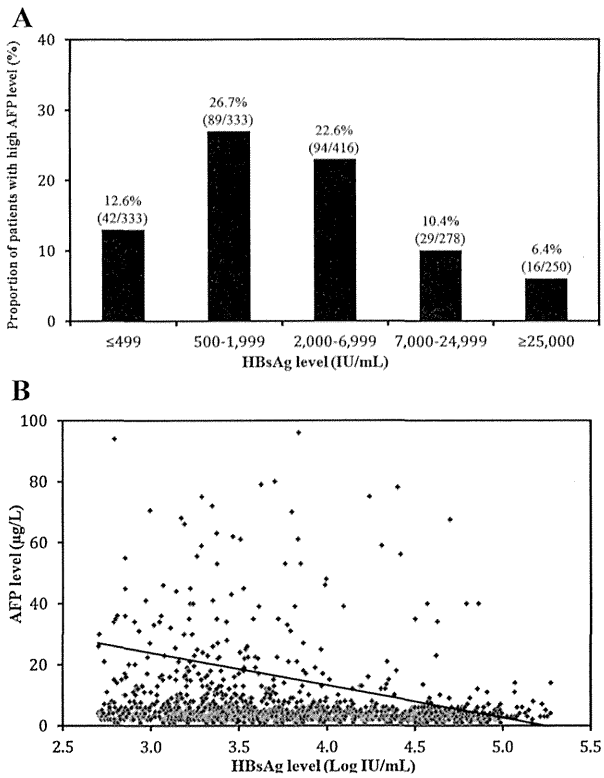

Fig. 2. **A**: Proportions of patients with the high AFP levels (≥11 μg/L) at the initial visit, stratified according to the HBsAg levels. Patients with the HBsAg levels above 500 IU/ml included a significantly lower proportions of patients with the high AFP levels and the HBsAg levels above 7,000 IU/ml (8.5%) than those with the HBsAg levels below 7,000 IU/ml (24.4%) ($P < 0.001$). **B**: Analysis of data of patients with the HBsAg levels above 500 IU/ml at the initial visit, showed a significant negative correlation between logarithmically transformed HBsAg and AFP levels ($r = -0.225$, $P < 0.001$).

the AFP levels at the initial visit suggested the presence of two distinct populations within the study group. Especially, in 1,277 patients with the HBsAg levels above 500 IU/ml, a significantly smaller proportion of patients with high AFP levels were noted among those with HBsAg of more than 7,000 IU/ml (8.5%) than those with the HBsAg levels less than 7,000 IU/ml (24.4%) ($P < 0.001$). Furthermore, the HBsAg levels correlated negatively but significantly with the AFP levels ($r = -0.225$, $P < 0.001$) (Fig. 2B).

The HBsAg Levels and the Platelet Count at the Initial Visit

Blood samples from all patients were analyzed to determine the relationship between the HBsAg levels and the platelet count at the initial visit. The median platelet counts among patients with the HBsAg levels below 500 IU/ml, from 500 to 1,999 IU/ml, from 2,000 to 6,999 IU/ml, from 7,000 to 24,999 IU/ml, and above 25,000 IU/ml were $19.1 \times 10^4/mm^3$, $17.2 \times 10^4/mm^3$, $18.0 \times 10^4/mm^3$, $20.9 \times 10^4/mm^3$, and $21.2 \times 10^4/mm^3$, respectively (Fig. 3A). The relationship between the HBsAg levels and the platelet count at the initial visit also suggested the presence of two distinct populations within the study group. Especially, in 1,277 patients with the HBsAg levels of more than 500 IU/ml, significantly higher platelet counts were noted among those with the HBsAg levels of more than 7,000 IU/ml (the median platelet count; $21.0 \times 10^4/mm^3$) than those with the HBsAg levels less than 7,000 IU/ml (the median platelet count; $17.6 \times 10^4/mm^3$) ($P < 0.001$). Furthermore, the HBsAg

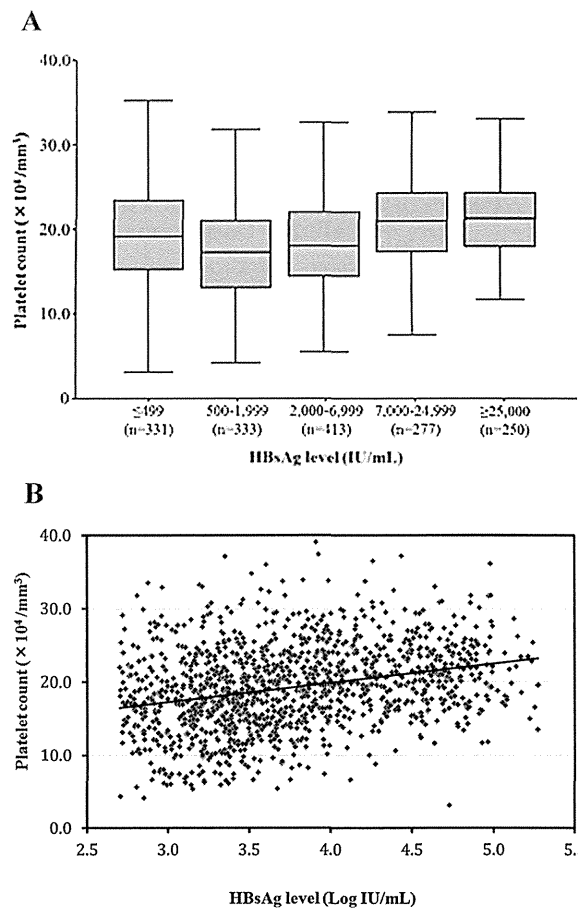

Fig. 3. **A**: The platelet count at the initial visit, stratified according to the HBsAg levels. Bars within the boxes indicate the median platelet count. The boxes denote the 25th to 75th percentiles, the lower and upper bars the 10th and 90th percentiles, respectively. Among patients with the HBsAg levels above 500 IU/ml at the initial visit, those with the HBsAg levels above 7,000 IU/ml had significantly higher platelet count (the median platelet count; $21.0 \times 10^4/mm^3$) compared to those with the HBsAg levels below 7,000 IU/ml (the median platelet count; $17.6 \times 10^4/mm^3$) ($P < 0.001$). **B**: Among patients with the HBsAg levels above 500 IU/ml at the initial visit, logarithmically transformed the HBsAg levels correlated significantly with the platelet count ($r = 0.293$, $P < 0.001$).

levels correlated significantly and positively with the platelet count ($r = 0.293$, $P < 0.001$) (Fig. 3B).

Clinical Profiles and Laboratory Data According to the HBsAg Level at the Initial Visit

Table II summarizes the clinical profiles and laboratory data according to the HBsAg level at the initial visit of 1,610 patients infected with HBV. Patients with the HBsAg levels below 500 IU/ml were significantly older and exhibited lower inflammatory activity (lower levels of AST and ALT), and had lower viral levels (they were HBeAg negative and had lower levels of HBcrAg/HBV DNA), compared to those with the HBsAg levels above 500 IU/ml ($P < 0.001$).

Factors Associated With High AFP Levels at the Initial Visit, Stratified According to the HBsAg Levels

Blood samples from all 1,610 patients were analyzed to determine the factors that affect the AFP level at the initial visit. Among 1,277 patients with the HBsAg levels more than 500 IU/ml at the initial visit, high AFP levels were detected in 228 (17.9%) patients. Univariate analysis identified 12 parameters that correlated significantly with a high AFP level at the initial visit. These included age (≥30 years; $P < 0.001$), AST (≥34 IU/L; $P < 0.001$), ALT (≥43 IU/L; $P < 0.001$), albumin (<3.9 g/dl; $P < 0.001$), GGT (≥50 IU/L; $P < 0.001$), total bilirubin (≥1.0 mg/dl; $P < 0.001$), platelet count ($<20.0 \times 10^4/mm^3$; $P < 0.001$), HBV genotype (C; $P < 0.001$), HBsAg levels (<7,000 IU/ml; $P < 0.001$), HBeAg (positive; $P < 0.001$), HBV DNA (≥5.0 log copies/ml; $P < 0.001$), and HBcrAg (≥3.0 log U/ml; $P < 0.001$). Multivariate analysis that included the above variables identified seven factors that influenced independently the elevated AFP level at the initial visit. These included HBsAg level (<7,000 IU/ml; OR 3.69, $P < 0.001$), albumin (<3.9 g/dl; OR 3.09, $P < 0.001$), platelet count ($<20.0 \times 10^4/mm^3$; OR 2.50, $P = 0.001$), GGT (≥50 IU/L; OR 2.28, $P = 0.001$), AST (≥34 IU/L; OR 2.77, $P = 0.003$), HBeAg (positive; OR 2.07, $P = 0.005$), and HBcrAg (≥3.0 log U/ml; OR 5.10, $P = 0.031$) (Table III).

Among 333 patients with the HBsAg levels less than 500 IU/ml, a high AFP at the initial visit was detected in 42 (12.6%) patients. Univariate analysis identified nine parameters that correlated significantly with a high AFP level at the initial visit. These included AST (≥34 IU/L; $P < 0.001$), ALT (≥43 IU/L; $P = 0.001$), albumin (<3.9 g/dl; $P < 0.001$), GGT (≥50 IU/L; $P < 0.001$), platelet count ($<20.0 \times 10^4/mm^3$; $P = 0.001$), HBV genotype (C; $P < 0.001$), HBeAg (positive; $P < 0.001$), HBV DNA (≥5.0 log copies/ml; $P = 0.001$), and HBcrAg (≥3.0 log U/ml; $P < 0.001$). Multivariate analysis that included the above variables identified three factors that influenced independently the elevated AFP level at the initial visit. These included albumin (<3.9 g/dl; OR 12.8, $P < 0.001$), GGT (≥50 IU/L; OR 6.95, $P = 0.002$), and HBcrAg (≥3.0 log U/ml; OR 5.62, $P = 0.010$) (Table III).

Factors Associated With High AFP Levels at the Initial Visit According to the HBsAg Levels in Patients With Low Transaminase Levels

To minimize the effect of inflammatory activity, we examined the data of 618 (among 1,610 patients) who

TABLE II. Profiles and Laboratory Data of Patients Infected With HBV According to the HBsAg Level at the Initial Visit

	HBsAg <500 IU/L	HBsAg ≥500 IU/L	*P*
Demographic data			
Number of patients	333	1,277	
Sex (male/female)	227/106	820/457	NS
Age (years)*	49 (18–75)	38 (18–83)	<0.001
Family history of liver disease[a]	130 (39.0%)	706 (55.3%)	<0.001
Lifetime cumulative alcohol intake (≥500 kg)	32 (9.6%)	80 (6.3%)	0.037
Laboratory data*			
Total bilirubin (mg/dl)	0.7 (0.2–2.9)	0.6 (0.1–2.9)	0.033
Aspartate aminotransferase (IU/L)	29 (12–175)	40 (5–220)	<0.001
Alanine aminotransferase (IU/L)	32 (7–289)	56 (5–297)	<0.001
Albumin (g/dl)	4.2 (1.1–5.6)	4.2 (1.0–5.5)	NS
Gamma-glutamyl transpeptidase (IU/L)	36 (2–2,370)	38 (4–1,638)	NS
Hemoglobin (g/dl)	14.4 (8.4–17.4)	14.6 (6.9–18.2)	NS
Platelet count ($\times 10^4/mm^3$)	19.1 (2.7–39.6)	19.2 (3.1–44.7)	NS
Alpha-fetoprotein (μg/L)	4 (1–968)	4 (1–1,770)	0.005
Virological data			
HBeAg (No. of positive)	37 (11.1%)	653 (51.1%)	<0.001
HBsAg (IU/ml)*	123 (0.09–498)	4,680 (503 to >125,000)	<0.001
HBcrAg (log U/ml)*	<3.0 (<3.0 to >6.8)	5.9 (<3.0 to >6.8)	<0.001
HBV DNA (log copies/ml)*	3.7 (<2.1 to >9.1)	6.6 (<2.1 to >9.1)	<0.001
HBV genotype (A/B/C/others/ND)	7/104/141/0/81	58/114/978/6/121	<0.001

NS; not significant.
Data are number/percentages of patients, except those denoted by *, which represent the median (range) values.
[a]Family history of positivity for hepatitis B surface antigen including third-degree relatives.

TABLE III. Results of Multivariate Logistic Analysis for Factors Associated With the High AFP Levels at the Initial Visit

Factor	Category	Risk ratio (95%CI)	*P*
Patients with the HBsAg levels above 500 IU/ml (n = 1,277)			
HBsAg (IU/ml)	1: ≥7,000	1	
	2: <7,000	3.69 (2.12–6.41)	<0.001
Albumin (g/dl)	1: ≥3.9	1	
	2: <3.9	3.09 (1.88–5.05)	<0.001
Platelet count ($\times 10^4$/mm^3)	1: ≥20.0	1	
	2: <20.0	2.50 (1.47–4.24)	0.001
Gamma-glutamyl transpeptidase (IU/L)	1: <50	1	
	2: ≥50	2.28 (1.40–3.72)	0.001
Aspartate aminotransferase (IU/L)	1: <34	1	
	2: ≥34	2.77 (1.42–5.39)	0.003
HBeAg	1: Negative	1	
	2: Positive	2.07 (1.24–3.45)	0.005
HBcrAg (log U/ml)	1: <3.0	1	
	2: ≥3.0	5.10 (1.16–22.4)	0.031
Patients with the HBsAg levels below 500 IU/ml (n = 333)			
Albumin (g/dl)	1: ≥3.9	1	
	2: <3.9	12.8 (4.02–41.7)	<0.001
Gamma-glutamyl transpeptidase (IU/L)	1: <50	1	
	2: ≥50	6.95 (2.06–23.5)	0.002
HBcrAg (log U/ml)	1: <3.0	1	
	2: ≥3.0	5.62 (1.51–21.0)	0.010

Low transaminase levels were defined as transaminase levels below the upper limit of normal.

had low transaminase levels (AST ≤33 IU/L and ALT ≤42 IU/L, i.e., below the upper limits of normal) to further determine those factors that determine the high level of AFP at the initial visit. High AFP was detected in 26 (6.1%) patients among 426 with the HBsAg levels above 500 IU/ml and low transaminase levels. Using the data of these patients, univariate analysis identified three parameters that correlated significantly with a high AFP level at the initial visit. These included albumin (<3.9 g/dl; $P = 0.004$), platelet count (<20.0 × 10^4/mm^3; $P = 0.012$), and HBsAg levels (<7,000 IU/ml; $P = 0.004$). Multivariate analysis that included the above variables identified albumin (<3.9 g/dl; OR 3.92, $P = 0.001$) and HBsAg levels (<7,000 IU/ml; OR 4.33, $P = 0.004$) as independent determinants of a high AFP level at the initial visit (Table IV).

Among 192 patients with the HBsAg levels below 500 IU/ml and low transaminase levels, high AFP levels were detected at the initial visit in 12 (6.3%). Univariate analysis identified three parameters that influenced significantly the elevated AFP level at the initial visit. These included albumin (<3.9 g/dl; $P = 0.010$), GGT (≥50 IU/L; $P = 0.011$), and platelet count (<20.0 × 10^4/mm^3; $P = 0.020$). Multivariate analysis that included these variables identified albumin (<3.9 g/dl; OR 7.19, $P = 0.004$) as the only independent determinant of a high AFP level at the initial visit (Table IV).

TABLE IV. Results of Multivariate Analysis for Factors Associated With the High AFP Levels at the Initial Visit

Factor	Category	Risk ratio (95%CI)	*P*
Patients with HBsAg >500 IU/ml and low transaminase levels (n = 426)			
Albumin (g/dl)	1: ≥3.9	1	
	2: <3.9	3.92 (1.71–9.01)	0.001
HBsAg (IU/ml)	1: ≥7,000	1	
	2: <7,000	4.33 (1.58–11.9)	0.004
Patients with HBsAg <500 IU/ml and low transaminase levels (n = 192)			
Albumin (g/dl)	1: ≥3.9	1	
	2: <3.9	7.19 (1.87–27.8)	0.004

Low transaminase levels were defined as transaminase levels below the upper limit of normal.

DISCUSSION

There is little information on the cutoff value of AFP that can be used to predict the future probability of HCC in patients with HBV infection. The present study followed-up patients naïve to antiviral therapy from the initial visit and showed that the rate of hepatocarcinogenesis was significantly higher in those with high AFP levels at the baseline than those with low levels. To our knowledge, the present study is the first to report the hepatocarcinogenesis rate stratified according to the AFP level in patients infected with HBV but free of HCC at the initial visit, based on a large-scale long-term follow-up cohort. The results indicated that patients with high AFP levels at the initial visit are at high risk of HCC, and emphasize the need to determine the factors that could affect the AFP level as surrogate markers of early hepatocarcinogenesis. Previous studies in patients with HCV infection indicated that suppression of the AFP level by treatment with interferon reduced the HCC risk even in those without complete eradication of HCV [Arase et al., 2007; Asahina et al., 2013]. However, there is little

evidence that suppression of the AFP level by antiviral therapy reduces the HCC risk in patients with HBV infection. Further prospective studies are needed to investigate this issue in detail.

In the present study, the relationship between the HBsAg levels and the AFP levels detected at the initial visit suggested the presence of two distinct groups within the study patients. Interestingly, in patients with the HBsAg levels above 500 IU/ml, a significant negative correlation was observed between the HBsAg and the AFP levels, and a significant positive correlation was observed between the HBsAg and the platelet count. Previous studies indicated that high serum AFP levels correlated with liver fibrosis Stage 3 and 4 [Bayati et al., 1998; Chu et al., 2001; Hu et al., 2002, 2004], and that lower thrombocytopenia was closely associated with advanced liver disease [Ikeda et al., 2009; Akuta et al., 2012]. Considered together, these results emphasize the importance of hyper-α-fetoproteinemia and thrombocytopenia in the prediction of severe liver fibrosis, respectively. Based on the present results and the recent reports suggesting the potential correlation between the HBsAg level and the stage of liver fibrosis [Seto et al., 2012; Martinot-Peignoux et al., 2013], it is possible that HBsAg levels could correlate with the stage of fibrosis in patients with the HBsAg levels above 500 IU/ml. Further studies are needed to determine the value of hyper-α-fetoproteinemia in patients with low and high HBsAgemia.

In addition to the HBsAg level, multivariate analysis also identified HBcrAg as another viral factor that influenced independently the AFP level at the baseline. HBcrAg comprises HBcAg, HBeAg and a 22-kDa precore protein coded with the precore/core gene [Kimura et al., 2002, 2005]. Previous studies reported a significant correlation between serum HBcrAg concentrations and intrahepatic levels of covalently closed circular DNA (cccDNA) [Wong et al., 2007; Suzuki et al., 2009]. Other studies indicated that HBcrAg is a useful predictor of HCC during antiviral therapy [Kumada et al., 2013], and post-treatment recurrence of HCC during antiviral therapy [Hosaka et al., 2010]. The present study, based on patients naïve to antiviral therapy showed that high serum HBcrAg concentrations also correlated with high AFP at the initial visit. This is the first report demonstrating the potential usefulness of HBcrAg as a surrogate marker for early hepatocarcinogenesis.

The impact of the HBsAg level on hepatocarcinogenesis is not clear at this stage. In this study, the effect of the HBsAg levels at the initial visit on HCC was assessed in 1,061 consecutive antiviral therapy-naive patients infected with HBV. Analysis of data of 794 patients with the HBsAg levels above 500 IU/ml at the initial visit (after exclusion of patients on antiviral therapy) showed a significantly lower cumulative HCC rate in patients with the HBsAg levels above 7,000 IU/ml than those with levels below 7,000 IU/ml ($P < 0.001$, Log-rank test, Fig. 4). This result suggests that HBsAg levels at the baseline do not only influence AFP, but also play a role in hepatocarcinogenesis. Further studies need to be performed to determine the pathomechanisms of HBsAg in hepatocarcinogenesis.

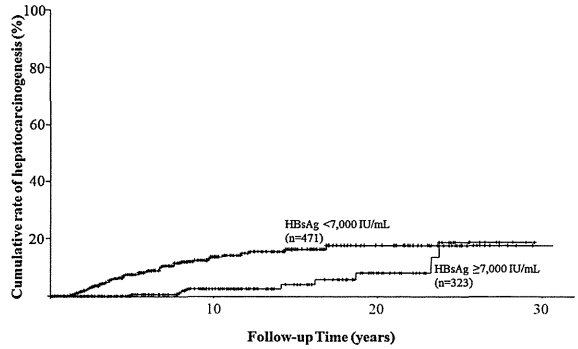

Fig. 4. Cumulative rate of hepatocarcinogenesis stratified according to the HBsAg levels at the initial visit in patients naïve to antiviral therapy from the initial visit until last visit. In a preliminary study based on 794 patients with the HBsAg levels above 500 IU/ml at the initial visit, the cumulative hepatocarcinogenesis rate for patients with the HBsAg levels more than 7,000 IU/ml was significantly lower than for those with levels below 7,000 IU/ml ($P < 0.001$; Log-rank test).

The present study has certain limitations. First, the study did not examine the effects of other genotypes, apart from HBV genotype B or C. Second, the study population was limited to Japanese and did not include other races, and thus generalization of the results to other races cannot be made based on the results. Third, the study did not investigate the effects of antiviral therapy (interferon and/or nucleot(s)ide analogs) on the outcome since such therapy suppressed the AFP levels and thus reduce the risk of HCC in patients with HBV infection.

In conclusion, the present studies demonstrated that the HBsAg level seem to influence the AFP levels and can be used as a surrogate marker for early hepatocarcinogenesis in patients with hepatitis B viral infection.

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