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Original Article

Effects of structural variations of *APOBEC3A* and *APOBEC3B* genes in chronic hepatitis B virus infection

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Aim: Human APOBEC3 deaminases induce G to A hypermutation in nascent DNA strand of hepatitis B virus (HBV) genomes and seem to operate as part of the innate antiviral immune system. We analyzed the importance of APOBEC3A (A3A) and APOBEC3B (A3B) proteins, which are potent inhibitors of adeno-associated-virus and long terminal repeat (LTR)-retrotransposons, in chronic HBV infection.

Methods: We focused on the common deletion polymorphism that spans from the 3′ part of *A3A* gene to the 3′ portion of *A3B* gene. An association study was carried out in 724 HBV carriers and 469 healthy control subjects. We also analyzed hypermutated genomes detected in deletion and insertion (non-deletion) homozygous patients to determine the effect of *APOBEC3* gene deletion. Further, we performed functional analysis of *A3A* gene by transient transfection experiments.

Results: The association study showed no significant association between deletion polymorphism and chronic HBV carrier state. Context analysis also showed a negligible effect for the deletion. Rather, mild liver fibrosis was associated with *APOBEC* gene deletion homozygosity, suggesting that *A3B* deletion is not responsible for chronic HBV infection. Functional analysis of A3A showed that overexpression of *A3A* induced hypermutation in HBV genome, although the levels of hypermutants were less than those introduced by *A3G*. However, overexpression of *A3A* did not decrease replicative intermediates of HBV.

Conclusion: These results suggest that A3A and A3B play little role in HBV elimination through anti-viral defense mechanisms. The significance of hypermutation induced by A3A should be investigated further.

Key words: APOBEC3A, APOBEC3B, APOBEC3G, deaminase, hypermutation, structural variation

INTRODUCTION

APOBEC3 CYTIDINE DEAMINASE family consists of at least seven tandem arrayed genes *APOBEC3A (A3A), A3B, A3C, A3DE, A3F, A3G,* and *A3H* on chromosome 22.[1,2] The anti-viral effect of A3G was initially identified in 2002 when it was found to inhibit the replication of human immunodeficiency virus (HIV).[3] Similarly, A3F, A3B and A3DE have been reported to inhibit HIV replication.[4-8]

APOBEC3 proteins also act on many other viruses such as simian immunodeficiency virus,[9] adeno-associated virus[10] and retrotransposons.[11-13] With regard to hepatitis B virus (HBV), A3G was also reported to inhibit HBV replication and induction of hypermutation, although the significance of the latter on viral inactivation is still controversial.[14-23] Among the APOBEC3 family members, A3B, A3C, A3G and A3F have been

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extensively analyzed in these reports for induction of hypermutation and inhibition of replication of HBV. In contrast, the function of A3A on HBV has not been evaluated despite its potent inhibitory effects on adeno-associated virus and retrotransposons.[9–13] Recently, Henry *et al.*[24] reported that, among the APOBEC3 family, A3A is the most efficient editor in induction of hypermutation in the HBV genome. This finding is not consistent with the previous reports. However, the relationship between genomic DNA editing by A3A and its effect on HBV replication have not been elucidated. This background prompted us to examine the effects of A3A on HBV replication and induction of hypermutation.

A recent study[25] identified a common deletion polymorphism of *APOBEC* gene spanning from the 3′ end of *A3A* gene to the 3′ portion of *A3B* gene (the segment extending from exon 5 of *A3A* to exon 8 of *A3B* was removed by the deletion, positions 37, 683, 131–37, 712, 716 on chromosome 22). The deletion results in complete elimination of the *A3B* coding region and the resultant fusion gene has a protein sequence identical to A3A, but has 3′ untranslated region of A3B. This polymorphism might modulate the expression levels of A3A peptide because the transcription levels and stability of this fusion mRNA could be altered by replacement of the 3′ untranslated region sequences. Analyzing the association between this deletion polymorphism and chronic HBV infection should clarify the effect of *A3B* on the establishment of chronic HBV carrier state.

The aims of the present study were to determine the association between *APOBEC3* gene deletion polymorphism and chronic HBV infection and the effect of *A3A*, which might be up- or down-regulated by the deletion polymorphism, on HBV replication and induction of hypermutation, by *in-vitro* overexpression experiments.

PATIENTS AND METHODS

Study subjects

BLOOD SAMPLES WERE obtained from 724 patients with chronic HBV infection at the hospitals of the Hiroshima Liver Study Group (http://home.hiroshima-u.ac.jp/naika1/hepatology/english/study.html) and Toranomon hospital. We also collected 469 control samples from healthy individuals who agreed to join the BioBank Japan Project at the Institute of Medical Science, the University of Tokyo. The study protocols were approved by the ethics committees of the University of Tokyo and the Center for Genomic Medicine, Riken. All participants were ethnically Japanese and provided written informed consent. Histological activity and fibrosis was assessed in liver biopsy specimens by the Metavir score.[26]

HBV markers

We measured DNA polymerase by the method of Robinson *et al.*[27] The quantity of HBV DNA was assessed by the following tests. Quantiplex HBV DNA probe assay (Chiron Corporation, Emeryville, CA), PCR (Amplicor Cobas TaqMan HBV Auto; Roche Molecular Diagnostic, Basel), transcription mediated amplification (TMA) assay (Fujirevio Diagnostic, Tokyo). The level of HBV in serum was assessed as high or low according to the following criteria (< 200 or ≥ 200 for DNA polymerase, < 200 or ≥ 200 for probe assay, < 6.0 or ≥ 6.0 for PCR assay, < 6.0 or ≥ 6.0 for TMA assay).

HBV-e antigen (HBeAg) and HBV-e antibody (HBeAb) were measured by commercially available chemiluminescent enzyme immunoassay kit (Abbott Laboratories, Chicago, IL). The cut off levels were 1.0 (cut off index) for HBeAg and 70% for HBeAb.

Genotyping

First, we genotyped genomic samples of 94 individuals by the PCR assay using the Deletion and Insertion specific primer sets reported by Kidd *et al.*[25] Since we observed some non-specific amplification, which was confirmed by sequencing analysis, we used the invader probes,[28] which specifically recognize *A3A* and *A3B*. These probes were designed and synthesized by Third Wave Technologies (Madison, WI). Deletion and two-insertion (non-deletion) PCR assays were performed separately as described previously,[25] then pooled (Deletion : Insertion1 : Insertion2 = 3:1:1), and subjected to Invader assay.

Cell culture and transfection

Human liver cancer cell line, HepG2, was purchased from RIKEN Cell Bank (Tsukuba). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C under 5% CO_2. Cells were seeded to semi-confluence in six-well tissue culture plates. Transient transfection experiments were performed using TransIT-LT1 (Mirus, Madison, WI) according to the instructions provided by the supplier.

Plasmid construction

The expression vector for hemagglutinin (HA)-tagged human A3G was kindly provided by Dr. Takaori (Kyoto University).[29] We constructed A3A cDNA expression

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plasmid by cloning DNA fragment, which was amplified by PCR from cDNA obtained from lymphocytes of a deletion homozygous patient, into pcDNA3.1/nV5-DEST (Invitrogen, Carlsbad, CA). Construction of the wild-type HBV 1.4 genome length plasmid, pTRE-HB-wt was described previously (Tsuge *et al.*;[30] GenBank accession no. AB206816).

Analysis of core-associated HBV DNA

The cells were harvested 4 days after transfection and lysed with 250 μl lysis buffer [10 mM Tris/HCl, pH 7.4, 140 mM NaCl and 0.5% (v/v) NP-40]. The lysate was then centrifuged for 2 min at 15 000 × *g*. The core particles were immunoprecipitated from the supernatant by mouse anti-core monoclonal antibody (anti-HBc determinant α, Institute of Immunology, Tokyo). Genomic DNA was separated from the core particles by SDS/proteinase K digestion followed by phenol extraction and ethanol precipitation. Quantitative analysis was performed using the above HBV DNA by RT-PCR using the RT-PCR system (Applied Biosystems, Foster City, CA). The primers and the probe used were described previously.[31] The real-time PCR was performed in a 25-μl reaction volume containing 2×TaqMan Gene Expression Master Mix, 0.9 μM of each primer, 0.25 μM probe and 1 μl DNA solution. The thermal profile was 50°C for 2 min, 95°C 10 min, followed by 40 cycles of amplification (denaturation at 95°C for 15 sec, annealing at 55°C for 30 sec and extension at 62°C for 90 sec).

Analysis of hypermutated HBV genomes by 3D-RT-PCR

Hypermutated genomes were detected and quantified by modified 3DRT-PCR using the primers, probe and reagents described previously.[31] The thermal profile was 50°C for 2 min, 95°C for 10 min followed by initial denaturation at 85°C for 20 min and 45 cycles of amplification (denaturation at 85°C for 15 sec, annealing at 50°C for 30 sec and extension at 62°C for 90 sec).

Detection of A3A-A3B fusion mRNA by RT-PCR

We extracted total RNA from lymphocytes of each allele patients using RNeasy Mini Kit (Qiagen, Hilden) and reverse-transcribed using ReverTra Ace (TOYOBO, Osaka) with random primer in accordance with the instructions supplied by the manufacturer. We then amplified cDNAs by 35 cycles of PCR using primers specific for exon 1 of *A3A* and 3′-untranslated region of *A3B* in a 25μl reaction volume containing 1 × KOD-Plus buffer [0.3 μM each primers, 0.2 mM $MgSO_4$, 1 μl DNA solution and 1 unit of KOD-Plus (TOYOBO Co.)]. The thermal profile was initial denaturation at 98°C for 2 min, followed by 35 cycles of amplification (denaturation at 98°C for 15 sec, annealing at 58°C for 15 sec and extension at 68°C for 60 sec). Nucleotide sequences of the amplified fusion cDNA sequences were confirmed by direct sequencing.

Western blot analysis

Cell lysates prepared as described above were separated by sodium dodecyl sulfate polyacrylamide electrophoresis on a 12% poly acrylamide gel and transferred to polyvinylidene fluoride (Pall Corporation, Pensacola, FL). The membranes were incubated with anti-V5 (Invitrogen), anti-hemagglutinin fusion epitope monoclonal anti-body (Roche) or with anti-β-actin monoclonal antibody (Sigma-Aldrich, St Louis, MO) followed by incubation with horseradish peroxidase-conjugated sheep anti-mouse antibody (GE Healthcare UK, Buckinghamshire). We detected signals using the ECL system (GE Healthcare).

Nucleotide sequencing analysis of hypermutated HBV genomes by 3D-PCR, cloning and nucleotide sequencing

We analyzed hypermutated HBV DNA genomes obtained from serum samples of each genotype patient by 3D PCR (denaturation at 85°C) and cloning and sequencing. The amplified DNA fragments were cloned into pGEM T Easy vector (Promega Corporation, Madison, WI) by TA cloning. Nucleotide sequences were determined using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The nucleotide sequences were compared with those obtained by direct sequencing of amplified PCR products by normal PCR protocol.

Statistical analysis

The allele frequencies was calculated and fit to Hardy-Weinberg equilibrium was tested by the chi-square test between cases and controls using Excel software (Microsoft, Redmond, WA).[32] We also compared differences in allele frequency and genotype distribution of the deletion between cases and controls with χ^2-test. Continuous data were compared by analysis of variance (ANOVA). Differences in categorical data were analyzed by the χ^2-test. Differences in core-associated HBV and hypermutated HBV genomes per 1×10^4 copies of HBV genomes, were analyzed by Student's *t*-test.

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Table 1 Characteristics of subjects

	Patients	Control	*P*-value
Number of patients	724	469	–
Sex			NS
Male	499	373	
Female	224	95	
Age (years)	53.1 (20.6–86.4)	55 (18–93)	NS
ALT	66 (5–3634)	–	–
Fibrosis stage		–	–
F0	13		
F1	80		
F2	149		
F3	114		
F4	46		
Activity		–	–
A0	1		
A1	50		
A2	125		
A3	47		
Platelet ($\times 10^4$/mm^3)	16.5 (2.2–29.8)	–	–
HBV DNA		–	–
High	137		
Middle	108		
Low	156		
HBeAg/HBeAb		–	–
+/–	207		
–/+	184		
Hepatocellular carcinoma	65	–	–

Data are number of patients or median (range) values. Differences in age between case and control were compared by Mann–Whitney *U*-test. The sex ratio was analyzed by the χ^2-test. ALT, alanine aminotransferase; HBVeAb, hepatitis B virus e antibody; HBVeAg, hepatitis B virus e antigen; NS, not significant.

RESULTS

Association between chronic HBV carriers, clinical parameters and the APOBEC3 gene deletion

TABLE 1 SUMMARIZES the clinicopathological features of the patients and control subjects. If A3B contributes to the prevention of chronic HBV infection, there should be an association between chronic HBV carrier state and *APOBEC* gene deletion polymorphism. However, we did not find any association between the two (Table 2). Furthermore, all clinical parameters, with the exception of the extent of liver fibrosis associated with chronic HBV, did not associate with the polymorphism (Tables 3,4). Advanced histopathological stages were associated with insertion homozygosity. These findings also suggest that A3B does not play any important role in anti-viral immunity in the development of chronic HBV infection.

Table 2 Case-control analysis of *APOBEC3B* deletion

	Frequency (%)		Additive mode		
	Ins	Del	*P*-value	OR	95% CI
HBV ($n = 724$)	0.709	0.291	0.599	0.964	0.624–1.489
Control ($n = 469$)	0.719	0.281			

P-values were calculated from case-control analysis by χ^2-test. OR, odds ratio; CI, confidence interval; Del, deletion homozygote; Ins, insertion homozygote.

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Table 3 Correlation between deletion and clinical parameters

	Genotype			*P*-value
	I/I	I/D	D/D	
Genotype frequency	0.50	0.42	0.08	NS
Age (years)	54.0 ± 12.8	52.0 ± 12.6	50.4 ± 13.3	NS
ALT	169.0 ± 320.6	149.5 ± 322.9	196.8 ± 309.3	NS
Platelets (×10⁴/mm³)	16.8 ± 5.2	16.6 ± 6.1	17.0 ± 5.8	NS

Data are number of patients or mean ± SD. Age, ALT and platelet count were compared by ANOVA. ALT, alanine aminotransferase; D/D, deletion homozygote; H, heterozygote; I/I, insertion homozygote; NS, not significant.

Context analysis of hypermutated genomes obtained from deletion homozygous and insertion homozygous patients

The amount of hypermutated genomes was not analyzed in this study because it is known to fluctuate during the clinical course.[33] Instead, we searched for the target context of G to A mutation in hypermutated HBV genomes using serum obtained from patients with deletion homozygotes and with insertion homozygotes. As shown Figure 1, multiple G to A hypermutations were observed in deletion homozygote and insertion homozygote patients. The results of context analysis showed no significant difference between the contexts obtained from deletion homozygotes and those form non-deletion homozygotes (Fig. 2). In fact, the preferred contexts were similar in all three deletion homozygous patients and one insertion homo patient (DD1-3 and II1 in Fig. 2) but slightly different from those of the remaining two (II2 and II3). These results suggest that the effect of deletion is not strong in these preferred context patterns.

Detection of A3A-A3B fusion mRNA

We then analyzed whether the resultant *A3A* and A3B fusion was actually transcribed. We designed primers specific for exon 1 of *A3A* and the 3′-untranslated region

Table 4 Association of clinical parameters and *APOBEC* gene polymorphism (categorical data)

	Genotype frequency				Additive mode	I/I vs I/D, D/D	D/D vs I/I, I/D
	I/I	I/D	D/D				
Sex (Male/Female)				*P* value	0.76	0.85	0.30
Male (*n* = 328)	154 (0.47)	143 (0.44)	31 (0.09)	OR	0.75	1.03	0.72
Female (*n* = 166)	78 (0.47)	74 (0.45)	14 (0.08)	95% CI	0.40–1.41	0.75–1.41	0.40–1.33
Fibrosis stage (F0-F1/F2-F4)				*P* value	0.0054	0.0019	0.48
F0-F1 (*n* = 62)	22 (0.35)	34 (0.55)	6 (0.10)	OR	0.51	0.47	0.74
F2-F4 (*n* = 187)	95 (0.51)	77 (0.41)	15 (0.08)	95% CI	0.21–1.24	0.30–0.76	0.31–1.73
Activity (A0-A1/A2-A3)				*P* value	0.31	0.46	0.30
A0-A1 (*n* = 51)	22 (0.43)	23 (0.45)	6 (0.12)	OR	0.56	0.80	0.60
A2-A3 (*n* = 168)	81 (0.48)	75 (0.45)	12 (0.07)	95% CI	0.20–1.56	0.45–1.44	0.22–1.60
HBV DNA (High/Low)				*P* value	0.12	0.12	0.47
High (*n* = 194)	82 (0.42)	94 (0.48)	18 (0.09)	OR	0.66	0.73	0.77
Low (*n* = 206)	103 (0.50)	88 (0.43)	15 (0.07)	95% CI	0.32–1.40	0.49–1.09	0.38–1.57
HBeAg/HBeAb ((+/–)/(–/+))				*P* value	0.52	0.34	0.84
+/– (*n* = 207)	89 (0.43)	99 (0.48)	19 (0.09)	OR	0.96	0.82	1.07
–/+ (*n* = 184)	88 (0.48)	78 (0.42)	18 (0.10)	95% CI	0.47–1.95	0.55–1.23	0.54–2.11
HCC				*P* value	0.85	0.89	0.64
(–) (*n* = 648)	323 (0.50)	266 (0.41)	59 (0.09)	OR	0.73	1.04	0.69
(+) (*n* = 65)	34 (0.52)	31 (0.47)	0 (0.00)	95% CI	0.25–2.13	0.62–1.73	0.24–1.98

ALT, alanine aminotransferase CI, confidence interval; D/D deletion homozygote; H, heterozygote; HBVeAg, hepatitis B virus e antibody; HBVeAg, hepatitis B virus e antigen; HCC, hepatocellular carcinoma; I/I, insertion homozygote; OR, odds ratio.

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direct_D/D3 2998 CACTGGCCAGAGGC AAATCAGGTAGGA GCGGGAGCATTC GGGCCAGGGGTCA CCCCACCA 3057
clone1_D/D3      .....A...A.AA.......AA..AA.A.AAA.A.....AAA...AAAT...........
clone2_D/D3      .........A.AA.......A......A.AAA.A.....AA....AAA............
clone3_D/D3      .....A...A.AA.......AA..AA.A.AAA.A.....AAA...AAAT...........

direct_D/D3 3058 CACGGAGGTCTTTTGGGGTGGAGCCCTCAGGCTCAGGGC ATATTGACAACAGTGCCAGTA 3117
clone1_D/D3      ...AA.A.......AAAA.AA.A......AA....AAA......A......A.A...A..
clone2_D/D3      ...AA.........AAAA.AA........AA....AAA......A......A.A...A..
clone3_D/D3      ...AA.A.......AAAA.AA.A......AA....AAA......A......A.A...A..

direct_D/D3 3118 GCACCTCCTCCTGCCTCCACCAATCGGCAGTCAGGAAGACAGCCTACTCCCATCTCTCCA 3177
clone1_D/D3      A............................AA.........................
clone2_D/D3      A.......................AA..A...AA..A...................
clone3_D/D3      A............................AA.........................

direct_D/D3 3278 CCTCTAAGAGACAGTCATCCTCAGGCCATGCAATGGAA 3215
clone1_D/D3      .......A.A.T.C...............A....AA..
clone2_D/D3      .......A.A...A.........AA....A..G.AA..
clone3_D/D3      .......A.A.T.C...............A....AA..


direct_I/I3 2998 CACTGGCCAGAGGCAAATCAGGTAGGAGTGGGAGCATTCGGGCCAGGGTTCACCCCACCA 3057
clone1_I/I3      ....AA...A.AA.......AA..AA.A.AAA.A.....AAA...AAA............
clone2_I/I3      ..T.AA...A.AA.......AA..AA.A.AAA.A.....AAA...AAA............
clone3_I/I3      ....AA...A.AA.......AA..AA.A.AAA.A.....AAA...AAA............

direct_I/I3 3058 CACGGCGGTCTTTTGGGGTGGAGCCCTCAGGCTCAGGGC ATATTGACAACAGTGCCAGCA 3117
clone1_I/I3      ....A.AA......AAAA.A...............AAA......A......A.A...A..
clone2_I/I3      ...AA.AA......A...............A....AAA......A......A.A...A..
clone3_I/I3      ......A.......AAAA.A...............AAA......A......A.A...A..

direct_I/I3 3118 GCACCTCCTCCTGCCTCCACCAATCGGCAGTCAGGAAGACAGCCTACTCCCATCTCTCCA 3177
clone1_I/I3      A............................AA..A......................
clone2_I/I3      A.......................TA......AA..A...A.................
clone3_I/I3      A.......................AA......AA........................

direct_I/I3 3278 CCTCTAAGAGACAGTCATCCTCAGGCCATGCAGTGGAA 3215
clone1_I/I3      .......A.A...A.........A.....A..A.AA..
clone2_I/I3      .......A.A.............A.....A..A.AA..
clone3_I/I3      .......A.A...A...............A..A.AA..
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Figure 1 Nucleotide sequences of hypermutated genomes detected from deletion homozygous and insertion homozygous patients. Nucleotide sequences of 3D-PCR amplified hebatitis B virus (HBV) DNA clones are compared with those obtained by usual PCR and direct sequencing. Upper panel, nucleotide sequences obtained from a deletion homozygous patient. Lower panel, nucleotide sequences obtained from a homozygous patient. Nucleotide numbers are those from GenBank accession no. AB206816.

of *A3B*, and performed RT-PCR using cDNAs obtained from patients of each genotype. We obtained amplified DNA fragments of expected size only from deletion homozygotes and heterozygotes (Fig. 3). These results confirmed the transcription of the fusion mRNA with the coding region of *A3A* and the 3′ untranslated region of *A3B*.

Inhibition of HBV replication and induction of hypermutation by A3A

We then analyzed the antiviral effect and induction of hypermutation by A3A. Although the expression of both A3A and A3G was confirmed by western Blot analysis (Fig. 4A), transient expression of A3A did not reduce the amount of the core-associated HBV DNA in HepG2 cells (Fig. 4B). However, A3A transfection increased the hypermutated genomes of HBV in a dose-dependent manner albeit the level of induction was much lower than that observed when transfected with A3G. These results suggest that A3A has negligible anti-viral effect although it induces hypermutation of HBV genomes.

DISCUSSION

THE MAIN FINDINGS of the present study were: (i) no association between *APOBEC3* deletion and chronic HBV infection (Table 2). (ii) Mild liver fibrosis and low alanine amino transferase (ALT) levels were associated with APOBEC gene deletion homozygous genotype. (iii) The absence of *A3B* is not responsible for chronic HBV carrier status, although A3B is known as a potent inhibitor of adeno-associated virus and retrotransposons.[12] This suggests different antiviral activities for APOBEC proteins against viruses and that A3B plays little role in inhibition of HBV. (iv) The preferred context analysis showed no differences between insertion homozygotes and deletion homozygotes. Only one of the six patients examined showed different context pattern (Fig. 2). These results suggest that A3B protein has only small effect on the formation of hypermutated genomes in the serum of chronic carriers. The protein has been reported to induce hypermutation on the negative and positive strands of HBV.[18] However, our results showed that the effect of *A3B* is almost negligible in

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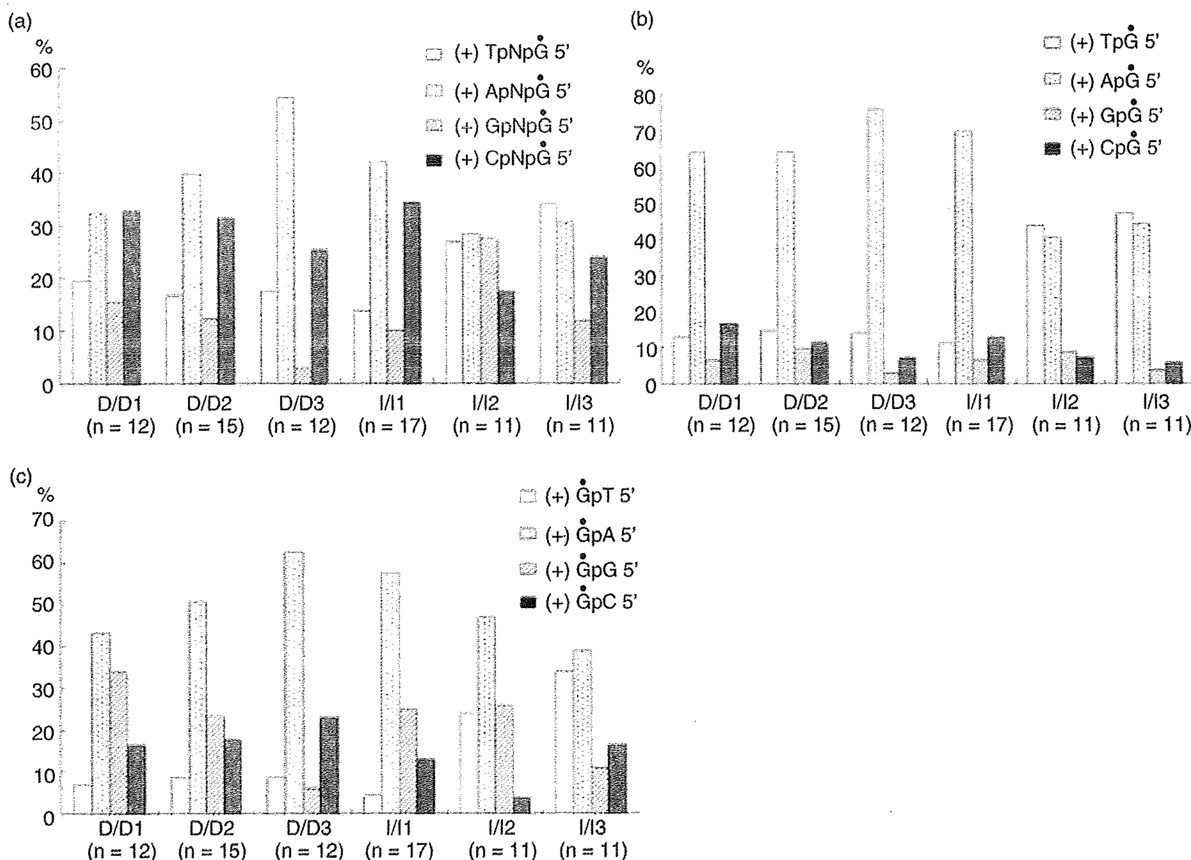

Figure 2 Context analysis of hypermutated genomes from deletion homozygous and insertion homozygous patients. Context of G to A hypermutation in hypermutated genome clones obtained from three deletion homozygous patients (D/D1, D/D2 and D/D3) and three insertion homozygous patients (I/I1, I/I2 and I/I3) were analyzed. Numbers after each patient represent the number of clones analyzed in each patient. (a) Two letters up-stream, (b) one letter upstream and (c) one letter downstream of mutated G residue were analyzed.

chronic HBV carriers compared to that of A3G. It is assumed that the other APOBEC3 family proteins mainly induce hypermutation of HBV genomes in HBV carriers to compensate for the function of deleted A3B. It is also assumed that the expression pattern of the remaining six APOBEC3 proteins is different from patient to patient.

As discussed above, our results suggest that A3B protein has almost no effect on prevention of chronic HBV infection and induction of hypermutation. It is thus assumed that A3B is not part of the innate anti-viral immune system against HBV. This is consistent with the finding that deletion is commonly seen in normal populations[25] irrespective of HBV carrier rates. Other association studies are required to clarify the role of A3B protein on other pathogens. The functional relevance of other APOBEC3 proteins on HBV infection as anti-viral immunity should be clarified further.

We also found that A3A protein induced hypermutation on the negative strand of HBV. However, the level of induction of hypermutation was much less than that of A3G (Fig. 4). Recent reports showed quite different effects for *A3A* on induction of hypermutation on HBV genomes. Henry *et al.*[24] reported that A3A is the most efficient editor of seven APOBEC3 proteins. In contrast, Zang *et al.*[23] did not detect induction of hypermutation on HBV. Although these different results might come from different cell lines and conditions used in each experiment, our results clearly showed that *A3A* induced hypermutation on the negative strand of HBV genome.

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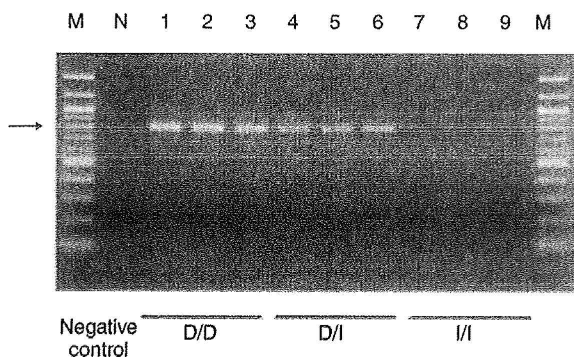

Figure 3 Detection of A3A-A3B fusion mRNA by PCR. A3A-A3B fusion mRNA was amplified by PCR using primers specific to A3A and A3B (see Materials and Methods) and detected by agarose gel electrophoresis. Lanes 1–3 are those from deletion homozygous patients, lanes 4–6 are from heterozygous patients and 7–9 are from insertion homozygous patients. M, molecular weight size marker (1 kb DNA Ladder; New England BioLabs, Ipswich, MA), N, negative control.

The deletion of APOBEC gene spans from 3′ end of the *A3A* gene to the 3′ portion of the *A3B* gene. This deletion results in the formation of a fusion gene that has *A3A* amino acid sequence and *A3B* 3′ untranslated region. The expression level of this protein may be different from the undeleted *A3A* due to different stability of RNA or different transcription levels. The results of context analysis also indicated that the effect of this alteration of expression levels of *A3A* is almost negligible compared with A3G. This is consistent with our results that showed lower levels of induction of hypermutation on HBV genome by *A3A* compared with *A3G*.

Our results indicated that the effect of both *A3A* and *A3B* is not significant in the development and progression of chronic hepatitis B. The two proteins also have only little influence on the hypermutation state of HBV in chronic HBV carriers. The results also showed higher induction levels of hypermutation by *A3G* than by *A3A*.

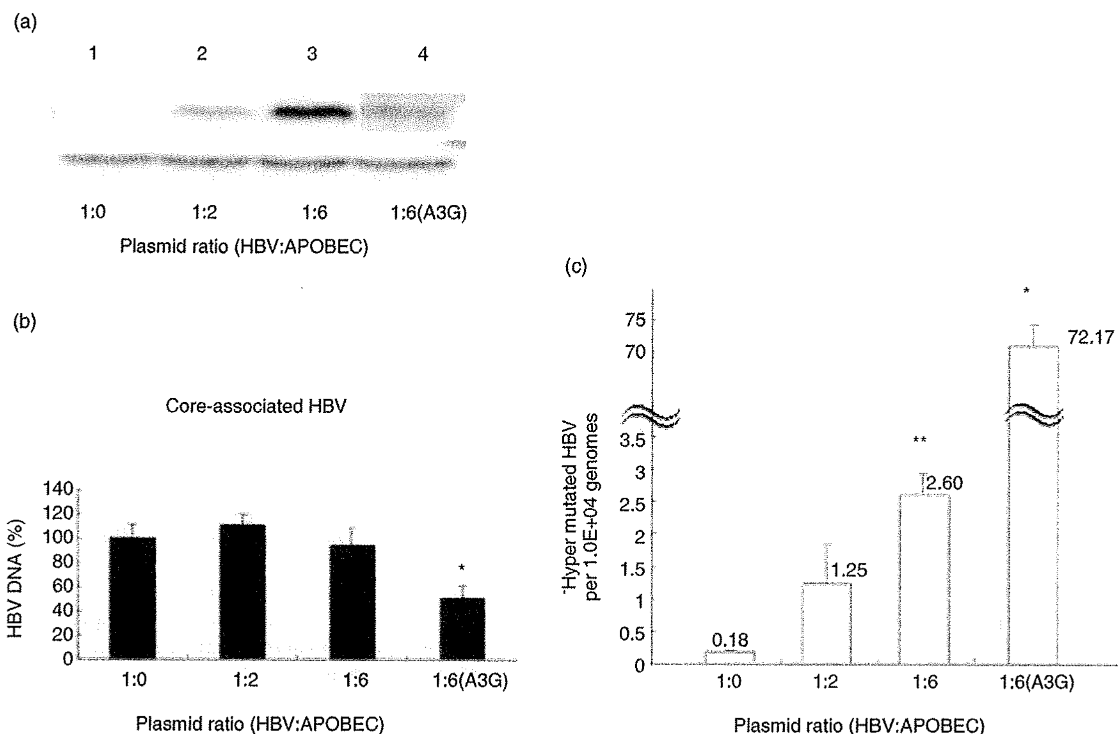

Figure 4 Analysis of inhibition of HBV replication and induction of hypermutation by A3A. HepG2 cells were transiently transfected with expression plasmid of A3A together with 1.4 genome length hepatitis B virus (HBV) expression vector. The indicated amounts of HBV and A3A (1:0, 1:2, 1:6) or A3G (1:6) expression plasmids were transfected into HepG2 cells. All experiments were performed more than twice with similar results. (a) APBEC3 gene expression levels were detected by western blot analysis. (b) The amounts of core associated replicative intermediates of HBV were measured by RT-PCR. (c) Quantitative measurement of hypermutated genomes by 3D real-time PCR. Data in (B) and (C) are mean ± SD. $^{*}P < 0.05$; $^{**}P < 0.01$

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Sufficient evidence indicates that *A3G* has anti-viral effects on HBV,[14-17] suggesting that some but not all APOBEC3 proteins operate as part of the anti-viral immune system against HBV infection. Further study is needed to clarify the functional role of each APOBEC3 protein for innate anti-viral immunity in chronic HBV infection.

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REFERENCES

1 Jarmuz A, Chester A, Bayliss J *et al.* An anthropoid-specific locus of orphan c to u rnaediting enzymes on chromosome 22. *Genomics* 2002; 79: 285–96.

2 Wedekind JE, Dance GS, Sowden MP, Smith HC. Messenger RNA editing in mammals: new members of the APOBEC family seeking roles in the family business. *Trends Genet* 2003; 19: 207–16.

3 Sheehy AM, Gaddis NC, Choi JD, Malim MH. Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. *Nature* 2002; 418: 646–50.

4 Bishop KN, Holmes RK, Sheehy AM, Davidson NO, Cho SJ, Malim MH. Cytidine deamination of retroviral DNA by diverse APOBEC proteins. *Curr Biol* 2004; 14: 1392–96.

5 Liddament MT, Brown WL, Schumacher AJ, Harris RS. APOBEC3F properties and hypermutation preferences indicate activity against HIV-1 in vivo. *Curr Biol* 2004; 14: 1385–91.

6 Wiegand HL, Doehle BP, Bogerd HP, Cullen BR. A second human antiretroviral factor, APOBEC3F, is suppressed by the HIV-1 and HIV-2 Vif proteins. *EMBO J* 2004; 23: 2451–8.

7 Zheng YH, Irwin D, Kurosu T, Tokunaga K, Sata T, Peterlin BM. Human APOBEC3F is another host factor that blocks human immunodeficiency virus type 1 replication. *J Virol* 2004; 78: 6073–6.

8 Dang Y, Wang X, Esselman WJ, Zheng YH. Identification of APOBEC3DE as another antiretroviral factor from the human APOBEC family. *J Virol* 2006; 80: 10522–33.

9 Cullen BR. Role and mechanism of action of the APOBEC3 family of antiretroviral resistance factors. *J. Virol* 2006; 80: 1067–76.

10 Chen H, Lilley CE, Yu Q *et al.* APOBEC3A is a potent inhibitor of adeno-associated virus and retrotransposons. *Curr Biol* 2006; 16: 480–5.

11 Bogerd HP, Wiegand HL, Doehle BP, Lueders KK, Cullen BR. APOBEC3A and APOBEC3B are potent inhibitors of LTR-retrotransposon function in human cells. *Nucleic Acids Res* 2006; 34: 89–95.

12 Bogerd HP, Wiegand HL, Hulme AE *et al.* Cellular inhibitors of long interspersed element 1 and Alu retrotransposition. *Proc Natl Acad Sci USA* 2006; 103: 8780–5.

13 Chen H, Lilley CE, Yu Q *et al.* APOBEC3A is a potent inhibitor of adeno-associated virus and retrotransposons. *Curr Biol* 2006; 16: 480–5.

14 Turelli P, Mangeat B, Jost S, Vianin S, Trono D. Inhibition of hepatitis B virus replication by APOBEC3G. *Science* 2004; 303: 1829.

15 Rösler C, Köck J, Malim MH, Blum HE, von Weizsäcker F. Comment on "inhibition of hepatitis B virus replication by APOBEC3G". *Science* 2004; 305: 1403a.

16 Turelli P, Jost S, Mangeat B, Trono D. Response to comment on "inhibition of hepatitis B virus replication by APOBEC3G". *Science* 2004; 305: 1403b.

17 Noguchi C, Ishino H, Tsuge M *et al.* G to A hypermutation of hepatitis B virus. *Hepatology* 2005; 41: 626–33.

18 Suspène R, Guétard D, Henry M, Sommer P, Wain-Hobson S, Vartanian JP. Extensive editing of both hepatitis B virus DNA strands by APOBEC3 cytidine deaminases in vitro and in vivo. *Proc Natl Acad Sci* 2005; 102: 8321–6.

19 Bonvin M, Achermann F, Greeve I *et al.* Interferon-inducible expression of APOBEC3 editing enzymes in human hepatocytes and inhibition of hepatitis B virus replication. *Hepatology* 2006; 43: 1364–74.

20 Xu R, Zhang X, Zhang W, Fang Y, Zheng S, Yu XF. Association of human APOBEC3 cytidine deaminases with the generation of hepatitis virus B x antigen mutants and hepatocellular carcinoma. *Hepatology* 2007; 46: 1810–20.

21 Jost S, Turelli P, Mangeat B, Protzer U, Trono D. Induction of antiviral cytidine deaminases does not explain the inhibition of hepatitis B virus replication by interferons. *J Virol* 2007; 81: 10588–96.

22 Bonvin M, Greeve J. Effects of point mutations in the cytidine deaminase domains of APOBEC3B on replication and hypermutation of hepatitis B virus in vitro. *J Gen Virol* 2007; 88: 3270–4.

23 Zhang W, Zhang X, Tian C *et al.* Cytidine deaminase APOBEC3B interacts with heterogeneous nuclear ribonucleoprotein K and suppresses hepatitis B virus expression. *Cell Microbiol* 2008; 10: 112–21.

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24 Henry M, Guétard D, Suspène R, Rusniok C, Wain-Hobson S, Vartanian JP. Genetic editing of HBV DNA by monodomain human APOBEC3 cytidine deaminases and the recombinant nature of APOBEC3G. *PLoS ONE* 2009; **4**: e4277.

25 Kidd JM, Newman TL, Tuzun E, Kaul R, Eichler EE. Population stratification of a common APOBEC gene deletion polymorphism. *PLoS Genet* 2007; **3**: e63.

26 Bedossa P, Poynard T. An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. *Hepatology* 1996; **24**: 289–93.

27 Price PM, Banerjee R, Jeffrey AM, Acs G. The mechanism of inhibition of hepatitis B virus replication by the carbocyclic analog of 2′-deoxyguanosine. *Hepatology* 1992; **16**: 8–12.

28 Ohnishi Y, Tanaka T, Ozaki K, Yamada R, Suzuki H, Nakamura Y. A high-throughput SNP typing system for genome-wide association studies. *J Hum Genet* 2001; **46**: 471–7.

29 Kobayashi M, Takaori-Kondo A, Shindo K, Abudu A, Fukunaga K, Uchiyama T. APOBEC3G targets specific virus species. *J Virol* 2004; **78**: 8238–44.

30 Tsuge M, Hiraga N, Takaishi H *et al.* Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis B virus. *Hepatology* 2005; **42**: 1046–54.

31 Noguchi C, Hiraga N, Mori N *et al.* Dual effect of APOBEC3G on hepatitis B virus. *J Gen Virol* 2007; **88**: 432–40.

32 Nielsen DM, Ehm MG, Weir BS. Detecting marker-disease association by testing for Hardy-Weinberg disequilibrium at a marker locus. *Am J Hum Genet* 1998; **63**: 1531–40.

33 Noguchi C, Imamura M, Tsuge M *et al.* G to A hypermutation in hepatitis B virus and clinical course of patients with chronic hepatitis B. *J Inf Dis* 2009; **199**: 1599–607.

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ORIGINAL ARTICLE

Antiviral activity, dose–response relationship, and safety of entecavir following 24-week oral dosing in nucleoside-naive Japanese adult patients with chronic hepatitis B: a randomized, double-blind, phase II clinical trial

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Abstract
Purpose A randomized, double-blind, multicenter study (ETV-047) was conducted to evaluate the dose–response relationship of entecavir and compare its antiviral activity and safety with lamivudine in Japanese patients with chronic hepatitis B (CHB).
Methods One hundred thirty-seven nucleoside-naive adult patients with CHB were randomized to once-daily oral doses of entecavir 0.01, 0.1, or 0.5 mg or lamivudine 100 mg for 24 weeks. The primary efficacy end point used to evaluate the dose–response relationship was mean change from baseline in serum hepatitis B virus (HBV) DNA level at week 22, as determined by polymerase chain reaction assay.
Results Entecavir demonstrated a clear dose–response relationship, with mean change from baseline in serum

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HBV DNA level of −3.11, −4.77, and −5.16 $\log_{10}$ copies/ml with entecavir 0.01, 0.1, and 0.5 mg, respectively. Entecavir 0.5 mg was superior to lamivudine 100 mg for the mean change in HBV DNA level (−5.16 vs. −4.29 $\log_{10}$ copies/ml; $P = 0.007$). The overall incidence of adverse events was comparable between treatment groups. Two patients discontinued treatment because of adverse events (one with liver cirrhosis [entecavir 0.5 mg] and one with grade 4 serum alanine aminotransferase (ALT) elevation, nausea, and malaise [lamivudine 100 mg]). Serum ALT flares were observed in four patients; flares were associated with 2 $\log_{10}$ reductions or more in HBV DNA level and resolved without dose interruption.

Conclusion Entecavir 0.01–0.5 mg is well tolerated and produces a dose-dependent reduction in viral load in nucleoside-naive Japanese patients with CHB. Compared with lamivudine 100 mg, entecavir 0.1 mg demonstrated noninferiority and entecavir 0.5 mg was superior in this population.

Keywords Chronic hepatitis B · Entecavir · Lamivudine · HBV DNA · ALT flare

Introduction

It is reported that more than 2 billion individuals worldwide have been infected with hepatitis B virus (HBV) and approximately 350 million people are long-term HBV carriers [1]. Chronic hepatitis B (CHB) is induced by chronic replication of HBV in the liver and has a poor prognosis, with 20–40% of infected individuals developing liver cirrhosis, noncompensated liver disorder, or hepatocellular carcinoma [2]. Treatment of CHB is aimed at sustained inhibition of HBV replication and remission of liver disease [3], ultimately preventing progression to liver cirrhosis or hepatocellular carcinoma [4].

Prior to the advent of the nucleoside analog lamivudine, interferon-α formed the mainstay of treatment, but this immunoregulatory cytokine requires parenteral administration and is poorly tolerated [5]. Lamivudine is well tolerated on oral administration and has been proven to be highly effective in the treatment of CHB, but the emergence of resistance mutations (including the YMDD motif) in the reverse-transcriptase domain of HBV polymerase frequently results in overt viral rebound and disease progression [6–9]. The novel nucleoside analog adefovir is effective against wild-type HBV and lamivudine-resistant strains and is well tolerated on long-term administration, but its clinical use is restricted by the need for renal monitoring in patients with impaired renal function [10].

Entecavir, a cyclopentylguanine-derived nucleoside analog and selective inhibitor of HBV replication, was approved by the U.S. Food and Drug Administration in 2005 for the treatment of CHB. Entecavir displays potent antiviral activity in the woodchuck and duck models of HBV infection [11, 12] and is reported to be 100- to 2,200-fold more potent than lamivudine and adefovir in inhibiting HBV replication in vitro [13, 14]. Phase II clinical trials of entecavir conducted in non-Japanese patients with CHB have demonstrated entecavir to be well tolerated and more effective than lamivudine [15, 16].

A global dose-finding study (ETV-005) conducted in lamivudine-naive patients with CHB compared three doses of entecavir (0.01, 0.1, and 0.5 mg once daily) with lamivudine 100 mg once daily over a 22-week treatment period. Entecavir showed a clear dose–response relationship and was well tolerated at all three dose levels; in addition, 0.1 and 0.5 mg of entecavir showed superior antiviral activity compared with 100 mg of lamivudine [15].

Phase I studies of single-dose (0.05–2.5 mg) and multiple-dose (0.1–1.0 mg daily) entecavir conducted in Japan have confirmed the drug's safety in healthy men. As in Caucasian populations, entecavir displayed linear plasma pharmacokinetics over a wide range of doses, including putative therapeutic doses (0.5 and 1.0 mg), in Japanese subjects; there was no evidence of significant ethnic differences in its pharmacokinetics and pharmacodynamics. Similar findings to those obtained in the global phase II clinical trials of entecavir might therefore be expected from corresponding studies conducted in Japanese patients.

To evaluate the dose–response relationship, the antiviral activity and safety of entecavir in Japanese CHB patients, we conducted a 24-week phase II study comparing entecavir (0.01, 0.1, and 0.5 mg daily) to lamivudine (100 mg daily).

Materials and methods

Study design

This randomized, double-blind, double-dummy study was conducted at 38 institutions in Japan from August 2003 to March 2005. Eligible patients comprised 20- to 75-year-old men and women with CHB who fulfilled the following criteria: (i) HBsAg-positive for 24 weeks or more or IgM HBcAb-negative with biopsy-confirmed CHB; (ii) HBeAg-positive or HBeAg-negative for 12 weeks or more; (iii) serum HBV DNA level 40 MEq/ml or more (143 pg/ml) by Quantiplex™ branched DNA hybridization method (bDNA assay) (≥ 7.6 $\log_{10}$ genome equivalent by the transcription-mediated amplification method or $\geq 10^{7.6}$ copies/ml by Roche Amplicor™ polymerase chain reaction method [PCR assay]) measured 2 weeks or more before screening and serum HBV DNA level 40 MEq/ml or more (by bDNA assay) at screening; (iv) serum alanine

aminotransferase (ALT) level 1.25–10 times the upper limit of normal (ULN); and (v) well-compensated liver disease with prothrombin time prolongation 3 s or less or international normalized ratio 1.5 or less, serum albumin level 3.0 g/dl or more, and total bilirubin 2.5 mg/dl or less (42.75 μmol/l). After a 6-week screening period, eligible patients were stratified according to HBeAg status and study site and randomized (1:1:1:1) to oral treatment with entecavir (0.01, 0.1, or 0.5 mg plus matching placebo capsule) or lamivudine (100 mg plus matching placebo tablet) once daily for 24 weeks. All doses were administered at fixed times of the day, avoiding the 2 h before and after meals. Pregnant women were excluded from the study, as were patients with liver cirrhosis, patients with a history or evidence of variceal bleeding, patients with hepatic encephalopathy or ascites requiring diuretics, or patients with paracentesis. Patients with other liver disease (e.g., autoimmune hepatitis) were excluded from the study. In addition, patients were excluded if they had a serum creatinine level more than 1.5 × ULN, hemoglobin level less than 10.0 g/dl, platelet count less than 70,000/mm^3, granulocyte count less than <1,500/mm^3 or plasma α-fetoprotein level more than 100 ng/ml, a history of allergy induced by nucleoside analog or exposure to nucleoside analogs, a recent history (previous 24 weeks) of treatment with immunosuppressives or interferon-α/β, or current treatment of CHB.

Treatment efficacy was assessed after 22 weeks, and all eligible patients who completed 24 weeks of blinded therapy were given the option of enrolling in a separate entecavir trial. Patients who discontinued therapy prematurely were followed up for 24 weeks postdosing. Patients began anti-HBV therapy as recommended by their physician during the postdosing follow-up period.

Informed consent was obtained from all patients in writing prior to their inclusion in the study. The study was conducted in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice guidelines and notifications were issued by the Ministry of Health and Labor.

Efficacy and safety assessment

The primary efficacy end point for the evaluation of the dose–response relationship of entecavir was the change from baseline in mean serum HBV DNA level at week 22, as determined by PCR assay. Secondary efficacy end points for the assessment of the noninferiority of entecavir at each dose to lamivudine included the change from baseline in mean serum HBV DNA level at week 22, as determined by PCR assay, the percentage of patients with a reduction in serum HBV DNA level 2 $\log_{10}$ copies/ml or more or a serum HBV DNA level below the limit of detection (400 copies/ml by PCR assay; 2.5 pg/ml or 0.7 MEq/ml by bDNA assay) at week 22, the percentage of patients with HBeAg loss, the percentage of patients with HBeAg seroconversion (HBeAg loss and appearance of HBe-antibody), the percentage of patients achieving ALT normalization (World Health Organization grade 0: <1.25 × ULN), and the percentage of patients achieving a protocol-defined response (HBV DNA level <0.7 MEq/ml by bDNA assay, HBeAg negativity and serum ALT level <1.25 × ULN for HBeAg-positive patients; HBV DNA level <0.7 MEq/ml by bDNA assay and serum ALT level <1.25 ULN for HBeAg-negative patients) at week 22. The incidence of genotypic drug resistance was also assessed in patients who had a 1 $\log_{10}$ copies/ml or more increase in HBV DNA by PCR from nadir while on study drug.

Based on the results of the global dose–response study of entecavir conducted in nucleoside-naive patients (ETV-005 study) [15], noninferiority of entecavir 0.1 or 0.5 mg compared with lamivudine (100 mg) was confirmed if the upper 95% confidence interval (CI) for the difference in mean HBV DNA levels at week 22 was 0.8 $\log_{10}$ copies/ml or less.

Assay methods

Serum HBV DNA level was determined by Roche Amplicor™ PCR assay (Roche Diagnostics K.K., Tokyo, Japan) and Quantiplex™ (Chiron) bDNA assay. Clinical laboratory tests, serum HBV DNA assays, and HBV serology were performed at the central clinical laboratory designated by the trial sponsor. Genotypic analysis of HBV isolates was performed using samples collected from patients on the first day of treatment. Genotypic analysis of HBV DNA polymerase was performed at SRL Inc. (Tokyo, Japan).

Statistical analysis

Numerical data were expressed by descriptive statistics. Serum HBV DNA level, a continuous variable, was analyzed after logarithmic transformation. For treatment group, comparisons of continuous variables, analysis of variance models, incorporating baseline HBV DNA level and HBeAg status as covariates were employed. For intertreatment comparisons of binary data, Cochran–Mantel–Haenszel tests were employed using baseline HBeAg status as a stratification factor. For analysis of dose–response relationships, Student's t test was applied to linear regression plots of serum HBV DNA level against log dose. A two-sided $P < 0.05$ was taken to indicate statistical significance. For analysis of dose–response relationships using efficacy data, a two-sided $P < 0.05/3$ was taken to

indicate statistical significance following Bonferroni adjustment.

Results

Study population and demographic characteristics

A total of 137 patients, including 20- to 73-year-old men and women, met the study eligibility criteria and were randomized to the following treatment groups: entecavir 0.01 mg ($n = 35$), entecavir 0.1 mg ($n = 34$), entecavir 0.5 mg ($n = 34$), and lamivudine 100 mg ($n = 34$). Three patients (two in the entecavir 0.5 mg group and one in the lamivudine 100 mg group) discontinued the study prematurely; the reasons for discontinuation were noncompliance (one patient in the entecavir 0.5 mg group) and adverse events (liver cirrhosis in one patient [entecavir 0.5 mg group] and grade 4 serum ALT elevation with nausea and malaise in one patient [lamivudine 100 mg group]). Accordingly, a total of 134 patients (entecavir 0.01 mg group, 35 patients; entecavir 0.1 mg group, 34 patients; entecavir 0.5 mg group, 32 patients; and lamivudine 100 mg group, 33 patients) completed 24 weeks of treatment and were included in the efficacy assessment.

The four treatment groups were matched with respect to gender, age, body weight, and proportion of HBeAg-positive patients (Table 1). Serum HBV DNA levels by PCR assay (mean ± SD) at baseline were 7.94 ± 0.87, 8.09 ± 1.05, 8.39 ± 0.73, and 7.94 ± 0.83 $\log_{10}$ copies/ml for the entecavir 0.01, 0.1, and 0.5 mg and lamivudine 100 mg groups, respectively. With regard to HBV genotype, 124 patients were genotype C, 6 patients were genotype A, 5 patients were genotype B, and 2 patients were genotype F. All patients were nucleos(t)ide-naive and none had been pretreated with interferon therapy.

Virologic response

Mean changes (from baseline) in serum HBV DNA level at week 22 were −3.11, −4.77, and −5.16 $\log_{10}$ copies/ml with entecavir 0.01, 0.1, and 0.5 mg, respectively (Fig 1; Table 2). Estimated differences in serum HBV DNA levels between the 0.1 and 0.5 mg entecavir groups and the low-dose entecavir group (0.01 mg) were determined after adjustment for baseline level and HBeAg status. Estimated intertreatment group differences (adjusted 95% CI) were −1.61 (−2.20 to −1.02) $\log_{10}$ copies/ml between the entecavir 0.01 and 0.1 mg groups and −1.95 (−2.53 to −1.37) $\log_{10}$ copies/ml between the entecavir 0.5 and 0.01 mg groups; both of these differences were statistically significant ($P < 0.0001$). In contrast, the difference in serum HBV DNA levels between the high-dose (0.5 mg) and medium-dose (0.1 mg) entecavir groups was not statistically significant (estimated difference [adjusted 95% CI] −0.23 [−0.69 to 0.23] $\log_{10}$ copies/ml). Taken together, these results demonstrate the superiority of high- and medium-dose entecavir (0.1 and 0.5 mg) compared with low-dose entecavir (0.01 mg) in terms of viral load reduction (Table 3). Linear regression analyses indicated a

Table 1 Baseline demographics and clinical characteristics of treated subjects

	ETV 0.01 mg ($n = 35$)	ETV 0.1 mg ($n = 34$)	ETV 0.5 mg ($n = 34$)	LVD 100 mg ($n = 34$)
Male, n (%)	25 (71.4)	23 (67.6)	23 (67.6)	28 (82.4)
Female, n (%)	10 (28.6)	11 (32.4)	11 (32.4)	6 (17.6)
Age (years), mean ± SD	42.0 ± 12.5	40.1 ± 9.8	39.8 ± 10.4	42.3 ± 12.6
Weight (kg), mean ± SD	66.2 ± 12.5	64.6 ± 11.9	65.3 ± 11.1	64.4 ± 9.0
Ethnicity Japanese, n (%)	35 (100)	34 (100)	34 (100)	34 (100)
HBV DNA ($\log_{10}$ copies/ml by PCR), mean ± SD	7.94 ± 0.87	8.09 ± 1.05	8.39 ± 0.73	7.94 ± 0.83
HBeAg positive, n (%)	30 (85.7)	30 (88.2)	30 (88.2)	31 (91.2)
ALT (IU/l), mean ± SD	150.1 ± 111.8	162.0 ± 127.1	142.4 ± 82.2	185.0 ± 130.8
AST (IU/l), mean ± SD	83.2 ± 40.0	114.3 ± 109.4	81.0 ± 43.0	121.6 ± 85.4
Total bilirubin (mg/dl), mean ± SD	0.65 ± 0.25	0.56 ± 0.15	0.66 ± 0.25	0.71 ± 0.28
HBV genotype (%)				
C	32 (91.4)	30 (88.2)	32 (94.1)	30 (88.2)
A	1 (2.86)	2 (5.88)	1 (2.94)	2 (5.88)
B	1 (2.86)	1 (2.94)	1 (2.94)	2 (5.88)
F	1 (2.86)	1 (2.94)	0	0

ETV entecavir; *LVD* lamivudine

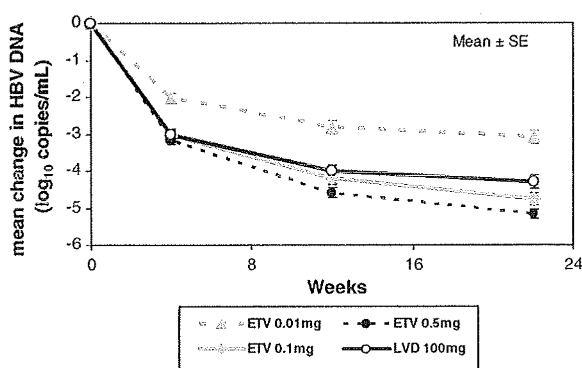

Fig. 1 Mean change from baseline in serum HBV DNA level by PCR assay through 22 weeks in patients treated with entecavir (ETV) 0.01, 0.1, and 0.5 mg and lamivudine 100 mg. Mean change in serum HBV DNA level was plotted as a function of time after the initiation of the protocol therapy (weeks). Data expressed as mean ± SE

significant dose–response relationship between $\log_{10}$ entecavir dose and reduction in $\log_{10}$ serum HBV DNA level ($P < 0.0001$).

Mean change (from baseline) in serum HBV DNA level at week 22 for the lamivudine 100 mg group was −4.29 $\log_{10}$ copies/ml (Fig. 1; Table 2). Estimated mean differences (95% CI) in serum HBV DNA level (after adjustment for baseline level and HBeAg status) were −0.39 (−0.83 to 0.05) $\log_{10}$ copies/ml between the entecavir 0.1 mg and lamivudine 100 mg groups and −0.62 (−1.06 to −0.18) $\log_{10}$ copies/ml between the entecavir 0.5 mg and lamivudine 100 mg groups, indicating the noninferiority of the entecavir 0.1 and 0.5 mg groups to the lamivudine 100 mg group and the superiority of the entecavir 0.5 mg group to the lamivudine 100 mg group ($P = 0.007$) (Table 2). In contrast, the entecavir 0.01 mg group was significantly inferior to the lamivudine 100 mg group (estimated mean difference = 1.20 [0.69–1.71]; $P < 0.0001$) (Table 2).

The secondary efficacy end point of a reduction in serum HBV DNA level 2 $\log_{10}$ copies/ml or more or HBV DNA level less than 400 copies/ml by PCR assay was achieved by 88.6% of patients in the entecavir 0.01 mg group and by 100% of patients in the entecavir 0.1 and 0.5 mg groups at week 22. Ninety-seven percent of patients in the lamivudine 100 mg group achieved this end point at week 22. HBV DNA level less than 0.7 MEq/ml by bDNA assay was achieved by 65.7%, 94.1%, and 100% of patients in the 0.01, 0.1, and 0.5 mg entecavir groups, respectively, and by 93.9% of patients in the lamivudine 100 mg treatment group.

Serologic response

Among HBeAg-positive patients, there was no significant difference between seroconversion rates at week 22 for the entecavir 0.01, 0.1, and 0.5 mg treatment groups (10.0%, 13.3%, and 3.6%, respectively) versus the lamivudine 100 mg treatment group (3.3%; Table 2). All patients who lost HBeAg also experienced HBeAg seroconversion.

Biochemical response

At baseline, elevated serum ALT levels (>1.25 × ULN) were present in more than 90% of patients in all four treatment groups. At week 22, normal serum ALT levels (World Health Organization grade 0, <1.25 × ULN) were recorded in similar proportions of patients in the entecavir 0.01, 0.1, and 0.5 mg treatment groups (75.0%, 85.3%, and 80.0% of patients, respectively) and the lamivudine treatment group (78.1% of patients), with no significant intergroup difference (Table 2).

Response

Response (HBV DNA level <0.7 MEq/ml by bDNA assay, HBeAg loss, and serum ALT level <1.25 × ULN for HBeAg-positive patients and HBV DNA level <0.7 MEq/ml by bDNA assay and serum ALT <1.25 × ULN for HBeAg-negative patients) was achieved by 14.3%, 20.6%, and 15.6% of patients in the entecavir 0.01, 0.1, and 0.5 mg

Table 2 Differences in HBV DNA levels between entecavir dose groups by PCR at week 22 in evaluable subjects

	0.1 mg ETV–0.01 mg ETV ($n = 34$, $n = 35$)	0.5 mg ETV–0.01 mg ETV ($n = 32$, $n = 35$)	0.5 mg ETV–0.1 mg ETV ($n = 32$, $n = 34$)
Estimated difference[a] ($\log_{10}$ copies/ml)	−1.61	−1.95	−0.23
Standard error	0.24	0.24	0.19
95% Confidence interval[b]	−2.20, −1.02	−2.53, −1.37	−0.69, 0.23
P-value	<0.0001	<0.0001	0.227

[a] Estimated differences are regression-adjusted for baseline serum HBV DNA and HBeAg status

[b] 95% Confidence interval is adjusted by modified Bonferroni procedures

ETV entecavir

Table 3 Virology and biochemical responses at week 22 and comparison of entecavir treatment groups with lamivudine in evaluable subjects

Response	ETV 0.01 mg (n = 35)	ETV 0.1 mg (n = 34)	ETV 0.5 mg (n = 32)	LVD 100 mg (n = 33)
HBV DNA by PCR assay				
Reduction from baseline at week 22 ($\log_{10}$ copies/ml), mean ± S.E.	−3.11 ± 0.18	−4.77 ± 0.17	−5.16 ± 0.13	−4.29 ± 0.18
HBV DNA estimated difference[a] (vs. LVD) ($\log_{10}$ copies/ml)	1.20	−0.39	−0.62	–
Standard error	0.26	0.22	0.22	–
95% Confidence interval	0.69, 1.71	−0.83, 0.05	−1.06, -0.18	–
P-value	<0.0001[b]	0.081	0.007[c]	–
HBV DNA by Roche Amplicor™ PCR assay				
Change in $\log_{10}$ HBV DNA reduction >2 or HBV DNA <400 copies/ml at week 22, n (%)	31 (88.6)	34 (100)	32 (100)	32 (97.0)
P-value (vs. LVD)	0.206	NR[d]	NR[d]	–
HBV DNA by Quantiplex assay				
HBV DNA <0.7 MEq/ml (2.5 pg/ml) at week 22, n (%)	23 (65.7)	32 (94.1)	32 (100)	31 (93.9)
P-value (vs. LVD)	0.002	1.000	NR[d]	–
Normalization of ALT levels[e]				
At week 22, *n/n* with abnormal baseline (%)	24/32 (75.0)	29/34 (85.3)	24/30 (80.0)	25/32 (78.1)
P-value (vs. LVD)	0.842	0.439	0.880	–
Loss of HBeAg and seroconversion at week 48[f]				
HBeAg loss, n/n HBeAg positive at baseline (%)	3/30 (10.0)	4/30 (13.3)	1/28 (3.6)	1/30 (3.3)
HBeAg seroconversion	3/30 (10.0)	4/30 (13.3)	1/28 (3.6)	1/30 (3.3)
P-value (vs. LVD)	0.605	0.350	1.000	–
Response[g] at week 22, n (%)	5 (14.3)	7 (20.6)	5 (15.6)	3 (9.1)
P-value (vs. LVD)	0.735	0.190	0.480	–

[a] Estimated differences are regression-adjusted for baseline HBV DNA and HBeAg status

[b] Two-sided test indicates inferiority of the entecavir 0.01 mg dose

[c] Two-sided test indicates superiority of the entecavir dose

[d] Not reported because expected counts <5

[e] WHO grade 0, ALT <1.25 × upper limit of normal

[f] Seroconversion was defined as disappearance of HBe-antigen and appearance of HBe-antibody

[g] Response was defined as HBV DNA levels <0.7 MEq/ml, HBeAg negativity and ALT <1.25 × ULN for HBeAg-positive patients and HBV DNA levels <0.7 MEq/ml and ALT <1.25 × ULN for HBeAg-negative patients

ETV entecavir

LVD lamivudine

treatment groups, respectively, and by 9.1% of patients in the lamivudine treatment group at week 22, and there were no significant differences in the rates of response between the four treatment groups (Table 2).

Resistance analysis

During the treatment period, serum HBV DNA level increased by 1 $\log_{10}$ copies/ml or more from its nadir in one patient in the entecavir 0.01 mg group and one patient in the lamivudine 100 mg group. Nucleotide sequence analysis of the DNA polymerase coding region, using viral samples collected from these two patients at day 1 and at week 22, revealed no lamivudine-resistance substitutions (rt180 and rt204 amino acid residues) [17, 18] or entecavir-resistance substitutions (rt184, rt202, and rt250 amino acid residues) [19].

Safety

During the study, adverse events were experienced by similar proportions of patients in the entecavir 0.01, 0.1, and 0.5 mg groups and the lamivudine 100 mg treatment group (97.1%, 97.1%, 91.2%, and 100.0%, respectively). Most adverse events were of mild or moderate intensity (grade 1/2) and transient. The most frequently reported adverse events (affecting ≥ 10% of patients in any one treatment group) included nasopharyngitis, headache, and

Table 4 Summary of adverse events and laboratory abnormalities during the 24-week blinded treatment phase

	ETV 0.01 mg (n = 35)	ETV 0.1 mg (n = 34)	ETV 0.5 mg (n = 34)	LVD 100 mg (n = 34)
Any adverse events	34 (97)	33 (97)	31 (91)	34 (100)
Most frequent clinical adverse events,[a] n (%)				
Nasopharyngitis	9 (25.7)	10 (29.4)	11 (32.4)	10 (29.4)
Headache	6 (17.1)	7 (20.6)	2 (5.9)	7 (20.6)
Diarrhea	1 (2.9)	1 (2.9)	4 (11.8)	4 (11.8)
Grade 3/4 clinical adverse events, n (%)	0	0	1 (2.9)	1 (2.9)
Grade 3/4 laboratory adverse events, n (%)	2 (5.7)	4 (11.8)	2 (5.9)	4 (11.8)
Any serious adverse events, n (%)	0	1 (2.9)	2 (5.9)	1 (2.9)
Discontinuations due to adverse events,[b] n (%)	0	0	1 (2.9)	1 (2.9)
ALT flares,[c] n (%)	0	1 (2.9)	1 (2.9)	2 (5.9)
Death, n (%)	0	0	0	0

[a] Occurring in at least 10% of patients

[b] One patient treated with ETV 0.5 mg discontinued the study drug due to hepatic cirrhosis. One patient treated with lamivudine discontinued due to increased ALT

[c] ALT flare defined ALT >2 × baseline and 10 × ULN

ETV entecavir

LVD lamivudine

diarrhea (Table 4). Grade 3/4 clinical adverse events occurred in one patient in the entecavir 0.5 mg group (colon carcinoma) and one patient in the lamivudine group (anal ulcer); neither of these events was considered to be related to the study drug. Serious adverse events were limited to the above-mentioned case of colon carcinoma, serum ALT elevation (entecavir 0.1 mg group [$n = 1$], entecavir 0.5 mg group [$n = 1$]), and serum aspartate aminotransferase (AST)/ALT elevation (lamivudine 100 mg group [$n = 1$]), but these were not considered to be causally related to the study drug and did not necessitate treatment discontinuation. Transient ALT flares (serum ALT >2 × baseline level and >10 × ULN) occurred in four patients (entecavir 0.1 mg group [$n = 1$], entecavir 0.5 mg group [$n = 1$], and lamivudine 100 mg group [$n = 2$]) and were associated with HBV DNA level decreases of 2 $\log_{10}$ copies/ml or more. None of the ALT flares were associated with hepatic decompensation and serum ALT and AST levels recovered to less than 1.25 × baseline level on continuation of the study treatment.

Discussion

The global ETV-005 study reported that entecavir was superior to lamivudine at reducing viral load in nucleoside-naive patients with CHB infection [15]. We conducted the present study, using an identical design to the ETV-005 study, to determine whether the findings from this earlier study are applicable to Japanese patients. In keeping with the previous findings, our results indicate that entecavir produces a dose-related reduction in serum HBV DNA level ($0.01 < 0.1 \leq 0.5$ mg) in nucleoside-naive Japanese patients with CHB; the log dose–response curves for the reduction in serum HBV DNA level with entecavir in the two studies were similar, with estimated regression curve slopes of –1.24 (Japanese study) and –1.32 (global study). In addition, both studies demonstrated the noninferiority of the entecavir 0.1 mg group compared with the lamivudine 100 mg group and the superiority of the entecavir 0.5 mg group compared with the lamivudine 100 mg group. The demonstration of a dose–response relationship for entecavir and the superiority of the entecavir 0.5 mg dose over lamivudine confirm that the antiviral activity of entecavir in Japanese patients is similar to that observed in study ETV-005. In a previous study, Ono et al. [14] demonstrated that the in vitro potency of entecavir was up to 2,200 times greater than that of lamivudine. The results presented here substantiate these earlier in vitro data and confirm the greater potency of entecavir over lamivudine in patients with CHB.

Serum ALT normalization rates with entecavir 0.5 mg and lamivudine 100 mg (~80%) were higher in the present study than those reported in the ETV-005 study (entecavir 0.5 mg, 69.0%; lamivudine 100 mg, 59.1%) [15]. In keeping with previous findings [20, 21], the incidence of entecavir-associated serum ALT flares in Japanese patients was low. The serum ALT flares occurred against a background of 2 $\log_{10}$ copies/ml or more reductions in serum

HBV DNA level, and serum ALT levels subsequently normalized without discontinuation of entecavir. Therefore, the serum ALT flare noted here may indicate recovery of the host's immune response arising from the reduction in HBV viral titer [22, 23]. ALT flares have been reported after the discontinuation of entecavir therapy [15, 16], thus necessitating long-term follow-up to identify possible posttreatment viral rebound.

In conclusion, the results of this dose-ranging study demonstrate a clear dose–response relationship for entecavir in terms of mean HBV DNA level reduction at week 22. Entecavir 0.5 mg was significantly more effective than lamivudine 100 mg in reducing HBV DNA levels in nucleoside-naive Japanese adult patients with CHB. At this dose level, entecavir treatment resulted in serum HBV DNA levels of less than 400 copies/ml in 100% of patients and normalization of serum ALT levels in 80% of patients after 22 weeks. Moreover, entecavir 0.5 mg once daily was well tolerated and showed a comparable safety profile to lamivudine.

References

1. Maddrey WC. Hepatitis B: an important public health issue. J Med Virol 2000;61:362–366. doi:10.1002/1096-9071(200007)61:3<362::AID-JMV14>3.0.CO;2-I
2. Kao JH, Chen DS. The natural history of hepatitis B virus infection. In Lai CL, Locarnini S, editors. Hepatitis B Virus. London: International Medical Press; 2002. 161–172
3. Lok AS, McMahon BJ. American Association for the Study of Liver Diseases (AASLD) practice guidelines. Chronic Hepatitis B. Hepatology 2007;45:507–539. doi:10.1002/hep.21513
4. Rivkin A. A review of entecavir in the treatment of chronic hepatitis B infection. Curr Med Res Opin 2005;21:1845–1856. doi:10.1185/030079905X65268
5. Wong DKH, Cheung AM, O'Rourke K, Naylor CD, Detsky AS, Heathcote J. Effect of alpha-interferon treatment in patients with hepatitis B e antigen-positive chronic hepatitis B. A meta-analysis. Ann Intern Med 1993;119:312–323
6. Allen MI, Deslauriers M, Andrews CW, Tipples GA, Walters KA, Tyrrell DL, et al. Identification and characterization of mutations in hepatitis B virus resistant to lamivudine. Lamivudine Clinical Investigation Group. Hepatology 1998;27:1670–1677. doi:10.1002/hep.510270628
7. Chang TT, Lai CL, Chien RN, Guan R, Lim SG, Lee CM, et al. Four years of lamivudine treatment in Chinese patients with chronic hepatitis B. J Gastroenterol Hepatol 2004;19(11):1276–1282. doi:10.1111/j.1440-1746.2004.03428.x
8. Liaw YF, Sung JJ, Chow WC, Farrell G, Lee CZ, Yuen H, et al. Lamivudine for patients with chronic hepatitis B and advanced liver disease. N Engl J Med 2004;351(15):1521–1531. doi:10.1056/NEJMoa033364
9. Omata M. Treatment of chronic hepatitis B infection. N Engl J Med 1998;339:114. doi:10.1056/NEJM199807093390209
10. Liaw YF, Leung N, Kao JH, Piratvisuth T, Gane E, Han KH, et al. Asian-Pacific consensus statement on the management of chronic hepatitis B: a 2008 update. Hepatol Int 2008;2:263–283. doi:10.1007/s12072-008-9080-3
11. Genovesi EV, Lamb L, Medina I, Taylor D, Seifer M, Innaimo S, et al. Efficacy of the carbocyclic 2′-deoxyguanosine nucleoside BMS-200475 in the woodchuck model of hepatitis B virus infection. Antimicrob Agents Chemother 1998;42:3209–3217
12. Marion PL, Salazar FH, Winters MA, Colonno RJ. Potent efficacy of entecavir (BMS-200475) in a duck model of hepatitis B virus replication. Antimicrob Agents Chemother 2002;46:82–88. doi:10.1128/AAC.46.1.82-88.2002
13. Colonno RJ, Rose R, Baldick CJ, Levine S, Pokornowski K, Yu CF, et al. Entecavir resistance is rare in nucleoside naïve patients with hepatitis B. Hepatology 2006;44:1656–1665. doi:10.1002/hep.21422
14. Ono SK, Kato N, Shiratori Y, Kato J, Goto T, Schinazi RF, et al. The polymerase L528M mutation cooperates with nucleotide binding-site mutations, increasing hepatitis B virus replication and drug resistance. J Clin Invest 2001;107:449–455. doi:10.1172/JCI11100
15. Lai CL, Rosmawati M, Lao J, Van Vlierberghe H, Anderson FH, Thomas N, et al. Entecavir is superior to lamivudine in reducing hepatitis B virus DNA in patients with chronic hepatitis B infection. Gastroenterology 2002;123:1831–1838. doi:10.1053/gast.2002.37058
16. Chang TT, Gish RG, Hadziyannis SJ, Cianciara J, Rizzetto M, Schiff ER, et al. A dose-ranging study of the efficacy and tolerability of entecavir in lamivudine-refractory chronic hepatitis B patients. Gastroenterology 2005;129:1198–1209. doi:10.1053/j.gastro.2005.06.055
17. Ling R, Mutimer D, Ahmed M, Boxall EH, Elias E, Dusheiko GM, et al. Selection of mutations in the hepatitis B virus polymerase during therapy of transplant recipients with lamivudine. Hepatology 1996;24:711–713. doi:10.1002/hep.510240339
18. Tipples GA, Ma MM, Fischer KP, Bain VG, Kneteman NM, Tyrrell DLJ. Mutation in HBV RNA-dependent DNA polymerase confers resistance to lamivudine in vivo. Hepatology 1996;24:714–717
19. Tenney DJ, Levine SM, Rose RE, Walsh AW, Weinheimer SP, Discotto L, et al. Clinical emergence of entecavir-resistant hepatitis B virus requires additional substitutions in virus already resistant to lamivudine. Antimicrob Agents Chemother 2004;48:3498–3507. doi:10.1128/AAC.48.9.3498-3507.2004
20. Chang TT, Gish R, de Man R, Gadano A, Sollano J, Chao YC, et al. A comparison of entecavir and lamivudine for HBeAg-positive chronic hepatitis B. N Engl J Med 2006;354:1074–1076. doi:10.1056/NEJMoa051285
21. Sherman M, Yurdaydin C, Sollano J, Silva M, Liaw Y-F, Cianciara J, et al. Entecavir is superior to continued lamivudine for the treatment of lamivudine-refractory. HBeAg-positive chronic hepatitis B. Gastroenterology 2006;130:2039–2049
22. Boni C, Bertoletti A, Penna A, Cavalli A, Pilli M, Urbani S, et al. Lamivudine treatment can restore T cell responsiveness in chronic hepatitis B. J Clin Invest 1998;102:968–975. doi:10.1172/JCI3731
23. Nair S, Perrillo RP. Serum alanine aminotransferase flares during interferon treatment of chronic hepatitis B: is sustained clearance of HBV DNA dependent on levels of pretreatment viremia? Hepatology 2001;34:1021–1026. doi:10.1053/jhep.2001.28459

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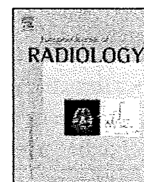

Evaluation of portosystemic collaterals by MDCT-MPR imaging for management of hemorrhagic esophageal varices

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ABSTRACT

Objective: To study the correlation between changes in portosystemic collaterals, evaluated by multidetector-row computed tomography imaging using multiplanar reconstruction (MDCT-MPR), and prognosis in patients with hemorrhagic esophageal varices (EV) after endoscopic treatment.
Methods: Forty-nine patients with primary hemostasis for variceal bleeding received radical endoscopic treatment: endoscopic injection sclerotherapy (EIS) or endoscopic variceal ligation (EVL). Patients were classified according to the rate of reduction in feeding vessel diameter on MDCT-MPR images, into the narrowing ($n = 24$) and no-change ($n = 25$) groups. We evaluated changes in portosystemic collaterals by MDCT-MPR before and after treatment, and determined rebleeding and survival rates.
Results: The left gastric and paraesophageal (PEV) veins were recognized as portosystemic collaterals in 100 and 80%, respectively, of patients with EV on MDCT-MPR images. The rebleeding rates at 1, 2, 3, and 5 years after endoscopic treatment were 10, 15, 23, and 23%, respectively, for the narrowing group, and 17, 24, 35, and 67%, respectively, for the no-change group ($P = 0.068$). Among no-change group, the rebleeding rate in patients with large PEV was significantly lower than that with small PEV ($P = 0.027$). The rebleeding rate in patients with small PEV of the no-change group was significantly higher than that in the narrowing group ($P = 0.018$). There was no significant difference in rebleeding rates between the no-change group with a large PEV and narrowing group ($P = 0.435$).
Conclusion: Changes in portosystemic collaterals evaluated by MDCT-MPR imaging correlate with rebleeding rate. Evaluation of portosystemic collaterals in this manner would provide useful information for the management of hemorrhagic EV.

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1. Introduction

Variceal bleeding is a serious adverse event in patients with liver cirrhosis. Patients surviving the first episode of variceal bleeding have a greater than 60% risk of recurrent hemorrhage within 1 year of the initial episode [1]. All patients surviving a variceal bleed should therefore receive radical treatment to prevent rebleeding. The combination therapy of pharmacological treatment and endoscopic variceal ligation (EVL) is generally considered to prevent variceal rebleeding [2–4]. However, some studies showed that narrowing of feeding vessels by embolization with endoscopic injection sclerotherapy (EIS) reduced the recurrence of esophageal varices (EV) [5–7].

Although some studies suggested a close relationship between changes to feeding vessels and EV recurrence after endoscopic therapy [8], little is known about the portosystemic collaterals and their association with rebleeding of hemorrhagic EV. New endoscopic methods to treat and monitor the treatment effect in these patients are clearly needed. The portal venous system has been evaluated by invasive methods such as angiography and percutaneous transhepatic portography (PTP). However, advances in computed tomography (CT) for diagnostic imaging allow useful information about portosystemic collaterals to be obtained by multidetector-row CT imaging (MDCT). Previous evaluation by MDCT-multiplanar reconstruction (MPR) imaging before and after endoscopic treatment showed a close relationship

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between changes in feeding vessels and prognosis after endoscopic therapy [9].

The present retrospective study was designed to determine the relationship between portosystemic collaterals and prognosis of hemorrhagic EV using MDCT-MPR imaging.

2. Materials and methods

2.1. Patients

Sixty consecutive patients with hemorrhaged EV were admitted to our institution from January 2000 to March 2007. Two patients died of hemorrhagic shock while 58 patients underwent emergency endoscopic examination after reaching a stable condition. Active bleeding was detected in 40 patients and in the remaining 18 patients spontaneous hemostasis was evaluated as a red plug in 4 patients and as a white plug in 14 patients. As a rule, primary hemostasis was induced at the bleeding point by endoscopic variceal ligation (EVL). All 40 patients treated by EVL achieved primary hemostasis. However, 2 refused radical treatment, one underwent liver transplantation after endoscopic hemostasis, and 6 patients died of liver failure. Thus, 49 patients (18 with spontaneous hemostasis and 31 with primary hemostasis) underwent radical treatment (Fig. 1). Radical endoscopic treatment was performed after estimating the general condition of the patient, liver function, renal function, portosystemic collaterals, and hepatocellular carcinoma (HCC) by MDCT-MPR imaging. Endoscopic injection sclerotherapy (EIS), in which the sclerosant (5% ethanolamine oleate) is injected into the varices, was generally used as the radical treatment. However, EVL was selected for patients with progressive HCC, poor liver function, poor renal function, and narrow EVs. Consequently, EIS was performed in 37 patients while EVL in 12 patients. MDCT-MPR imaging was also used to evaluate the effect of endoscopic treatment. None of the 49 patients was on β-blocker medication during the follow-up period. Fig. 1 shows the algorithm for selection of patients with acute variceal bleeding for treatment. Fig. 2 shows examples of variceal bleeding and hemostasis with EVL.

Table 1 lists the clinical characteristics of patients. Endoscopic findings of the EVs were evaluated according to the classifica-

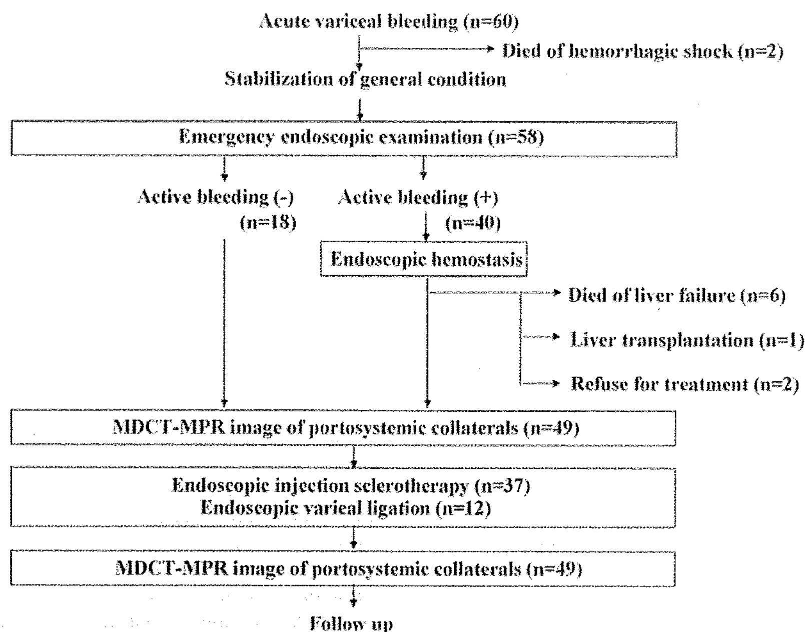

Fig. 1. Algorithm for selection of patients with acute variceal bleeding for treatment.

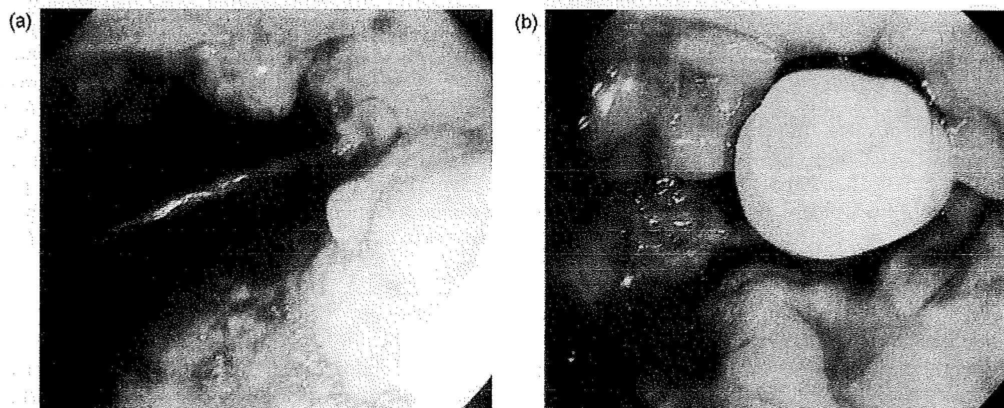

Fig. 2. Endoscopic findings of hemorrhagic esophageal varices. (a) spurting bleeding and (b) endoscopic variceal ligation.

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