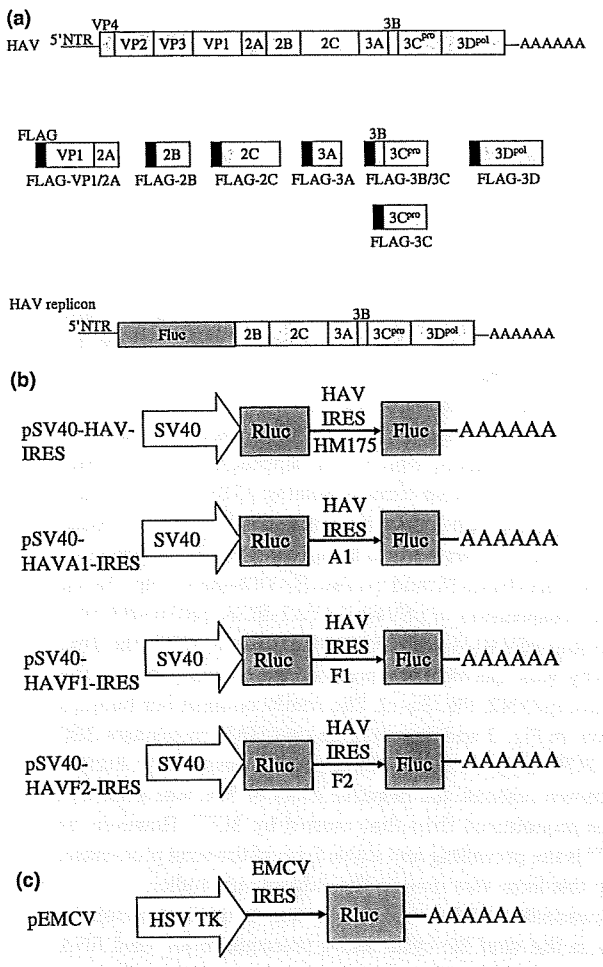

Fig. 1 Schematic representation of the hepatitis A virus (HAV) constructs used in this study. (a) Structures of the HAV genome (upper panel), FLAG-tagged HAV proteins (middle panel; ref. 17) and HAV replicon pT7-18f-luciferase (lower panel; ref. 13). AAAAAA, poly A tail. (b) Structure of the bicistronic plasmids used. pSV40-HAV-internal ribosome entry site (IRES) encodes the Renilla luciferase, the IRES of HAV strain HM175 and the firefly luciferase (Fluc) under the control of the simian virus 40 promoter (SV40) (ref. 16). pSV40-HAVA1-IRES, pSV40-HAVF1-IRES and pSV40-HAVF2-IRES encode IRES elements derived from an acute hepatitis and two fulminant hepatitis cases, respectively. (c) Structure of plasmid pEMCV.

competes with cap-dependent translation of host proteins [1,12]. In this study, we show that HAV $3C^{pro}$ cleaved PTB and suppressed cap-independent translation initiation. The data indicate that the viral proteinase might play an important role in the regulation of HAV IRES-mediated cap-independent translation by targeting noncanonical translation factors.

MATERIALS AND METHODS

Cell lines

Huh-7, a human hepatoma cell line, and its stably transformed derivative Huh-T7 that expresses the T7 RNA polymerase [3] were grown in Dulbecco's modified Eagle medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated foetal bovine serum with or without G418 sulfate (400 μg/mL; Promega, Madison, WI, USA), in addition to penicillin and streptomycin.

Plasmids

pT7-18f-luciferase (LUC), a replication-competent HAV replicon, containing an open-reading frame with the firefly luciferase (Fluc) flanked by the first four amino acids of the HAV polyprotein and by 12 C-terminal amino acids of VP1, followed by the P2 and P3 domains of the HAV polyprotein (HAV strain HM175 18f, GenBank Accession No. M59808), and pT7-18f-LUCmut, a replication-deficient replicon, were described previously [13] (Fig. 1a).

The constructs encoding the simian virus 40 (SV40) promoter-driven *Renilla reniformis* luciferase (Rluc), the IRES derived from the cell culture adapted HAV strain HM175 [14], and Fluc, named pSV40-HAV-IRES, was prepared as described previously [2,15] (Fig. 1b). To investigate the specific effect exerted by the HAV IRES sequences, bicistronic reporter constructs (pSV40-HAVA1-IRES, pSV40-HAVF1-IRES and pSV40-HAVF2-IRES; Kanda *et al.*, manuscript in preparation) were prepared, which included the IRES of clinical specimens. Construction of HAV protein expression plasmids was described previously [16]. Briefly, seven regions of the HAV genome were amplified by reverse transcription-polymerase chain reaction (PCR) with HAV region-specific primers [16]. These regions were HAV VP1-2A, 2B, 2C, 3A, 3BC, 3C and 3D. The amplified products were cloned into pCXN2 (kindly provided by Prof. J. Miyazaki, Osaka University, Japan), a mammalian expression vector with a beta-actin-based CAG promoter and a SV40 origin [17], to generate pCXN2-FLAG-VP1-2A, 2B, 2C, 3A, 3BC, 3C, 3D expressing FLAG-tagged proteins [16] (Fig. 1a). To control for the target specificity, pEMCV, which contains the encephalomyocarditis virus (EMCV) IRES upstream of Rluc, was generated (Fig. 1c). Transient expression of $3C^{pro}$ using vaccinia virus, pGEM-3C, and pEXT7-HAV3C was described before [12].

Transfection and protein analyses

Approximately 60% confluent Huh-7 cells, grown in 6-well culture plates, were transfected with 0.3 μg of the LUC reporter plasmid and 0.1 μg of each HAV protein-expressing plasmid using Effectene transfection reagent (Qiagen, Tokyo, Japan). Forty-eight hours after transfection, cell

extracts were prepared, and a LUC assay kit (Toyo Ink, Tokyo, Japan) was used according to the manufacturer's instructions. LUC activity was measured in relative light units with a luminometer (AB-2200-R; ATTO, Tokyo, Japan). The assays were adjusted to protein amount and were conducted, on average, in duplicate [18]. To determine cleavage of the host proteins PTB and poly(A)-binding protein (PABP), extracts of transfected cells were analysed for viral antigen and host proteins, as described previously [12]. Viral proteins were identified using anti-FLAG and anti-HAV 3C antibodies. PTB was recognized by the monoclonal antibody BB7 [19].

RESULTS

HAV proteinases 3BC and 3C suppress IRES-dependent translation

Translation of the HAV polyprotein is initiated cap-independently and is driven by an IRES. As a first approach to assess the role of HAV proteins, we examined cap-independent and cap-dependent translation using the bicistronic reporter constructs depicted in Fig. 1. pSV40-HAV-IRES, which contains the IRES of HAV strain HM175 (Fig. 1b), was transfected into Huh-7 together with various expression vectors encoding FLAG-tagged HAV protein (Fig. 1a). The expression of these proteins was confirmed by Western blotting with anti-FLAG antibodies (data not shown and ref. 16). Compared to the control (pCXN2) and to the other HAV proteins tested (VP1-2A, 2B, 2C, 3A and 3D), expression of HAV 3BC or 3C specifically inhibited cap-independent translation initiated by the HAV IRES as determined by the Fluc activity (Fig. 2).

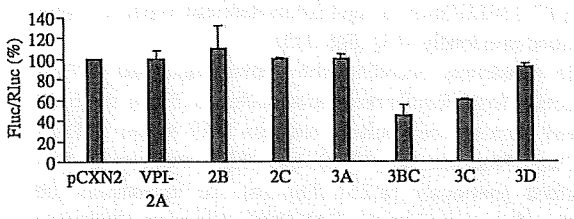

Fig. 2 Effects of hepatitis A virus (HAV) proteins on HAV internal ribosome entry site (IRES)-dependent translation. Relative luciferase activities are indicated (IRES/Cap; firefly luciferase/*Renilla reniformis* luciferase, %). Luciferase activities were determined in three independent experiments. Error bars represent standard errors of the mean.

To corroborate the observed suppression of HAV IRES-independent translation, we next examined the effect of $3C^{pro}$ on translation, which was dependent on HAV IRES elements derived from clinical isolates; IRES A1 was taken from an acute self-limited hepatitis (pSV40-HAVA1-IRES), and F1 and F2 were derived from fulminant HAV infections (pSV40-HAVF1-IRES and pSV40-HAVF2-IRES) (Fig. 3a–c). After coexpression of pSV40-HAVA1-IRES, pSV40-HAVF1-IRES and pSV40-HAVF2-IRES with 3BC or $3C^{pro}$, the Fluc activity was specifically suppressed when compared to the control (pCXN2, Fig. 3a–c). The results confirm our findings shown in Fig. 2 and demonstrate that HAV proteinases 3BC and $3C^{pro}$ suppress HAV IRES-dependent translation. For yet unknown reasons, the negative effect of 3BC was generally more pronounced than that exerted by $3C^{pro}$. However, as $3C^{pro}$ is the prevailing and stable form of the viral proteinase, only this form was used in the subsequent studies.

Translation of the viral polyprotein is the first metabolic step in the viral life cycle and a prerequisite for viral RNA synthesis. It can be assumed that a negative effect on

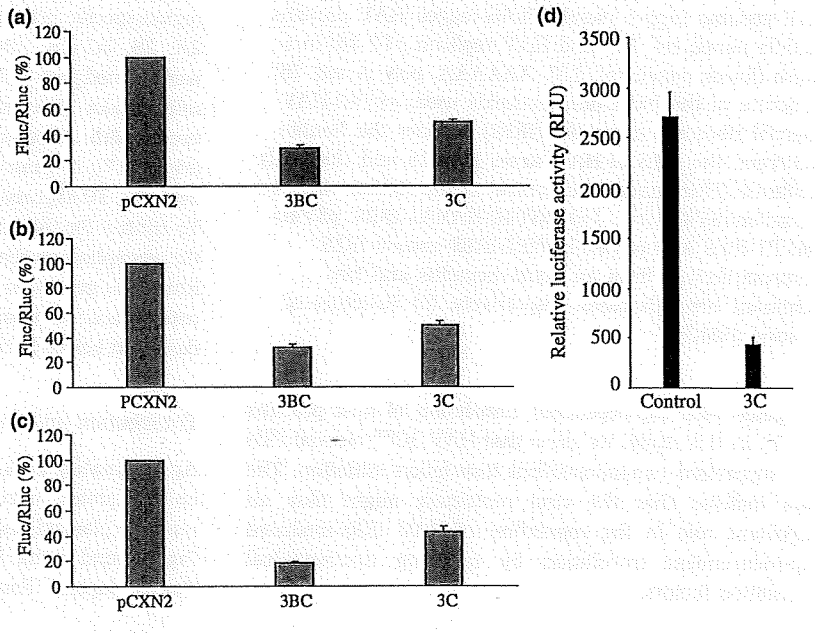

Fig. 3 Effects of hepatitis A virus (HAV) 3BC or $3C^{pro}$ on HAV internal ribosome entry site (IRES)-dependent translation (a–c) and on expression of the HAV replicon [pT7-18f-luciferase (LUC)] (d). HAV was derived from clinical isolates: (a) acute hepatitis; (b) and (c) two forms of fulminant hepatitis. Relative luciferase activities are indicated (IRES/Cap; firefly luciferase/*Renilla reniformis* luciferase, %) (a–c). LUC activities are presented as an average of three independent experiments. Error bars represent standard errors of the mean. RLU, relative light units.

translation might indirectly cause a reduction in viral genome production. HAV replication was efficiently studied using the viral replicon (see Fig. 1a, lower panel) with Fluc as reporter gene in place of the viral structural proteins [13]. The reporter gene activity is directly proportional to viral RNA synthesis. To investigate whether 3C-mediated suppression of translation affects genome replication, we cotransfected HAV replicon RNA with the 3C-expression or control vector into Huh-T7 cells (Fig. 3d). HAV replicon replication was monitored by reporter assay 72 h post-transfection. Compared to the replication-deficient replicon (pT7-18f-LUCmut), the reporter activity at this point was derived from newly synthesized viral genomes and therefore represents viral genome synthesis [13]. Compared to the control, HAV replication was significantly suppressed in the presence of excess 3C, indicating that 3C-mediated inhibition of translation restrained HAV genome replication in human hepatoma cells.

To assess the specificity of the inhibitory effect exerted by HAV proteinase $3C^{pro}$, translation initiated at the EMCV IRES was compared with the HAV IRES. For this, HAV $3C^{pro}$ was coexpressed with pEMCV (Fig. 1c), and the Rluc activity of the cell extracts collected 48 h post-transfection was determined. Compared to the HAV IRES tested in parallel experiments, the EMCV IRES activity was similar in the presence and absence of coexpressed HAV $3C^{pro}$ [118 ± 29 (%)]. Combined and in light of the results described in the following, these findings suggest that an essential ITAF was cleaved by HAV $3C^{pro}$. As shown in the following, HAV $3C^{pro}$ partially cleaved PTB, whose active role in picornaviral IRES-dependent translation has been demonstrated previously [6,8]. As EMCV IRES translation was unaffected by HAV $3C^{pro}$, PTB is not an essential ITAF for this IRES, confirming an earlier report [20]. Intriguingly, an excess of PTB even suppressed EMCV IRES-driven translation [21]. The abundance and distribution of PTB varies significantly among cell types [6,8]. Large amounts of PTB were found in the cytoplasmic fraction of Huh-7 cells that were used in our studies. Based on these observations, the results reported here suggest that HAV $3C^{pro}$ reduced the cytoplasmic levels of intact PTB to such a degree that only the activity of the HAV IRES was affected, but not that of the EMCV IRES.

$3C^{pro}$ cleaves PTB

It has been reported that the HAV IRES is associated with La autoantigen, GAPDH, PTB, PABP and PCBP [6,8,12–14]. The latter two proteins were cleaved by HAV $3C^{pro}$ [12,13]. Furthermore, it was shown that PTB is cleaved by polioviral $3C^{pro}$ and that PTB fragments inhibit polioviral IRES-dependent translation [22]. To assess whether the observed suppression of HAV IRES translation might be because of 3C-mediated cleavage of PTB, we tested the levels of endogenous PTB after transient expression of $3C^{pro}$ in Huh-7 cells. As GAPDH was found to suppress HAV IRES translation and to antagonize the enhancing effect of PTB [8], GAPDH levels were tested in parallel. As control for the proteolytic activity of $3C^{pro}$ *in vivo*, cleavage of the poly(A)-binding protein was also analysed. Recombinant $3C^{pro}$ was identified by immunoblot with anti-3C (Fig. 4, left panel) [12,13], and PABP was partially cleaved as demonstrated earlier (Fig. 4, middle panel). Whereas the levels of GAPDH were unchanged (data not shown), a PTB cleavage product of approximately 45 kDa and a slightly faster migrating polypeptide were clearly detectable when HAV $3C^{pro}$ was expressed (Fig. 4, right panel). The extent of host protein cleavage significantly depended on the amount of 3C expressed (compare lanes 1 and 3). Specific PTB cleavage was also observed when the extracts used in Fig. 2 were tested (not shown). Moreover, PTB of Huh-7 cells, the rabbit

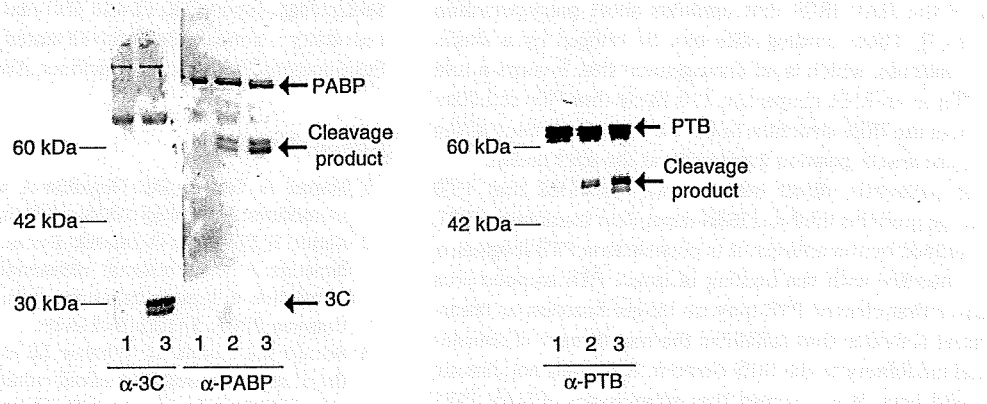

Fig. 4 Hepatitis A virus (HAV) $3C^{pro}$ cleaves the polypyrimidine tract-binding protein (PTB). Huh-7 cells were transfected with pGEM (lanes 1), pGEM-3C (lanes 2) and pEXT7-HAV3C (lanes 3) and infected with vaccinia virus T7. Cell lysates were collected 24 h post-transfection and subjected to immunoblot using anti-3C, anti-poly(A)-binding protein and anti- PTB. As less $3C^{pro}$ was produced by pGEM-3C in comparison with pEXT7-HAV3C, cleavage of host proteins was more pronounced in lanes 3 when compared to lanes2.

reticulocyte lysate, and recombinant PTB produced in *E. coli* was substrate to cleavage-mediated *in vitro* by purified recombinant HAV $3C^{pro}$ (data not shown). Combined with its well-documented translation enhancing effect and binding specificity to stem-loop IIIa of the HAV IRES [6–8], the results strongly suggest that the inhibitory effect of HAV $3C^{pro}$ on HAV IRES translation is because of proteolytic cleavage of PTB.

DISCUSSION

The expression level of the viral proteinase was found to substantially affect the detection of PTB cleavage products (see Fig. 4). Neither in HAV-infected cells nor in cells expressing the HAV replicon were PTB cleavage fragments detectable (not shown). A similar discrepancy was observed for PCBP, another ITAF that is essential for picornaviral translation and the molecular switching to RNA replication [9,23,24]. Whereas PCBP cleavage by recombinant HAV $3C^{pro}$ was clearly shown, PCBP-processing products were not apparent in extracts of HAV-infected cells [9]. Combined, our findings on HAV-3C-mediated cleavage of PCBP and PTB suggest that because of the protracted replication of HAV, very low quantities of $3C^{pro}$ are present in infected cells and cleavage of these host proteins is not discernible. This is in clear contrast to poliovirus whose highly efficient replication resulted in obvious cleavage of both PCBP and PTB [22,24].

The functional domains of PTB are four RNA recognition motifs that all bind short pyrimidine-rich sequences. By binding to different sites on the same RNA molecule, PTB can lead to distinctive RNA restructuring. Such conformational changes are thought to be critical in enabling the ribosomal recruitment in IRES-driven translation initiation. Our constructs do not include the 1–138 nt region of 5′NTR, in which a pyrimidine-rich-tract exists. PTB interacts with stem-loop IIIa of the HAV IRES that contains short polypyrimidine tracts [7]. These binding sites can be bridged by a single PTB molecule, which is an arrangement that favours a role for PTB as an RNA chaperone. It is likely that PTB stabilizes or alters the IRES structure to enable the recruitment of the ribosome and to position it correctly at the start codon.

For poliovirus, direct evidence was provided that PTB cleavage products inhibited IRES-dependent translation [22]. As outlined by the authors, it is possible that PTB fragments may interfere with the binding of intact PTB to poliovirus IRES or that cleaved PTB may no longer function as translational activator that facilitates the recruitment of translational machinery to the IRES element. Although not directly assessed here, it is assumed that suppression of HAV IRES translation is induced by similar mechanism(s). Moreover, in poliovirus-infected Hela cells, PTB cleavage fragments are redistributed to the cytoplasm [22]. As abundant quantities of PTB are present in the cytoplasm of Huh-7 cells used in our study [8], PTB redistribution might not be essential for the effect of PTB cleavage on HAV translation. Yet it is attractive to speculate that the PTB fragment(s) might have altered RNA-binding specificity. For poliovirus IRES translation, an attractive model was put forward for the participation of PTB and PCBP in the molecular switch from viral translation to RNA replication [22]. Supposedly, after viral 3C-mediated cleavage, PTB and PCBP lose their enhancing function. Once IRES translation is stalled, replication of the viral RNA consequently is turned on. Taken together with our earlier observations [9], HAV translation is inhibited indirectly by its own product, $3C^{pro}$, through the proteolytic cleavage of PCBP and PTB.

The HAV 3B and 3C proteins are 23 and 219 amino acids in length, respectively [25]. The 3B moiety was found to be essential for the 3AB interaction with 3CD [26]. It seems that 3BC was more suppressive than 3C in cap-independent translation. Further studies will reveal the 3B function in the interaction with PTB and 3BC. In conclusion, HAV proteinase 3C cleaved PTB and suppressed HAV IRES-dependent translation.

ACKNOWLEDGEMENT

The authors thank Dr S.U. Emerson and Dr J. Miyazaki for pHM175 and pCXN2, respectively.

STATEMENT OF PERSONAL INTERESTS

None of the authors have personal interests relevant to this research to declare.

DECLARATION OF FUNDING INTERESTS

This work was supported by grants 21590829, 21590828 and 21390225 from the Japan Science and Technology Agency, Ministry of Education, Culture, Sports, Science and Technology, Japan (TK, FI and OY) and a grant from Chiba University Young Research-Oriented Faculty Member Development Program in Bioscience Areas (TK).

REFERENCES

1 Martin A, Lemon SM. Hepatitis A virus: from discovery to vaccines. *Hepatology* 2006; 43(2 Suppl. 1): S164–S172.
2 Kanda T, Yokosuka O, Imazeki F *et al.* Amantadine inhibits hepatitis A virus internal ribosomal entry site-mediated translation in human hepatoma cells. *Biochem Biophys Res Commun* 2005; 331(2): 621–629.
3 Schultz DE, Honda M, Whetter LE *et al.* Mutations within the 5′ nontranslated RNA of cell culture adapted hepatitis A virus which enhance cap-independent translation in cultured African green monkey kidney cells. *J Virol* 1996; 70(2): 1041–1049.
4 Semler BL, Waterman ML. IRES-mediated pathways to polysomes: nuclear versus cytoplasmic routes. *Trends Microbiol* 2008; 16(1): 1–5.

5 Cordes S, Kusov Y, Heise T, Gauss-Müller V. La autoantigen suppresses IRES-dependent translation of the hepatitis A virus. *Biochem Biophys Res Commun* 2008; 368(4): 1014–1019.
6 Gosert R, Chang KH, Rijnbrand R *et al.* Transient expression of cellular polypyrimidine-tract binding protein stimulates cap-independent translation directed by both picornaviral and flaviviral internal ribosome entry sites in vivo. *Mol Cell Biol* 2000; 20(5): 1583–1595.
7 Schultz DE, Hardin CC, Lemon SM. Specific interaction of glyceraldehyde 3-phosphate dehydrogenase with the 5′-nontranslated RNA of hepatitis A virus. *J Biol Chem* 1996; 271(24): 14134–14142.
8 Yi M, Schultz DE, Lemon SM. Functional significance of the interaction of hepatitis A virus RNA with GAPDH: opposing effects of GAPDH and polypyrimidine tract binding protein on internal ribosome entry site function. *J Virol* 2000; 74(14): 6459–6468.
9 Zhang B, Seitz S, Kusov Y *et al.* RNA interaction and cleavage of poly(C)-binding protein 2 by hepatitis A virus protease. *Biochem Biophys Res Commun* 2007; 364(4): 725–730.
10 Sawicka K, Bushell M, Spriggs KA, Willis AE. Polypyrimidine-tract-binding protein: a multifunctional RNA-binding protein. *Biochem Soc Trans* 2008; 36(Pt 4): 641–647.
11 Kolupaeva VG, Hellen CU, Shatsky IN. Structural analysis of the interaction of the pyrimidine tract-binding protein with the internal ribosomal entry site of encephalomyocarditis virus and RNAs. *RNA* 1996; 2(12): 1199–1212.
12 Zhang B, Morace G, Gauss-Müller V, Kusov Y. Poly(A) binding protein, C-terminally truncated by the hepatitis A virus proteinase 3C, inhibits viral translation. *Nucleic Acids Res* 2007; 35(17): 5975–5984.
13 Gauss-Müller V, Kusov YY. Replication of a hepatitis A virus replicon detected by genetic recombination in vivo. *J Gen Virol* 2002; 83(Pt 9): 2183–2192.
14 Emerson SU, Lewis M, Govindarajan S *et al.* cDNA clone of hepatitis A virus encoding a virulent virus: induction of viral hepatitis by direct nucleic acid transfection of Marmosets. *J Virol* 1992; 66(11): 6649–6654.
15 Kanda T, Zhang B, Kusov Y *et al.* Suppression of hepatitis A virus genome translation and replication by siRNAs targeting the internal ribosomal entry site. *Biochem Biophys Res Commun* 2005; 330(4): 1217–1223.
16 Kanda T, Yokosuka O, Kato N *et al.* Hepatitis A virus VP3 may activate serum response element associated transcription. *Scand J Gastroenterol* 2003; 38(3): 307–313.
17 Niwa H, Yamamura K, Miyazaki J. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 1991; 108(2): 193–200.
18 Kanda T, Steele R, Ray R, Ray RB. Hepatitis C virus core protein augments androgen-receptor mediated signaling. *J Virol* 2008; 88(22): 11066–11072.
19 Chou MY, Underwood JG, Nikolic J *et al.* Multisite RNA binding and release of polypyrimidine tract binding protein during the regulation of c-src neural-specific splicing. *Mol Cell* 2000; 5(6): 949–957.
20 Kaminski A, Jackson RJ. The polypyrimidine tract binding protein (PTB) requirement for internal initiation of translation of cardiovirus RNAs is conditional rather than absolute. *RNA* 1998; 4(6): 626–638.
21 Kim YK, Jang SK. La protein is required for efficient translation driven by encephalomyocarditis virus internal ribosomal entry site. *J Gen Virol* 1999; 80(Pt 12): 3159–3166.
22 Back SH, Kim YK, Kim WJ *et al.* Translation of polioviral mRNA is inhibited by cleavage of polypyrimidine tract-binding proteins executed by polioviral $3C^{pro}$. *J Virol* 2002; 76(5): 2529–2542.
23 Gamarnik AV, Andino R. Switch from translation to RNA replication in a positive-stranded RNA virus. *Genes Dev* 1998; 12(15): 2293–2304.
24 Perera R, Daijogo S, Walter BL *et al.* Cellular protein modification by poliovirus: the two faces of poly(rC)-binding protein. *J Virol* 2007; 81(17): 8919–8932.
25 Totsuka A, Moritsugu Y. Hepatitis A virus proteins. *Intervirology* 1999; 42(2–3): 63–68.
26 Beneduce F, Ciervo A, Kusov Y *et al.* Mapping of protein domains of hepatitis A virus 3AB essential for interaction with 3CD and viral RNA. *Virol* 1999; 264(2): 410–421.

Human Pathology (2009) 40, 1304–1311

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Original contribution

Distinct expression of polycomb group proteins EZH2 and BMI1 in hepatocellular carcinoma

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Received 2 August 2008; revised 20 January 2009; accepted 30 January 2009

Keywords:
EZH2;
BMI1;
Hepatocellular carcinoma;
Short hairpin RNA;
MTS assay

Summary Polycomb gene products play a crucial role in the development of highly malignant phenotypes and aggressive cancer progression in a variety of cancers; however, their role in hepatocellular carcinoma remains unclear. First, we analyzed the impact of EZH2 and BMI1 modulation on cell growth of HepG2 cells. 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium assays revealed marked growth inhibition after *EZH2* or *BMI1* knockdown. In addition, simultaneous knockdown of these 2 genes further augmented cell growth inhibitory effects. Next, we conducted immnuohistochemical assessment of 86 hepatocellular carcinoma surgical specimens, evaluating the correlation between EZH2 and BMI1 protein expression and clinicopathologic features. High-level EZH2 and BMI1 expression was detected in 57 (66.3%) and 52 tumor tissues (60.5%), respectively. Among these, 48 tumor tissues (55.8%) showed colocalization of EZH2 and BMI1 in almost all tumor cells. The cumulative recurrence rate, but not survival rate, was significantly higher in patients positive for EZH2 ($P = .029$) and BMI1 ($P = .039$) than in their negative counterparts, as determined by Kaplan-Meier analysis. These data indicate that EZH2 and BMI1 may cooperate in initiation and progression of hepatocellular carcinoma.

1. Introduction

Cell-type–specific gene expression profiles are stabilized by changes in chromatin structure and DNA methylation patterns. Polycomb group proteins form multiprotein complexes and serve as a cellular memory system through epigenetic chromatin modifications [1,2]. So far, 2 major polycomb group complexes have been well characterized. Polycomb repressive complex 1 includes BMI1, RNF110/MEL18, HPH, CBX2/HPC1, Ring1A, and Rind1B, and polycomb repressive complex 2 includes EED, EZH2, and SUZ12. The polycomb repressive complex 1 and polycomb repressive complex 2 possess H2A-K119 ubiquitin E3 ligase activity and H3-K27 methyltransferase activity, respectively. BMI1, which is part of the polycomb repressive complex 1, contributes to the enhancement of RING1-mediated H2A-K119 ubiquitin E3 ligase activity. EZH2, a key molecule of

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0046-8177/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.humpath.2009.01.017

polycomb repressive complex 2, possesses histone methyltransferase activity and causes methylation at lysine residues of histone H3 (H3-K27). Both histone modifications contribute to polycomb group gene silencing. Although no physical associations have been demonstrated between the 2 complexes, H3-K27 methylation serves as a binding site for the recruitment of the polycomb repressive complex 1. Thus, the 2 polycomb group complexes could function in a cooperative manner to maintain gene silencing [1,2].

Polycomb repressive complex 1 has been implicated in stem cell self-renewal [1,2], a process by which epigenetic cellular memory is precisely inherited by daughter cells through cell division. Among polycomb repressive complex 1 components, we have demonstrated that BMI1 plays a central role in the inheritance of self-renewal of hematopoietic stem cells in both loss-of-function and gain-of-function analyses [3]. It is well recognized that polycomb repressive complex 1 transcriptionally represses tumor suppressor genes such as the Ink4b-Arf-Ink4a locus, which functions as a barrier to eliminate oncogenic cells by triggering apoptosis or cellular senescence [1,4]. We have demonstrated that tight repression of Ink4a-Arf in hematopoietic stem cells is essential to maintain the self-renewing capacity of hematopoietic stem cells [5]. On the other hand, there is increasing evidence that up-regulation of polycomb group proteins is deeply involved in tumor development. EZH2 is reportedly involved in the pathogenesis of malignant lymphoma and multiple myeloma [6,7]. BMI1 was initially identified as a c-myc–cooperating protooncogene in murine lymphomas [8], and increased levels of BMI1 expression has been implicated not only in human lymphoma but also in several types of leukemia [9-11]. Moreover, coexpression of EZH2 and BMI1 appears to be associated with the degree of malignancy in B-cell non–Hodgkin lymphoma [12]. Of importance, recent studies demonstrated that the increased expression of EZH2 proteins correlates with progression and poor prognosis of solid tumors such as prostate cancer and breast cancer [13-15].

In the present study, we first examined the cell growth activity of hepatocellular carcinoma cells stably expressing short hairpin RNAs against EZH2 and BMI1 in culture. Next, we conducted immunohistochemical analyses to estimate the expression levels of polycomb group proteins EZH2 and BMI1 in hepatocellular carcinoma. The cumulative survival rates and recurrent rates were analyzed using the Kaplan-Meier method to ask whether these molecules could be novel biologic markers.

2. Materials and methods

2.1. Cell culture

The hepatocellular carcinoma cell line HepG2 was cultured in Dulbecco's modified Eagle's medium (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen).

2.2. Lentiviral production and transduction

Lentiviral vectors (CS-H1-short hairpin RNA EF-1α-EGFP and CS-CDF-RfA-ERP) expressing short hairpin RNA that targets human EZH2 (target sequence: 5′-GGAAA-GAACGGAAATCTTA-3′), human BMI1 (target sequence: 5′-GAGAAGGAATGGTCCACTT-3′), and luciferase were constructed. Recombinant lentiviruses were produced as described elsewhere [3]. The cells were transduced with viruses in the presence of protamine sulfate.

2.3. Immunocytochemical analyses

HepG2 cells were fixed with methanol. After blocking in 10% goat serum, the cells were stained with a primary rabbit anti-EZH2 antibody (Zymed, San Francisco, CA) and a primary mouse anti-BMI1 antibody (Upstate Biotechnology, Lake Placid, NY) for 12 hours at 4°C. The cells then were washed and incubated with Alexa-555–conjugated goat anti-rabbit immunoglobulin G (IgG) (Molecular Probes, Eugene, OR) or Alexa-488–conjugated goat anti-mouse IgG (Molecular Probes), as appropriate, for 2 hours at room temperature. After being washed in phosphate-buffered saline (PBS), the cells were coverslipped with a mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) dihydrochloride (Vector Laboratories, Burlingame, CA).

2.4. Measurement of cell proliferation by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay

Cell growth of HepG2 cells after stable knockdown of EZH2 and/or BMI1 was measured by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. For this, we seeded cells in a 6-well plate at a density of 1×10^4 cells per well. MTS assays were performed in triplicate using the Cell Titer 96 Aqueous One Solution Cell Proliferation kit (Promega, Madison, WI) at culture days 2, 3, 4, and 5.

2.5. Patients and surgical specimens

Eighty-six patients who underwent surgical resection for hepatocellular carcinoma at Chiba University, Chiba, Japan, hospital were analyzed in this study. Informed consent for research use of the specimens was obtained for all cases. There were 73 male patients and 13 female patients with an average age of 63 ± 11 years (52-80 years). Among these, 32 cases had liver cirrhosis and 50 cases showed chronic hepatitis with mild to moderate activities. Liver damage of the patients was due to autoimmune disorder (n = 1), hepatitis B virus (HBV) (n = 19), hepatitis C virus (HCV)

(n = 38), both HBV and HCV (n = 2), and unknown etiology (n = 26). Paraffin-embedded sections of the tumors and surrounding nontumor liver tissues were examined by hematoxylin-eosin (H&E) staining and immunohistochemistry.

2.6. Immunohistochemical analyses

After deparaffinization and inhibition of endogenous peroxidase activities, the pathologic specimens of surgically resected tumors were stained with rabbit anti-EZH2 (1:100, Zymed), mouse anti-BMI1 (1:100, Upstate Biotechnology) or mouse anti–Ki-67 (1:50; Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. Before incubation with the primary antibodies, antigen retrieval was performed with ethylenediaminetetraacetic acid buffer (pH 8.0) in a pressure cooker at 100°C for 1 minute. The proteins were detected using ImmPRESS peroxidase micropolymer staining kits (Vector Laboratories) and 3,3′-diaminobenzidine substrate according to the manufacturer's instructions. To investigate the colocalization of EZH2 and BMI1, we simultaneously stained the clinical specimens with indicated antibodies. Subsequently, they were stained with Alexa-555–conjugated goat anti-rabbit IgG (Molecular Probes) and Alexa-488–conjugated goat anti-mouse IgG (Molecular Probes), respectively.

2.7. Statistical analyses

Statistical differences between 2 groups in MTS assays were analyzed using the Student t test. The correlations between the expression of polycomb group proteins and clinicopathologic features were assessed using the χ^2 test and Student t test to analyze qualitative and quantitative data, respectively. The cumulative survival and recurrent rates were analyzed by the Kaplan-Meier method, and the statistical significance between the 2 groups positive and

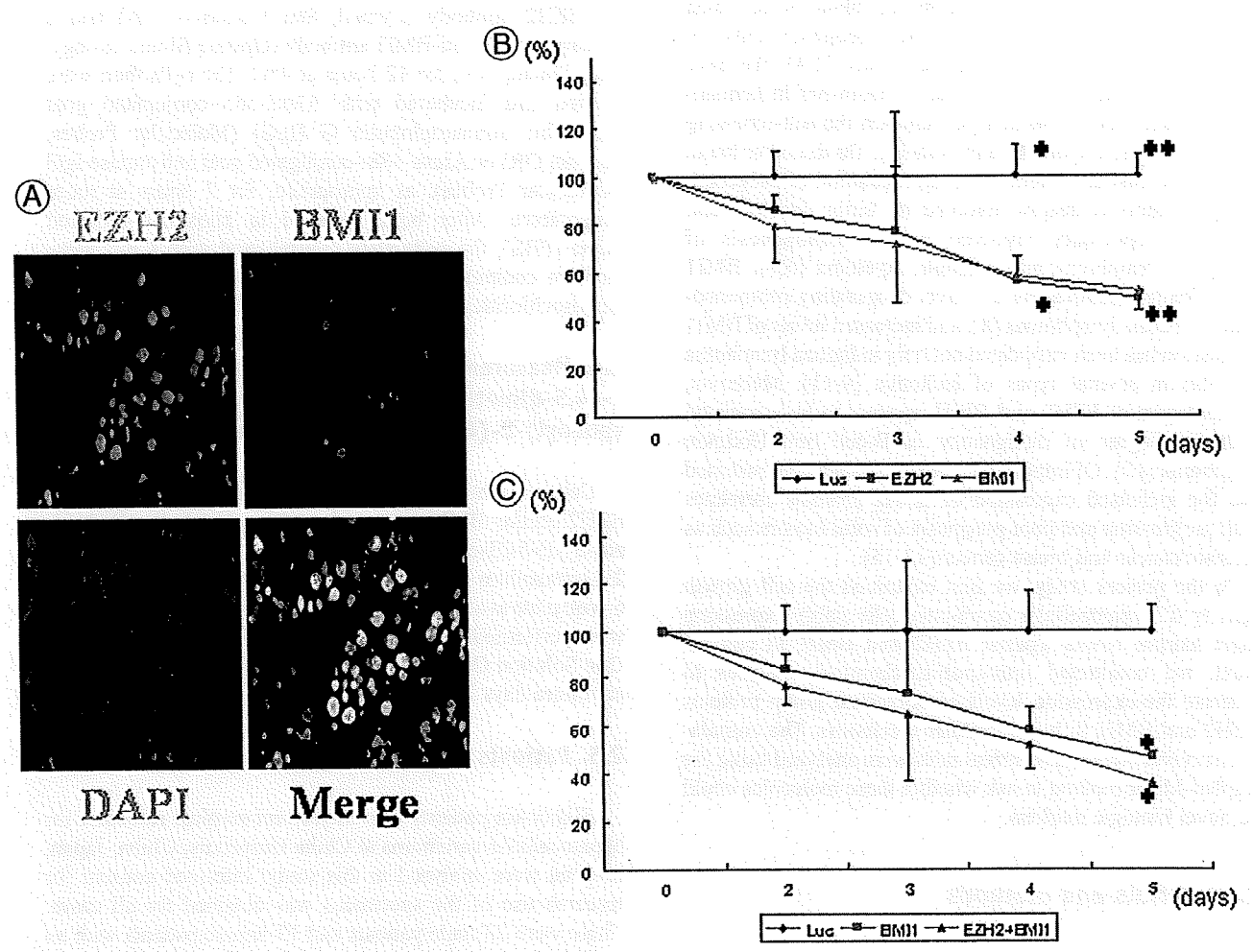

Fig. 1 Loss-of-function assays of EZH2 and BMI1 in HepG2 cells. (A) Immunocytochemical analyses of EZH2 and BMI1. Immunofluorescent labeling of EZH2 (green), BMI1 (red), and nuclear DAPI staining (blue) is merged. (B) Cell growth inhibition of EZH2 or BMI1 knockdown HepG2 cells compared with luciferase knockdown HepG2 cells as a control. Mean ± SD values are shown for each group, $*P < .05$, $**P < .005$. (C) Augmented growth inhibition of both EZH2 and BMI1 knockdown HepG2 cells. Mean ± SD values are shown for each group, $*P < .005$.

negative for polycomb group immunostaining was analyzed using the Wilcoxon test. Cox proportional regression analysis was performed to estimate rate ratios for the effect of EZH2 and BMI1 staining in tumors for recurrence. Potential risk factors assessed for recurrence included the following variables: BMI1 in tumors (presence versus absence), EZH2 in tumors (presence versus absence), sex (male versus female), age (>65 versus ≤65 years), size of tumors (>45 versus ≤45 mm), differentiation (poorly versus well, moderately), TNM stage [16] (I + II versus III + IVa), protein induced by vitamin K absence II (PIVKA II) level (>100 versus ≤100 mAU/mL), α-fetoprotein (AFP) level (>40 versus ≤40 mg/mL), etiology (HBV and HCV versus HBV alone, HCV alone, non-HBV, and non-HCV), chronic hepatitis (versus liver cirrhosis), aspartate aminotransferase (AST) level (>50 versus ≤50 IU/L), alanine aminotransferase (ALT) level (>50 versus ≤50 IU/L), total bilirubin level (>1.0 versus ≤1.0 mg/dL), albumin level (>3.8 versus ≤3.8 g/dL), platelet count (>15 × 10^9/L versus ≤15 × 10^9/L), prothrombin time (>80% versus ≤80%), the presence of portal invasion (versus no portal invasion), and tumor number (>2 versus ≤2). Variables statistically significant by univariate Cox proportional regression analysis were further studied by multivariate analysis. The differences were considered significant at $P < .05$.

3. Results

3.1. Stable short hairpin RNA-mediated knockdown of EZH2 and BMI1 mediated growth inhibition in HepG2 cells

To examine the basal expression of EZH2 and BMI1 in hepatocellular carcinoma cells, we conducted dual immunocytochemical analyses in HepG2 cells. EZH2 and BMI1 were widely detected in the nucleus and were coexpressed in more than 70% of HepG2 cells (Fig. 1A). To gain insight into the role of the polycomb gene products in HepG2 cells, we performed loss-of-function assays using lentivirus-mediated knockdown techniques. MTS assays showed that cell growth activity was consistently repressed, after EZH2 and BMI1 knockdown, for the 5 days of the observation period (Fig. 1B). Cell growth activity at days 2, 3, 4, and 5 was 86% ± 6.2%, 77% ± 27%, 56% ± 10%, and 49% ± 2.4%, respectively, after EZH2 knockdown and 79% ± 15%, 72% ± 25%, 58% ± 2.5%, and 52% ± 8.3%, respectively, after BMI1 knockdown compared with the luciferase knockdown cells, with there being a statistically significant difference at day 4 ($P < .05$) and 5 ($P < .005$) in the case of EZH2 or BMI1 knockdown. Intriguingly, simultaneous knockdown of EZH2 and BMI1 mediated an even greater degree of growth inhibition of HepG2 cells (Fig. 1C). Cell growth activity at days 2, 3, 4, and 5 was 77% ± 7.9%, 65% ± 27%, 52% ± 10%, and 36% ± 1.8%, respectively, after simultaneous knockdown compared with luciferase knockdown cells, with there being a statistically significant difference at day 5 ($P < .005$) between knockdown of both EZH2 and BMI1 and that of BMI1 alone.

Table 1 EZH2 and BMI1 expression in hepatocellular carcinoma and equivalent nontumor tissues

	Tumor	Nontumor
EZH2	57/86 (66.3%)*	5/86 (5.8%)*
BMI1	52/86 (60.5%)**	4/86 (4.7%)**

*, **$P < .05$.

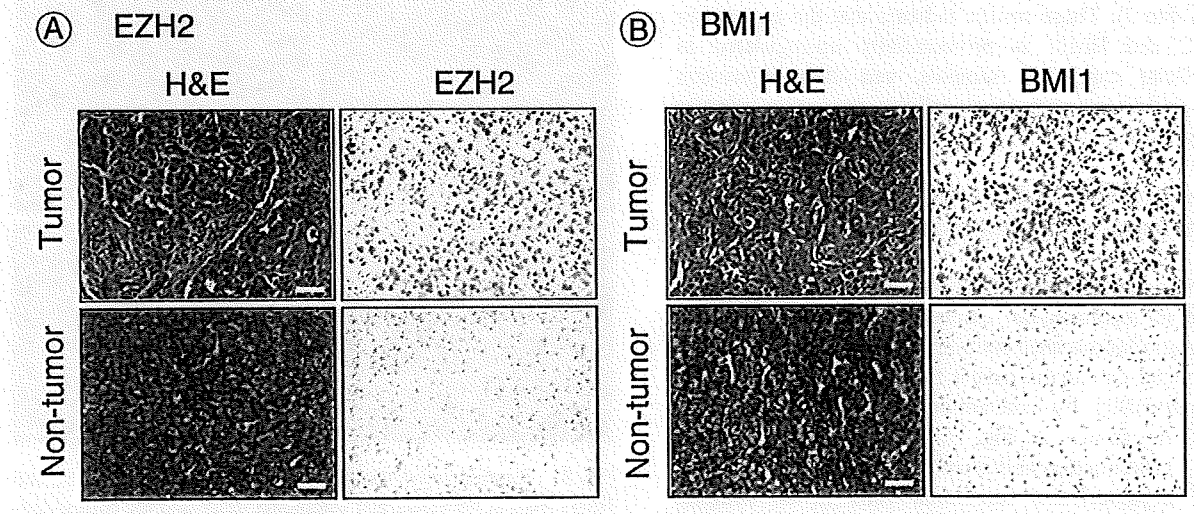

Fig. 2 Representative histopathologic analyses of EZH2 and BMI1. (A) H&E staining and immunohistochemical analysis of EZH2 in hepatocellular carcinoma tissue and the corresponding nontumor tissue. (B) H&E staining and immunohistochemical analysis of BMI1 in hepatocellular carcinoma tissue and the corresponding nontumor tissue (scale bar = 50 μm).

Table 2 The expression pattern of EZH2 and BMI1 in tumor and nontumor tissues

	Tumor				Nontumor			
	BMI1+	BMI1−	Total	*P*	BMI1+	BMI1−	Total	*P*
EZH2+	48	9	57	<.01	0	5	5	>.99
EZH2−	3	26	29		4	77	81	
Total	51	35	86		4	82	86	

3.2. Preferential expression of EZH2 and BMI1 in hepatocellular carcinoma samples

We evaluated the expression levels of polycomb group proteins EZH2 and BMI1 in hepatocellular carcinoma tissues and the corresponding nontumor tissues obtained from 86 patients by histopathologic analyses. Representative immunohistochemical staining of EZH2 and BMI1 is shown in Fig. 2A and B. The expression of EZH2 and BMI1 was diffusely detected in the nuclei of cancer cells, and no patients showed focal expression of these proteins in tumor tissues.

Fifty-seven (66.3%) of 86 hepatocellular carcinoma samples were positive for EZH2, whereas only 5 (5.8%) of 86 nontumor samples showed EZH2 expression ($P < .01$) (Table 1). On the other hand, BMI1 expression was detected in 52 (60.5%) of 86 hepatocellular carcinoma samples, whereas its expression was detected in only 4 (4.7%) of 86 nontumor samples ($P < .01$) (Table 1). Of importance, 48 (55.8%) of 86 hepatocellular carcinoma samples expressed both EZH2 and BMI1 (Table 2). Consequently, 26 (30.2%) of 86 samples did not express either protein. These results showed a statistically significant difference with respect to EZH2-positive ratio between BMI1-positive and BMI1-negative tumor tissues ($P < .01$). In contrast, 77 (89.5%) of 86 nontumor samples were negative for both EZH2 and BMI1 (Table 2). These results indicate that the expression of EZH2 and BMI1 is preferentially up-regulated in hepatocellular carcinoma samples, and their expression patterns could segregate hepatocellular carcinoma samples into subgroups.

3.3. Coexpression of EZH2 and BMI1 in hepatocellular carcinoma tissues

To determine whether EZH2 and BMI1 are coexpressed in the same hepatocellular carcinoma cells, we performed dual immunofluorescence staining on 10 randomly selected hepatocellular carcinoma tissues that were positive for both EZH2 and BMI1 by conventional immunohistochemical analysis. As shown in Fig. 3, EZH2 and BMI1 were detected in the nucleus and were coexpressed in more than 70% of hepatocellular carcinoma cells in all samples analyzed. These results strongly indicate cooperative contributions of EZH2 and BMI1 to the development and progression of hepatocellular carcinoma.

3.4. Correlation between the expression of EZH2 or BMI1 and that of Ki-67

We performed immunohistochemical analysis of Ki-67 in tumor and nontumor tissues and compared the results with those of EZH2 and BMI1 (Table 3). EZH2 was positive in 47 (80%) of 59 Ki-67–positive tumors and in 10 (37%) of 27 Ki-67–negative tumors ($P = .0002$). BMI1 was also positive in 44 (75%) of 59 Ki-67–positive tumors and in 8 (30%) of 27 Ki-67–negative tumors ($P = .0001$). In nontumor tissues, the staining of Ki-67 was found only in 11 cases, and we found no correlation between these 2 proteins and Ki-67 (Table 3).

3.5. Relationship between EZH2 or BMI1 expression and clinicopathologic features

We next evaluated the relationship between increased expression of polycomb group proteins and clinicopathologic parameters of the 86 hepatocellular carcinoma patients (Table 4). Significant correlation was observed between increased EZH2 expression in hepatocellular carcinoma tissues and hypoalbuminemia ($P = .01$) or advanced TNM stage ($P = .04$). In contrast, increased BMI1 expression in the

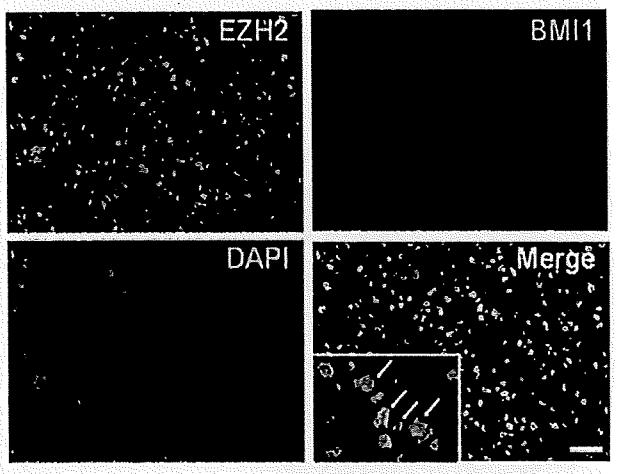

Fig. 3 Representative dual immunostaining of EZH2 (green) and BMI1 (red) in hepatocellular carcinoma tissue and the corresponding nontumor tissue. The nuclei were stained with DAPI (blue). Arrows indicate a typical nucleus positive for EZH2, BMI1, and DAPI (scale bar = 50 μm).

Table 3 Correlation between the expression of EZH2 or BMI1 and that of Ki-67

	Tumor				Nontumor			
	Ki-67+	Ki-67−	Total	*P*	Ki-67+	Ki-67−	Total	*P*
EZH2+	47	10	57	.0002	1	4	5	>.50
EZH2−	12	17	29		10	71	81	
Total	59	27	86		11	75	86	
BMI1+	44	8	52	.0001	1	3	4	.43
BMI1−	15	19	34		10	72	82	
Total	59	27	86		11	75	86	

hepatocellular carcinoma samples had no significant correlation with clinicopathologic factors.

3.6. Prognostic value of EZH2 or BMI1 expression in hepatocellular carcinoma patients

To elucidate the role of polycomb group proteins in hepatocellular carcinoma progression, we performed prognostic analyses of 61 hepatocellular carcinoma patients in whom curative operation was conducted and whose postoperative course could be followed. The cumulative recurrent rates and survival rates were analyzed by the Kaplan-Meier method (Fig. 4A-D). During the follow-up period (27.2 ± 19.6 months), 39 patients developed a recurrence of hepatocellular carcinoma and 19 patients died from relapsed hepatocellular carcinoma. Our results demonstrated higher cumulative recurrence rates in EZH2-positive patients than in EZH2-negative patients (P = .029). In contrast, the cumulative survival rates showed no significant differences between EZH2-positive and EZH2-negative patients. Of interest, results of the prognostic analysis correlating BMI1 expression were similar to those of the EZH2 expression-based analysis. The cumulative recurrence rates were higher in BMI1-positive patients than in BMI1-negative patients (P = .039), although the difference in survival rates between the 2 groups was not significant. Given that EZH2 and BMI1 are frequently coexpressed in the same samples, these prognostic findings appear quite reasonable.

Table 4 Correlation between polycomb group protein expression and clinicopathologic background in 86 hepatocellular carcinoma samples

Variables	EZH2 expression			BMI1 expression		
	Negative (n = 29)	Positive (n = 57)	*P*	Negative (n = 34)	Positive (n = 52)	*P*
Sex (male/female)	27/2	46/11	.13	31/3	42/10	.46
Age (y)	64.7 ± 9.8	62.6 ± 12.5	.45	63.6 ± 11.6	63.1 ± 11.8	.86
Liver function						
AST (IU/L)	66.8 ± 79.4	94.8 ± 126.0	.30	67.4 ± 75.0	97.2 ± 131.2	.25
ALT (IU/L)	71.4 ± 64.8	90.4 ± 122.3	.46	71.9 ± 64.3	92.0 ± 126.8	.42
Total bilirubin (mg/dL)	1.00 ± 0.38	1.12 ± 0.64	.39	0.96 ± 0.36	1.15 ± 0.66	.15
Albumin (g/dL)	4.02 ± 0.48	3.70 ± 0.53	.01[a]	3.92 ± 0.50	3.74 ± 0.55	.14
Prothrombin time (%)	84.4 ± 12.9	84.7 ± 19.3	.95	83.6 ± 12.5	85.1 ± 20.0	.76
Platelet ($\times 10^4$/mL)	15.60 ± 6.62	15.89 ± 6.66	.85	15.94 ± 6.88	15.70 ± 6.5	.88
PIVKA II (mAU/mL)	4027.0 ± 9934.8	5040.6 ± 12 873.8	.76	5654.6 ± 13 844.7	4250.1 ± 11 063.9	.65
AFP (mg/mL)	108.0 ± 181.6	9570.6 ± 37 690.7	.21	3710.9 ± 19 937.8	8283.1 ± 36 691.8	.53
Tumor factors						
Tumor size (mm)	47.4 ± 33.5	51.4 ± 33.6	.63	51.3 ± 34.5	49.4 ± 33.0	.81
No.	1.19 ± 0.51	1.40 ± 0.61	.18	1.26 ± 0.59	1.38 ± 0.58	.40
VP (+) (%)	3/19 (15.8%)	17/50 (34%)	.23	7/24 (29.2%)	13/45 (28.9%)	>.99
Well/moderate/poorly[b]	4/16/4	6/36/9	.84	5/20/4	5/32/9	.64
TNM (I/II/III/IVa)	5/15/4/0	5/25/20/8	.04[a]	4/17/8/0	6/23/16/8	.14
Etiology						
AIH/B/C/B and C/BC (−)	0/5/12/0/12	1/14/26/2/14	.63	1/6/14/0/13	0/13/24/2/13	.42
CH/LC	16/11	34/21	.81	21/11	29/21	.64

Abbreviations: VP, invasion to portal vein; AIH, autoimmune hepatitis; CH, chronic hepatitis; LC, liver cirrhosis; B, hepatitis B; C, hepatitis C.
NOTES: Reference range—AST, 10 to 35 IU/L; ALT, 5 to 40 IU/L; total bilirubin, 0.4 to 1.2 mg/dL; albumin, 3.9 to 5.2 g/dL; prothrombin time, 70% to 140%; platelet, 15 to 35 × 10^4/mL; PIVKA II, <40 mAU/mL; AFP, <20 ng/mL.

[a] Statistically significant.

[b] Histologic differentiation of the tumor.

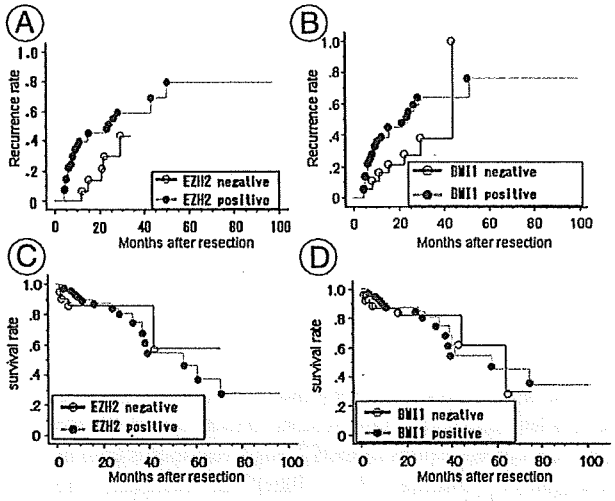

Fig. 4 Kaplan-Meier curves for the prognostic analyses. A and B, Cumulative recurrence rate with regard to EZH2 expression (A) and BMI1 expression (B). C and D, Cumulative survival rate with regard to EZH2 expression (C) and BMI1 expression (D).

The potential risk factors affecting tumor recurrence were analyzed by univariate Cox proportional hazards regression, and variables of P value less than 0.1 (the presence of BMI1, risk ratio, 2.3, $P = .053$; the presence of EZH2, risk ratio, 2.5, $P = .060$; age ≤65 years, risk ratio, 2.4, $P = .018$; AFP >40 mg/mL, risk ratio, 2.5, $P = .013$; albumin ≤3.8 mg/dL, risk ratio, 2.3, $P = .020$; the presence of portal vein invasion, risk ratio, 2.1, $P = .050$; TNM stage I + II, risk ratio, 0.40, $P = .015$) were further studied by multivariate analysis. However, we found that no variables examined in this multivariate analysis showed a statistically significant correlation with recurrence (the presence of BMI1, risk ratio, 1.4, $P = .51$; the presence of EZH2, risk ratio, 1.6, $P = .47$; age ≤65 years, risk ratio, 2.4, risk ratio, 2.0, $P = .07$; AFP >40 mg/mL, risk ratio, 2.0, $P = .12$; albumin ≤3.8 mg/dL, risk ratio, 2.2, $P = .11$; the presence of portal vein invasion, risk ratio, 0.64, $P = .43$; TNM stage I + II, risk ratio, 0.52, $P = .10$).

We found no effect on survival and recurrence in tumors that simultaneously expressed both EZH2 and BMI1 as compared with those that expressed only one of these proteins ($P = .61$ for recurrence and $P = .58$ for survival, as determined by Kaplan-Meier analysis).

4. Discussion

Hepatocellular carcinoma is one of the most common malignancies, and chronic viral infection by hepatitis B or C virus is well recognized as a major risk factor [17]. Therapeutic advancements such as nucleotide analogues and interferon has successfully improved hepatitis viremia and reduced the incidence of hepatocellular carcinoma, but the mortality rate of hepatocellular carcinoma remains high in spite of recent advances in cancer therapy [18]. In hepatocarcinogenesis, it appears that repeated injury and regeneration of damaged liver cells induce genetic and epigenetic dysregulation, which ultimately contribute to cancer development [19,20]. Recent studies highlight the pivotal role of polycomb group proteins in cancer [1]. However, our knowledge on their implications in hepatocarcinogenesis remains limited [21].

At first, we examined the growth activity of HepG2 cells in loss-of-function assays. The MTS assays showed that EZH2 or BMI1 knockdown mediated significant cell growth inhibition. This finding appears to be consistent with previous observations suggesting a role for EZH2 or BMI1 in a variety of immortalized and transformed cells [6,7,22]. Interestingly, knockdown of both EZH2 and BMI1 augmented cell growth inhibition, which indicated that the function of EZH2 might partly differ from that of BMI1 in molecular mechanisms underlying proliferation of hepatocellular carcinoma cells.

On the other hand, immunohistochemical analyses showed that increased expression of either EZH2 or BMI1 protein was observed in 60 (69.8%) of 86 hepatocellular carcinoma samples. Meanwhile, only 5.8% and 4.7% of surrounding nontumor tissues expressed EZH2 and BMI1, respectively. These results unveil preferential up-regulation of polycomb group protein expression during hepatocarcinogenesis and might implicate a special role for polycomb group proteins in the development and progression of hepatocellular carcinoma. Given that EZH2 and BMI1 were frequently coexpressed in the same samples, there might be functional crosstalk between EZH2 and BMI1 in the pathogenesis of hepatocellular carcinoma. Conversely, 26 (30.2%) of 86 samples exhibited expression of neither protein, which might be reflective of inherent heterogeneity with respect to origin of hepatocellular carcinoma and/or underlying molecular mechanisms.

Analyses of EZH2 mRNA expression in hepatocellular carcinoma samples based on real-time reverse transcriptase polymerase chain reaction have previously documented no significant correlation between EZH2 expression and disease-free survival [21]. Recently, another group reported that overexpression of EZH2 and BMI1 is associated with aggressive biologic behavior including vascular invasion and lymph node metastasis [23,24]. To examine whether protein expression of EZH2 and BMI1 are good biomarkers for recurrence and survival in our samples, we conducted prognostic analyses. The present analyses demonstrate that increased expression of both EZH2 and BMI1 proteins significantly correlated with recurrence after hepatectomy ($P = .029$ and $P = .039$, respectively), although there was no significant correlation between the expression of these polycomb group proteins and survival. Analyses of clinicopathologic parameters showed lower levels of serum albumin and advanced stage of TNM in EZH2-positive patients compared with EZH2-negative patients, which might indicate advanced liver dysfunction and tumor stage in EZH2-positive patients. In contrast, the significant

correlation between high EZH2 expression and portal vein invasion of the tumor, which was previously reported by mRNA expression-based analyses [21], was not detected in this study ($P = .23$).

In conclusion, our studies showed that polycomb proteins, in particular, EZH2 and BMI1, can have a strong effect on proliferation of hepatocellular carcinoma cells and that simultaneous knockdown of EZH2 and BMI1 has more marked effect on cell growth inhibition than knockdown of either protein alone. Immunohistochemical analyses further demonstrated a clear association between EZH2 and BMI1 expression and the development and progression of hepatocellular carcinoma, as well as recurrence after curative surgery. Thus, EZH2 and BMI1 could be target molecules in the development of new treatment strategies against hepatocellular carcinoma.

Acknowledgments

The authors thank Dr Huroyuki Miyoshi (RIKEN, Tsukuba, Japan) for providing lentiviral vectors and Dr Yoh Zen (Kanazawa University, Kanazawa, Japan) for technical assistance in immunohistochemical analyses.

References

[1] Valk-Lingbeek ME, Bruggeman SWM, van Lohuizen M. Stem cells and cancer: the polycomb connection. Cell 2004;118:409-18.
[2] Sparmann A, van Lohuizen M. Polycomb silencers control cell fate, development and cancer. Nat Rev Cancer 2006;6:846-56.
[3] Iwama A, Oguro H, Negishi M, et al. Enhanced self-renewal of hematopoietic stem cells mediated by the polycomb gene product, Bmi-1. Immunity 2004;21:843-51.
[4] Gil J, Peters G. Regulation of the INK4b-ARF-INK4a tumour suppressor locus: all for one or one for all. Nat Rev Mol Cell Biol 2006;7:667-77.
[5] Oguro H, Iwama A, Morita Y, Kamijo T, van Lohuizen M, Nakauchi H. Differential impact of Ink4a and Arf on hematopoietic stem cells and their bone marrow microenvironment in Bmi1-deficient mice. J Exp Med 2006;203:2247-53.
[6] Visser HP, Gunster MJ, Kluin-Nelemans HC, et al. The Polycomb group protein EZH2 is upregulated in proliferating, cultured human mantle cell lymphoma. Br J Haematol 2001;112:950-8.
[7] Croonquist PA, Van Ness B. The polycomb group protein enhancer of zeste homolog 2 (*EZH2*) is an oncogene that influences myeloma cell growth and the mutant ras phenotype. Oncogene 2005;24:6269-80.
[8] van Lohuizen M, Verbeek S, Scheijen B, Wientjens E, van der Gulden H, Berns A. Identification of cooperating oncogenes in Eμ-myc transgenic mice by provirus tagging. Cell 1991;65:737-52.
[9] Smith KS, Chanda SK, Lingbeek M, et al. Bmi-1 regulation of INK4A-ARF is a downstream requirement for transformation of hematopoietic progenitors by E2a-Pbx1. Mol Cell 2003;12:393-400.
[10] Mohty M, Yong AS, Szydlo RM, Apperley JF, Melo JV. The polycomb group BMI1 gene is a molecular marker for predicting prognosis of chronic myeloid leukemia. Blood 2007;110:380-3.
[11] Chowdhury M, Mihara K, Yasunaga S, Ohtaki M, Takihara Y, Kimura A. Expression of Polycomb-group (PcG) protein BMI-1 predicts prognosis in patients with acute myeloid leukemia. Leukemia 2007;21:1116-22.
[12] van Kemenade FJ, Raaphorst FM, Blokzijl T, et al. Coexpression of BMI-1 and EZH2 polycomb-group proteins is associated with cycling cells and degree of malignancy in B-cell non–Hodgkin lymphoma. Blood 2001;97:3896-901.
[13] Varambally S, Dhanasekaran SM, Zhou M, et al. The polycomb group protein EZH2 is involved in progression of prostate cancer. Nature 2002;419:624-9.
[14] Kleer CG, Cao Q, Varambally S, et al. EZH2 is a marker of aggressive breast cancer and promotes neoplastic transformation of breast epithelial cells. Proc Natl Acad Sci U S A 2003;100:11606-11.
[15] Kim JH, Yoon SY, Jeong SH, et al. Overexpression of Bmi-1 oncoprotein correlates with axillary lymph node metastases in invasive ductal breast cancer. Breast 2004;13:383-8.
[16] Liver Cancer Study Group of Japan. The general rules for the clinical and pathological study of primary liver cancer. 2nd English ed. Tokyo: Kanehara & Co., Ltd.; 2003.
[17] Wang BE, Ma WM, Sulaiman A, et al. Demographic, clinical, and virological characteristics of hepatocellular carcinoma in Asia: survey of 414 patients from four countries. J Med Virol 2002;67:394-400.
[18] Colombo M, Donato MF. Prevention of hepatocellular carcinoma. Semin Liver Dis 2005;25:155-61.
[19] Bruix J, Boix L, Sala M, Llovet JM. Focus on hepatocellular carcinoma. Cancer Cell 2004;5:215-9.
[20] Roberts LR, Gores GJ. Hepatocellular carcinoma: molecular pathways and new therapeutic targets. Semin Liver Dis 2005;25:212-25.
[21] Sudo T, Utsunomiya T, Mimori K, et al. Clinicopathological significance of EZH2 mRNA expression in patients with hepatocellular carcinoma. Br J Cancer 2005;92:1754-8.
[22] Chen Y, Lin MC, Yao H, et al. Lentivirus-mediated RNA interference targeting enhancer of zeste homolog 2 inhibits hepatocellular carcinoma growth through down-regulation of stathmin. Hepatology 2007;46:200-8.
[23] Sasaki M, Ikeda H, Itatsu K, et al. The overexpression of polycomb group proteins Bmi1 and EZH2 is associated with the progression and aggressive biological behavior of hepatocellular carcinoma. Lab Invest 2008;00 00:1-10.
[24] Sasaki M, Yamaguchi J, Itatsu K, Ikeda H Nakanuma Y. Over-expression of polycomb group protein EZH2 relates to decreased expression of $P16^{INK4a}$ in cholangiocarcinogenesis in hepatolithiasis. J Pathol 2008;215:175-83.

doi:10.1111/j.1440-1746.2008.05593.x

HEPATOLOGY

Efficacy and safety of entecavir in nucleoside-naive, chronic hepatitis B patients: Phase II clinical study in Japan

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Key words
chronic hepatitis B, entecavir, Japanese.

Accepted for publication 2 July 2008.

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Abstract

Background and Aim: Entecavir has demonstrated clinical efficacy for chronic hepatitis B. This study evaluated the efficacy and safety of entecavir in nucleoside-naive Japanese chronic hepatitis B patients.

Methods: In this multicenter, double-blind study, 66 nucleoside-naive Japanese chronic hepatitis B patients were randomized to 0.1 mg entecavir ($n = 32$) or 0.5 mg entecavir ($n = 34$) daily for 52 weeks. The primary endpoint was the proportion of patients whose serum hepatitis B virus (HBV) DNA decreased from baseline by ≥ 2 $\log_{10}$ copies/mL or became undetectable (<400 copies/mL by polymerase chain reaction assay) at week 48.

Results: One hundred percent of patients in both treatment groups achieved the primary efficacy endpoint, with 81% and 68% of patients achieving undetectable HBV DNA in the 0.1 mg and 0.5 mg treatment groups, respectively. Mean changes from baseline in HBV DNA were -4.49 $\log_{10}$ and -4.84 $\log_{10}$ copies/mL for the 0.1 mg and 0.5 mg groups, respectively. Significant improvements in necroinflammation were seen in both groups, as assessed by Knodell and New Inuyama classifications. Most adverse events were transient and classified as grade 1 or 2. There were no clinically significant differences in adverse events across the two treatment groups and no discontinuations due to adverse events in either group.

Conclusions: In Japanese nucleoside-naive patients with chronic hepatitis B, 0.1 mg or 0.5 mg entecavir daily provided excellent efficacy and was well tolerated. The 0.5 mg dose was selected for the treatment of nucleoside-naive patients.

Introduction

It has been reported that 350–400 million people worldwide are chronically infected with hepatitis B virus (HBV)[1,2] despite the widespread use of HBV vaccination for prevention of this disease. HBV infection is particularly prevalent in Asia–Pacific countries, with an estimated 75% of all chronically infected patients living in the region.[3] Prevalence rates reported in 2000 indicated that 0.8% of the Japanese population were hepatitis B virus surface antigen (HBsAg) positive with 36% of infected individuals being chronically infected.[4] Among those chronically infected, 20–40% will develop cirrhosis, decompensated liver disease or hepatocellular carcinoma.[5] In Asia, HBV is the leading cause of chronic hepatitis, cirrhosis and hepatocellular carcinoma.[4]

Treatment for chronic hepatitis B has evolved markedly over the last decade. Interferon-α was the only available treatment for many years, but this cytokine is efficacious in only approximately 35% of patients,[6] and is poorly tolerated by many patients due to adverse effects. Lamivudine, a cytosine analog, was the first oral anti-HBV nucleoside analog developed, and has demonstrated efficacy for treatment of chronic hepatitis B during short-term administration.[7,8] However, viral breakthrough due to emergence of lamivudine-resistant strains of HBV with amino acid substitutions in the YMDD (tyrosine–methionine–aspartate–aspartate) motif of reverse transcriptase result in loss of clinical benefit.[9–11] Long-term follow up of hepatitis B e antigen (HBeAg)-negative patients treated with adefovir, the second approved oral anti-HBV, have shown cumulative probabilities of genotypic

resistance of 29% at 5 years[12] and some studies have reported that 20–50% of patients receiving a 10 mg dose of adefovir have primary non-response[13] indicating that the approved dose of adefovir may be suboptimal.[14]

Entecavir is a deoxyguanosine analog that has more than 300 times greater potency than lamivudine *in vitro*.[15,16] Entecavir inhibits all three steps of HBV DNA replication: (i) priming of the HBV DNA polymerase; (ii) reverse transcription of negative-strand HBV DNA from pre-genomic messenger RNA; and (iii) synthesis of positive-strand HBV DNA.[17] In woodchuck models of HBV infection, entecavir reduced viral loads by up to 9 $\log_{10}$ copies/mL and prevented the onset of hepatocellular carcinoma.[18] In early clinical studies, entecavir was demonstrated to be safe and efficacious when given for 28 days.[19] In a 24-week, phase II international clinical trial, Lai *et al.* demonstrated a dose–response relationship for entecavir, and showed that entecavir was superior to lamivudine at doses of 0.1 mg and 0.5 mg for HBV DNA reduction.[20] Subsequently, two phase III international trials showed that 0.5 mg entecavir daily for 48 weeks achieved superior histological, virological and biochemical improvement in HBeAg-positive and HBeAg-negative nucleoside-naive patients compared with lamivudine, with comparable safety and no emergence of resistance without prior existence of the amino acid substitutions rtL180M and rtM204V/I/S which are associated with lamivudine resistance.[21,22] Entecavir was approved by the US regulatory authorities in March 2005. Study AI463047 evaluated 0.01 mg, 0.1 mg and 0.5 mg entecavir and 100 mg lamivudine in nucleoside-naive Japanese patients and established the 0.5 mg dose of entecavir as the optimal dose in this patient population. The current phase II dose-ranging trial, which commenced before the completion of AI463047, evaluated the efficacy and safety of 0.1 mg and 0.5 mg entecavir daily for 52 weeks in nucleoside-naive chronic hepatitis B patients in Japan. This study's primary objective was to demonstrate that entecavir has antiviral activity as indicated by the proportion of subjects who achieve a reduction from baseline in HBV DNA by ≥ 2 $\log_{10}$ copies/mL or to <400 copies/ml at week 48.

Methods

Study design

This was a randomized, double-blind, multicenter trial of 0.1 mg entecavir once daily and 0.5 mg entecavir once daily for 52 weeks in nucleoside-naive patients with HBeAg-positive or -negative chronic hepatitis B. Patients were randomized via a central registration procedure. A total of 66 patients were enrolled in this study, including men and women ranging in age from 27–68 years who were determined to be eligible for the study during the 6-week screening period. Following randomization, patients received either a 0.1 mg entecavir tablet plus a 0.5 mg placebo tablet ($n = 32$) or a 0.5 mg entecavir tablet plus a 0.1 mg placebo tablet ($n = 34$) orally once daily for 52 weeks. After 52 weeks of blinded dosing, patients were given the option of enrolling in an entecavir rollover study. All patients who discontinued blinded dosing early, or who completed the protocol but did not enroll in the entecavir rollover study, were followed for 24 weeks post-dosing, and could receive marketed anti-HBV therapy as recommended by their physician.

The study was conducted in compliance with the ethical principles of the Declaration of Helsinki, Good Clinical Practice guidelines, and Articles/Notifications of the Ministry of Health, Labor and Welfare in Japan. Written informed consent was obtained from all patients.

The study's primary efficacy objective was to demonstrate that 0.1 mg and 0.5 mg doses of entecavir had antiviral activity as indicated by the proportion of patients who achieve a reduction in HBV DNA of ≥ 2 $\log_{10}$ copies/mL or to below the limit of quantification (LOQ, 400 copies/mL) by polymerase chain reaction (PCR) assay (Roche Amplicor, Hoffmann-La Roche Ltd, Basel, Switzerland) at week 48. Secondary endpoints included the mean change from baseline in HBV DNA by PCR assay at week 48, and proportions of patients who achieved the following at week 48: (i) HBV DNA less than 400 copies/mL; (ii) serum alanine aminotransferase (ALT) normalization (<1.25 times the upper limit of normal [ULN], World Health Organization [WHO] toxicity grade 0); (iii) HBeAg loss and HBeAg seroconversion (HBeAg loss and appearance of anti-HBe) among patients who were HBeAg-positive at baseline; and (iv) complete response, defined as HBV DNA less than 400 copies/mL by PCR assay plus ALT less than 1.25 × ULN plus HBeAg negativity for those who were HBeAg-positive at baseline. The incidence of amino acid substitutions associated with entecavir resistance in patients who experienced a virological breakthrough, defined as an increase in HBV DNA of ≥ 1 $\log_{10}$ copies/mL from nadir, was also determined. Among patients with evaluable baseline and week 48 liver biopsies, the proportion of patients with histological improvement was determined. Histological improvement was defined as a $\geq$2-point decrease in the Knodell necroinflammatory score and no worsening of fibrosis (worsening was defined as a $\geq$1-point increase in the Knodell fibrosis score) from baseline to week 48. Liver biopsies were also evaluated using the New Inuyama classification system. The biopsy reading committee was blinded to treatment and sequence.

The primary safety endpoint was the proportion of patients in each treatment group who discontinued study medication due to adverse events. Secondary safety endpoints included incidence of adverse events, serious adverse events, laboratory abnormalities, grade 3–4 clinical adverse events and grade 3–4 laboratory abnormalities. ALT flares were defined as ALT >2 × baseline and >10 × ULN.

Study population

Patients were eligible for enrollment if they met the following inclusion criteria: (i) hepatitis B surface antigen (HBsAg)-positive for 24 weeks or more prior to screening or HBsAg-positive for less than 24 weeks prior to screening, negative for immunoglobulin M anti-hepatitis B core antibody and confirmation of chronic hepatitis on liver biopsy; (ii) HBeAg-positive for more than 12 weeks prior to screening or HBeAg-negative and positive for anti-HBe; (iii) active viral replication as evidenced by HBV DNA of $\geq 10^5$ copies/mL by PCR assay at screening; (iv) serum ALT ranging 1.3–10 times the ULN; and (v) compensated liver disease, as indicated by a prothrombin time $\leq$3 s longer than the normal control value or international normalized ratio of $\leq$1.5, serum albumin of $\geq$3.0 g/dL (30 g/L) and total bilirubin of $\leq$2.5 mg/dL (42.75 μmol/L). Women of childbearing potential underwent

Journal of Gastroenterology and Hepatology 24 (2009) 255–261 © 2009 The Authors
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contraceptive procedures as appropriate to avoid pregnancy during the trial period and for up to 8 weeks after completion of the trial.

The following patients were excluded from the study: pregnant and nursing women; patients diagnosed with cirrhosis, or with a history or evidence of variceal bleeding, encephalopathy or ascites requiring diuretics or paracentesis; patients with other forms of liver disease or suspected hepatic tumors; patients diagnosed with HIV infection; patients with a history of pancreatitis within 24 weeks prior to initiation of protocol therapy; and patients with an increased risk of hepatic toxicity or pancreatitis. In addition, patients who received immunosuppressive therapy (including systemic administration of corticosteroid-derivative agents) or who were treated with interferon-α or -β, within 24 weeks prior to initiation of protocol therapy, were excluded from the study. Patients treated with anti-HBV nucleoside analogs for more than 12 weeks were also excluded.

Assay methods

Serum HBV DNA was determined by Roche Amplicor PCR assay (LOQ, 400 copies/mL; Roche Diagnostics, Tokyo, Japan)[23] in a central laboratory. Clinical laboratory tests, PCR assays for HBV DNA, and serological tests for HBV were performed at SRL Inc. (Tokyo, Japan), the central clinical laboratory designated by the trial sponsor. Liver biopsy was performed within 6 weeks of initiation of study therapy; or, if a liver biopsy had been previously obtained within 52 weeks before initiation of protocol therapy, it was used as the baseline specimen for histological evaluation. Baseline biopsies were evaluated using the Knodell Histological Activity Index (HAI) and Knodell fibrosis scores,[24] and the New Inuyama classifications.[25] Genotype analysis of HBV strains was performed on samples from all patients at baseline using a PCR-restriction fragment length polymorphism assay (SRL). All samples were also analyzed at baseline for evidence of amino acid substitutions associated with lamivudine resistance (rtM204V/I) using a PCR-enzyme-linked minisequence assay (Medical & Biological Laboratories, Aichi, Japan). In addition, patients who experienced virological breakthrough (increase in HBV DNA of ≥1 $\log_{10}$ copies/mL from nadir of treatment) had baseline and on-treatment samples analyzed for amino acid substitutions associated with entecavir resistance (rtT184, rtS202 and rtM250) using a HBV DNA polymerase sequence assay at SRL.

Statistical analysis

Analyses of efficacy endpoints were based on treated patients. The primary objective would be demonstrated if the lower limit of the 95% confidence interval for the proportion of patients who achieved a reduction in HBV DNA from baseline by ≥2 $\log_{10}$ copies/mL or to less than 400 copies/mL by PCR assay at week 48 in either treatment arm was at least 60%. Parameters represented by continuous variables were summarized by the mean and standard error. In the analysis of binary endpoints, patients with missing week 48 measurements were treated as missing (non-completer = missing). All reported *P*-values are two-sided. For comparison of liver biopsy specimens before and after treatment, a Wilcoxin signed-rank test was performed.

Table 1 Baseline demographics and characteristics

	0.1 mg entecavir $n=32$	0.5 mg entecavir $n=34$
Men, *n* (%)	24 (75)	26 (76)
Women, *n* (%)	8 (25)	8 (24)
Age (years), mean ± SD	44.1 ± 11.4	46.6 ± 10.1
Weight (kg), mean ± SD	67.6 ± 18.2	65.6 ± 13.1
Ethnicity		
Japanese, *n* (%)	32 (100)	34 (100)
HBV DNA, mean ± SD		
$\log_{10}$ copies/mL by PCR	7.26 ± 1.08	7.68 ± 0.97
HBeAg-positive, *n* (%)	26 (81)	27 (79)
ALT (IU/L), mean ± SD	159.6 ± 210.4	141.0 ± 91.9
AST (IU/L), mean ± SD	97.7 ± 103.2	93.1 ± 60.6
Total bilirubin (mg/dL), mean ± SD	0.58 ± 0.22	0.62 ± 0.25
Knodell HAI score, no. of biopsy	31	31
pairs performed, mean ± SE	8.5 ± 0.5	8.7 ± 0.5
Prior treatment		
Interferon, *n* (%)	0	0
Lamivudine, *n* (%)	0	1†
Nucleside/nucleotide analogs, *n* (%)	0	0
HBV genotype *n* (%)		
C	29 (91)	33 (97)
B	2 (6.3)	0
Unknown	1 (3)	1 (3)

†Less than 12 weeks of therapy, consistent with protocol.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; HAI, Histological Activity Index; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; PCR, polymerase chain reaction; SD, standard deviation; SE, standard error.

Results

Study population

Of 102 patients enrolled and screened, 66 were randomized and treated. Thirty-two patients were assigned 0.1 mg entecavir and 34 patients 0.5 mg entecavir. The two treatment groups were well balanced at baseline for demographic and disease-related characteristics (Table 1). Approximately 80% of patients in both groups were HBeAg-positive, and mean HBV DNA at baseline were 7.26 and 7.68 $\log_{10}$ copies/mL for the 0.1 mg and 0.5 mg groups, respectively. Overall, 62 patients were infected with HBV genotype C, and two patients were infected with HBV genotype B. In two patients, HBV genotype was not identified. All patients completed protocol therapy for 52 weeks and were assessed for efficacy and safety. Compliance, measured by the volume of unused product returned from subjects to the institution, was reported to be between 95% and 100%. After completion of the protocol therapy, all patients entered an entecavir rollover study.

Virological response

One hundred percent of patients in both treatment groups achieved the primary efficacy endpoint (a reduction from baseline in HBV DNA of ≥2 $\log_{10}$ copies/mL or to <400 copies/mL by PCR assay

Table 2 Virological, biochemical and serological responses at weeks 24 and 48

Response	0.1 mg entecavir $n=32$	0.5 mg entecavir $n=34$
Primary efficacy endpoint		
Reduction in HBV DNA ≥2.0 $\log_{10}$ copies/mL or to >400 copies/mL by PCR assay at week 48	32 (100)	34 (100)
Other virological endpoints		
Mean change from baseline by PCR ($\log_{10}$ copies/mL), mean ± SE		
Week 24	−4.43 ± 0.16	−4.79 ± 0.14
Week 48	−4.49 ± 0.16	−4.84 ± 0.14
HBV DNA <400 copies/mL by PCR, *n* (%)		
Week 24	20 (63)	19 (56)
Week 48	26 (81)	23 (68)
Normalization of ALT†		
Week 24, *n/n* with abnormal baseline (%)	24/28 (86)	27/32 (84)
Week 48, *n/n* with abnormal baseline (%)	27/28 (96)	30/32 (94)
HBeAg seroconversion at week 48‡	8/26 (31)	8/27 (30)
Complete response§ at week 48, *n* (%)	12 (38)	13 (38)

†World Health Organization grade 0: ALT of <1.25 × ULN.
‡Loss of HBeAg and gain of anti-HBe.
§HBV DNA <400 copies/mL, HBeAg-negative and ALT of <1.25 × ULN.
ALT, alanine aminotransferase; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; PCR, polymerase chain reaction; SE, standard error.

at week 48; Table 2). By week 4, 94% and 91% of patients in the 0.1 mg and 0.5 mg groups, respectively, achieved the primary endpoint; 100% of patients in both groups had achieved it by week 8 and that proportion was maintained through to the end of treatment (week 48). Mean serum HBV DNA declined rapidly in both groups through week 4, and thereafter declined more slowly (Fig. 1). Mean change from baseline in HBV DNA at week 24 was −4.43 $\log_{10}$ copies/mL for the 0.1 mg group and −4.79 $\log_{10}$ copies/mL for the 0.5 mg group. Between weeks 24 and 48, across both groups, only slight decreases in mean HBV DNA occurred. At week 48, mean change from baseline in HBV DNA was −4.49 $\log_{10}$ copies/mL for the 0.1 mg group and −4.84 $\log_{10}$ copies/mL for the 0.5 mg group (P = non-significant [NS]; Fig. 1, Table 2). Eighty-one percent of patients receiving 0.1 mg entecavir and 68% of patients receiving 0.5 mg entecavir achieved HBV DNA of less than 400 copies/mL by PCR assay at week 48 (P = NS, Table 2).

Biochemical response

Approximately 90% of patients demonstrated abnormal ALT (≥1.25 × ULN) at baseline. Among patients with abnormal baseline ALT, the proportions achieving ALT normalization (<1.25 × ULN; WHO toxicity grade 0) at week 48 were 96% (27/28) for patients receiving 0.1 mg entecavir and 94% (30/32) for patients receiving 0.5 mg entecavir (P = NS, Table 2).

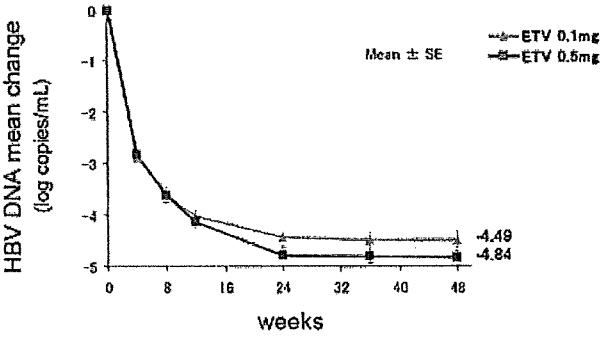

Figure 1 Mean change from baseline in hepatitis B virus (HBV) DNA through week 48 by polymerase chain reaction assay ($\log_{10}$ copies/mL) in patients treated with 0.1 mg and 0.5 mg entecavir (ETV). Data expressed as mean ± standard error.

Serological response

Among patients who were HBeAg-positive at baseline, the proportions achieving HBeAg loss at week 48 were 31% (8/26) in the 0.1 mg group and 30% (8/27) in the 0.5 mg group (Table 2). All patients who demonstrated HBeAg loss also showed acquisition of anti-HBe, thus rates of HBeAg seroconversion at week 48 were also 31% and 30% for the 0.1 mg group and 0.5 mg groups, respectively (Table 2).

Complete response

At week 48, the proportions of patients achieving complete response (defined as HBV DNA <400 copies/mL by PCR assay plus ALT <1.25 × ULN plus HBeAg negativity if they were HBeAg-positive at baseline) were 38% (12/32) for patients receiving 0.1 mg entecavir and 38% (13/34) for patients receiving 0.5 mg entecavir (P = NS, Table 2).

Histological response

Ninety-one percent (29/32) of patients in the 0.1 mg entecavir group and 88% (30/34) of patients in the 0.5 mg entecavir group had evaluable biopsy pairs from baseline and week 48 (Table 3). Histological improvement, defined using the Knodell classification system, occurred in 72% (21/29) and 80% (24/30) of patients in the 0.1 mg and the 0.5 mg groups, respectively. Mean change in Knodell HAI scores were −3.2 and −4.6 for the 0.1 mg and the 0.5 mg groups, respectively. For both groups, the change from baseline in HAI score was significant ($P < 0.0001$ for both groups). In patients who received 0.5 mg entecavir, 29% (9/31) of patients experienced an improvement or no worsening of Knodell fibrosis score, and the mean change from baseline in Knodell fibrosis score from baseline was significant ($P = 0.004$). According to New Inuyama classification, grading of necrotic/inflammatory findings improved for 64% (20/31) of patients in the 0.1 mg group and 74% (23/31) of patients in the 0.5 mg group, while no patient demonstrated worsening. For both groups, the improvement from baseline was significant ($P < 0.0001$ for both comparisons)

Table 3 Histological improvement at week 48 by Knodell scores and New Inuyama classification, relative to baseline

	0.1 mg entecavir $n=32$	0.5 mg entecavir $n=34$
Performed biopsy pairs, *n* (%)	31 (97)	31 (91)
Biopsy pairs evaluable for fibrosis, *n* (%)	29 (91)	30 (88)
Histological improvement (Knodell scores), *n* (%)†	21/29 (72)	24/30 (80)
Knodell HAI scores, reduction from baseline at Week 48, mean ± SE	−3.2 ± 0.5*	−4.6 ± 0.5*
New Inuyama classification, *n* (%)		
Grading (necroinflammation)‡	20/31 (64)*	23/31 (74)*
Staging (fibrosis)		
Improvement	7/29 (24)**	12/30 (40)***
No change	18/29 (62)**	17/30 (57)***
Worsening	4/29 (14)**	1/30 (3)***

Statistical significance relative to baseline. *$P<0.0001$, Wilcoxin signed-rank test (two patients in the 0.1 mg group and one in the 0.5 mg group are evaluable for necroinflammation but not fibrosis). **$P=0.432$, Wilcoxin signed-rank test. ***$P=0.003$, Wilcoxin signed-rank test.
†≥2-point decrease in Knodell necroinflammatory score with no worsening of Knodell fibrosis score.
‡Proportions with improvement.
HAI, Histological Activity Index; SE, standard error.

Table 4 Summary of safety

	No. of subjects (%)	
	0.1 mg entecavir $n=32$	0.5 mg entecavir $n=34$
Any adverse event *n* (%)	32 (100)	34 (100)
Clinical adverse events *n* (%)	31 (97)	34 (100)
Most frequent clinical adverse events,† *n* (%)		
Nasopharyngitis	15 (47)	17 (50)
Headache	9 (28)‡	10 (29)§
Abdominal pain upper	0	7 (21)
Tachycardia	0	5 (15)
Diarrhea	3 (9)	5 (15)
Laboratory adverse events *n* (%)	30 (94)	28 (82)
Grade 3–4 clinical adverse events *n* (%)	2 (6)	2 (6)
Grade 3–4 laboratory adverse events *n* (%)	5 (16)	6 (18)
Serious adverse events *n* (%)	3 (9.4)	3 (8.8)
Discontinuations due to adverse events *n* (%)	0	0¶
Death *n* (%)	0	0
ALT flare *n* (%)	2 (6)	2 (6)

†Occurring in at least 15% of patients of either treatment group.
‡Five cases related to study drug.
§Eight cases related to study drug.
¶One patient temporarily discontinued, but treatment resumed after 5 days when adverse event was judged unrelated to study drug.
ALT, alanine aminotransferase.

(Table 3). According to the New Inuyama fibrosis staging system, improvement in fibrosis occurred in 24% (7/29) of patients in the 0.1 mg group (P = NS) and in 40% (12/30) of patients in the 0.5 mg group ($P=0.003$, Table 3).

Resistance analysis

During the treatment period, two patients who received 0.5 mg entecavir experienced virological breakthrough at week 36. Both patients achieved undetectable HBV DNA. The first patient, with a baseline HBV DNA of 8.4 $\log_{10}$ copies/mL, experienced an increase in HBV DNA to 3.6 $\log_{10}$ copies/mL which was maintained at 48 weeks. The second patient, with a baseline HBV DNA of 8.5 $\log_{10}$ copies/mL, experienced an increase in HBV DNA to 4.5 $\log_{10}$ copies/mL and a subsequent decrease to 3.1 $\log_{10}$ copies/mL at week 48. Neither of the patients experienced ALT flares or other clinically relevant events. Genotypic analysis of HBV DNA polymerase was performed on samples from these two patients. Neither L180M nor M204V/I/S (which are associated with lamivudine resistance)[17,26,27] nor amino acid substitutions associated with entecavir resistance (at positions T184, S202 and M250) were detected.[28] Genotypic analysis of virus from all patients was carried out at baseline using methods with a sensitivity cut-off of 25%. Neither rtL180M nor rtM204V/I/S was detected in any patient. At week 48, the amino acid substitution rtM204I (associated with lamivudine resistance) was detected in two patients, one in the 0.1 mg group and one in the 0.5 mg group. However, entecavir was efficacious in the presence of the rtM204I variants and neither patient demonstrated virological breakthrough (HBV DNA decreased by 3.1 $\log_{10}$ copies/mL in the patient in the 0.1 mg group, and reduced to <2.6 $\log_{10}$ copies/mL in one patient in the 0.5 mg group at week 48) or elevation of ALT.

Safety

All 66 patients treated with the study drug completed 52 weeks of dosing. Adverse events were reported for all patients, but most were transient and mild or moderate (grade 1–2) in severity (Table 4). Adverse events were not considered to be related to the study drug, except for a number of cases of headache (five in the 0.1 mg arm and eight in the 0.5 mg arm), all of which were grade 1 or 2. Grade 3–4 clinical adverse events were observed in two patients (6%) in each treatment group, none of which was related to the study drug (one case of enteritis and one of spondylolisthesis in the 0.1 mg arm; one case of enteritis and one of intervertebral disc herniation in the 0.5 mg arm). Grade 3–4 laboratory adverse events occurred in five (16%) and six (18%) of patients in the 0.1 mg and 0.5 mg groups, respectively (two cases of ALT and AST elevations, two of lipase elevations and one of blood glucose elevation in the 0.1 mg arm; two cases of ALT and AST elevations, one of ALT elevation, one of lipase elevation, one of blood glucose elevation and one of amylase elevation in the 0.5 mg arm). There were no deaths in the study.

Serious adverse events occurred in three (9%) of patients in each treatment group (one case of infectious enterocolitis, one of acquired spondylolisthesis and one of ALT elevation in the 0.1 mg arm; one case of duodenal ulcer hemorrhage, one of ligament damage and one of intervertebral disc protrusion in the 0.5 mg arm). No serious adverse event was judged by the investigator

to be related to study medication. The protocol therapy was temporarily discontinued for one patient who developed duodenal ulcer hemorrhage, but therapy was restarted after 5 days when causal relationship with the test medication was ruled out. Except for this temporary interruption, no patient discontinued therapy for adverse events.

Alanine aminotransferase flares (defined as ALT of >2 times baseline and of >10 × ULN: grade 4) occurred in two patients (6%) in the 0.1 mg entecavir group and two patients (6%) in the 0.5 mg entecavir group. All ALT flares were transient, resolved on treatment, and were associated with a ≥2 $\log_{10}$ copies/mL reduction in HBV DNA. No ALT flare was associated with signs or symptoms of hepatic decompensation.

Discussion

The current study demonstrates that entecavir dosing for 52 weeks in Japanese patients was highly effective in reducing HBV DNA and normalizing ALT. The primary objective of this study was to demonstrate that entecavir has antiviral activity as indicated by the proportion of subjects who achieve a reduction from baseline in HBV DNA by ≥2 $\log_{10}$ copies/mL or to <400 copies/mL at week 48. All patients in both treatment groups achieved this primary efficacy endpoint, underscoring entecavir's potent anti-HBV efficacy. In both treatment groups, serum HBV DNA declined rapidly through week 4, then declined more slowly through week 24, and continued to decline through week 48 (mean change from baseline of −4.49 ± 0.16 and −4.84 ± 0.14 $\log_{10}$ copies/mL for 0.1 mg and 0.5 mg entecavir, respectively). This profile confirms the typical multiphasic pattern of antiviral action against HBV, similar to that observed in an international phase II 24-week trial of entecavir.[20]

Entecavir treatment also resulted in ALT normalization (ALT of <1.25 × ULN: WHO grade 0 toxicity) and HBeAg seroconversion concurrent with the observed declines in HBV DNA. Overall (across both treatment groups), approximately 95% of patients achieved ALT normalization, and approximately 30% of HBeAg-positive patients achieved HBeAg seroconversion. These results are similar to those reported in other international clinical trials of entecavir.[21,22] Specifically, Chang *et al.* reported that 48 weeks of entecavir 0.5 mg daily in nucleoside-naive, HBeAg-positive patients resulted in ALT normalization (ALT of ≤1.0 × ULN) in 68% and HBeAg seroconversion in 21% of these patients.[21] In the same study, 67% of patients achieved undetectable HBV DNA at week 48, which is comparable to the results of the present study. However, the mean change from baseline in HBV DNA in the study by Chang *et al.* (−6.9 $\log_{10}$ copies/mL) was greater than was observed in the current study (−4.84 $\log_{10}$ copies/mL for the entecavir 0.5 mg group). This difference is accounted for by the higher baseline HBV DNA of patients in the international trial (9.6 $\log_{10}$ copies/mL vs 7.7 $\log_{10}$ copies/mL for the 0.5 mg group in the present study).

The ultimate goal of chronic hepatitis B treatment is to arrest or reverse liver disease progression associated with HBV infection. This parameter is most directly and reliably measured by histological evaluation. In the current study, entecavir at doses of both 0.1 mg and 0.5 mg daily resulted in high rates of histological improvement (72% and 80%, respectively) when assessed by the Knodell scoring system. As the New Inuyama classification system is most often used to grade and stage liver disease progression in Japan, we also employed this method of histological evaluation, and obtained results consistent with the results of the Knodell evaluations; that is, there was significant improvement in necrosis/inflammation across both entecavir treatment groups and significant improvement in fibrosis for the 0.5 mg entecavir group. Entecavir's demonstrated histological benefit after 1 year of treatment suggests that its potent viral suppression might also reduce the risk of progression to cirrhosis and end-stage liver disease among chronic hepatitis B patients.

A high barrier to resistance among nucleoside-naive patients has been demonstrated with entecavir.[28,29] The combination of potent viral suppression and the requirement for multiple amino acid substitutions in the HBV reverse transcriptase to confer resistance to entecavir suggests that resistance emergence will be a rare event during long-term administration of entecavir. In phase III international clinical trials, less than 1% of patients treated with entecavir through 2 years experienced a virological breakthrough due to the emergence of entecavir resistance.[29] Phenotypic analyses have demonstrated that entecavir-resistant strains do not emerge in the absence of amino acid substitutions associated with lamivudine resistance (rtL180M and/or rtM204V/I/S).[29] In the present study, no amino acid substitutions at T184, S202 or M250 (all of which can mediate resistance in the presence of rtL180M + rtM204V/I/S) were detected. rtM204I emerged in two patients, one in the 0.1 mg entecavir group and one in the 0.5 mg entecavir group. In both patients, entecavir continued to suppress HBV replication and virological breakthroughs were not observed.

Entecavir was generally well tolerated in the current study. There were no clinically significant differences in the incidence or severity of adverse events between the two treatment groups, indicating that entecavir was well tolerated at a daily dose of 0.5 mg. ALT flares were infrequent, and those flares that did occur were associated with reductions in HBV DNA, and resolved without treatment interruption. These results are consistent with the safety and tolerability profile of entecavir reported in international trials.[20–22]

Entecavir's potent antiviral efficacy, good tolerability and high barrier to resistance offer the potential for long-term treatment of chronic hepatitis B with the objective of halting or reversing liver disease progression. The mean reduction in HBV DNA from baseline at week 48 and histological improvement observed in this trial, together with the results of previously published international trials, support the selection of the 0.5 mg dose of entecavir as an appropriate choice of primary therapy for treatment of nucleoside-naive Japanese patients with chronic hepatitis B infection.

Acknowledgments

This work was carried out with a grant from Bristol-Myers Squibb Company. T. S. is an employee of Bristol-Myers Squibb Company. M. O. serves as an advisor for Bristol-Myers Squibb Company. Thanks go to Namiki Izumi, Etsuro Orito, Takashi Kumada, Mitsuhiko Kawaguchi, Masanori Miyake, Kouzo Fujio, Yasuyuki Araki and Masaharu Ando who were investigators in this study and Chifumi Sato, Kendo Kiyosawa, Kyuichi Tanikawa who participated on the efficacy and safety committee for this study. The work was carried out at: Chiba University Hospital, Chiba; Musashino Red Cross Hospital, Tokyo; Nagoya City University Hospital, Aichi; Ogaki Municipal Hospital, Gifu; Okayama University

Hospital, Okayama; Okayama Saiseikai General Hospital, Okayama; Tsuyama Chuo Hospital, Okayama; Kurashiki Central Hospital, Okayama; Fukuyama City Hospital, Hiroshima; Hiroshima City Hospital, Hiroshima; Kagawa Prefectural Central Hospital, Kagawa; and Mitoyo General Hospital, Kagawa, Japan.

References

1 *Fact Sheet WHO/204 Hepatitis B. 2000.* Geneva, Switzerland: World Health Organization, 2003; 10–9.
2 Purcell RH. The discovery of the hepatitis viruses. *Gastroenterology* 1993; **104**: 955–63.
3 Mohamed R, Desmond P, Suh DJ *et al.* Practical difficulties in the management of hepatitis B in the Asia-Pacific region. *J. Gastroenterol. Hepatol.* 2004; **19**: 958–69.
4 Merican I, Guan R, Amarapuka D *et al.* Chronic hepatitis B virus infection in Asian countries. *J. Gastroenterol. Hepatol.* 2000; **15**: 1356–61.
5 Kao JH, Chen DS. The natural history of hepatitis B virus infection. In: Lai CL, Locarnini S, eds. *Hepatitis B Virus.* London: International Medical Press, 2002; 161–72.
6 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**: 312–23.
7 Dienstag JL, Schiff ER, Wright TL *et al.* Lamivudine as initial treatment for chronic hepatitis B in the United States. *N. Engl. J. Med.* 1999; **341**: 1256–63.
8 Lai CL, Chien RN, Leung NWY *et al.* A one-year trial of lamivudine for chronic hepatitis B. *N. Engl. J. Med.* 1998; **339**: 61–8.
9 Allen MI, Deslauriers M, Andrews CW *et al.* Identification and characterization of mutations in hepatitis B virus resistant to lamivudine. Lamivudine Clinical Investigation Group. *Hepatology* 1998; **27**: 1670–77.
10 Zoulim F. Detection of hepatitis B virus resistance to antivirals. *J. Clin. Virol.* 2001; **21**: 243–53.
11 Lau DT, Khokhar MF, Doo E *et al.* Long-term therapy of chronic hepatitis B with lamivudine. *Hepatology* 2000; **32**: 828–34.
12 Hadziyiannis S, Tassopolous N, Heathcote J *et al.* Long-term therapy with adefovir dipivoxil for HBeAg-negative chronic hepatitis B for up to 5 years. *Gastroenterology* 2006; **131**: 1743–51.
13 Fung S, Chae HB, Fontana R *et al.* Virologic response and resistance to adefovir in patients with chronic hepatitis B. *J. Hepatol.* 2006; **44**: 283–90.
14 Lok AS, McMahon BJ. Chronic hepatitis B. *Hepatology* 2007; **45**: 507–39.
15 Chang TT, Gish RG, Hadziyiannis SJ, Cianciara J, Rizzetto M, Schiff ER. A dose-ranging study of the efficacy and tolerability of entecavir in lamivudine-refractory chronic hepatitis B patients. *Gastroenterology* 2005; **129**: 1198–1209.
16 Ono SK, Kato N, Shiratori Y *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**: 449–55.
17 Seifer M, Hamatake RK, Colonno RJ, Standring DN. *In vitro* inhibition of hepadnavirus polymerases by the triphosphates of BMS-200475 and lobucavir. *Antimicrob. Agents Chemother.* 1998; **42**: 3200–8.
18 Colonno RJ, Genovesi EV, Medina I *et al.* Long-term entecavir treatment results in sustained antiviral efficacy and prolonged life span in the woodchuck model of chronic hepatitis infection. *J. Infect. Dis.* 2001; **184**: 1236–45.
19 de Man RA, Wolters LM, Nevens F *et al.* Safety and efficacy of oral entecavir given for 28 days in patients with chronic hepatitis B virus infection. *Hepatology* 2001; **34**: 578–82.
20 Lai CL, Rosmawati M, Lao J *et al.* Entecavir is superior to lamivudine in reducing hepatitis B virus DNA in patients with chronic hepatitis B infection. *Gastroenterology* 2002; **123**: 1831–38.
21 Chang TT, Gish RG, de Man R *et al.* A comparison of entecavir and lamivudine for HBeAg-positive chronic hepatitis B. *N. Engl. J. Med.* 2006; **354**: 1001–10.
22 Lai CL, Shouval D, Lok A *et al.* BEHoLD AI463027 Study Group. Entecavir versus lamivudine for patients with HBeAg-negative chronic hepatitis B. *N. Engl. J. Med.* 2006; **354**: 1011–20.
23 Matsuyama K, Hayashi K, Miura T *et al.* The Quantitative assay for HBV-DNA and the detection of HBV-DNA point mutation by Polymerase Chain Reaction -"AMPLICOR HBV MONITOR Test" and "HBV pre Core / Core Promoter Mutation Detection kit"-. *Kan Tan Sui* 2000; **41**: 59–71.
24 Knodell RG, Ishak KG, Black WC *et al.* Formulation and application of a numerical scoring system for assessing histological activity in asymptomatic chronic active hepatitis. *Hepatology* 1981; **1**: 431–35.
25 Ichida F, Tsuji T, Omata M *et al.* New Inuyama classification; new criteria for histological assessment of chronic hepatitis. *Int. Hepatology. Commun.* 1996; **6**: 112–19.
26 Ling R, Mutimer D, Ahmed M *et al.* Selection of mutations in the hepatitis B virus polymerase during therapy of transplant recipients with lamivudine. *Hepatology* 1996; **24**: 711–13.
27 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**: 714–17.
28 Tenney DJ, Levine SM, Rose RE *et al.* Clinical emergence of entecavir-resistant hepatitis B virus requires additional substitutions in virus already resistant to lamivudine. *Antimicrob. Agents Chemother.* 2004; **48**: 3498–7.
29 Colonno R, Rose R, Baldick CJ *et al.* Entecavir resistance is rare in nucleoside naïve patients. *Hepatology* 2006; **44**: 1656–65.