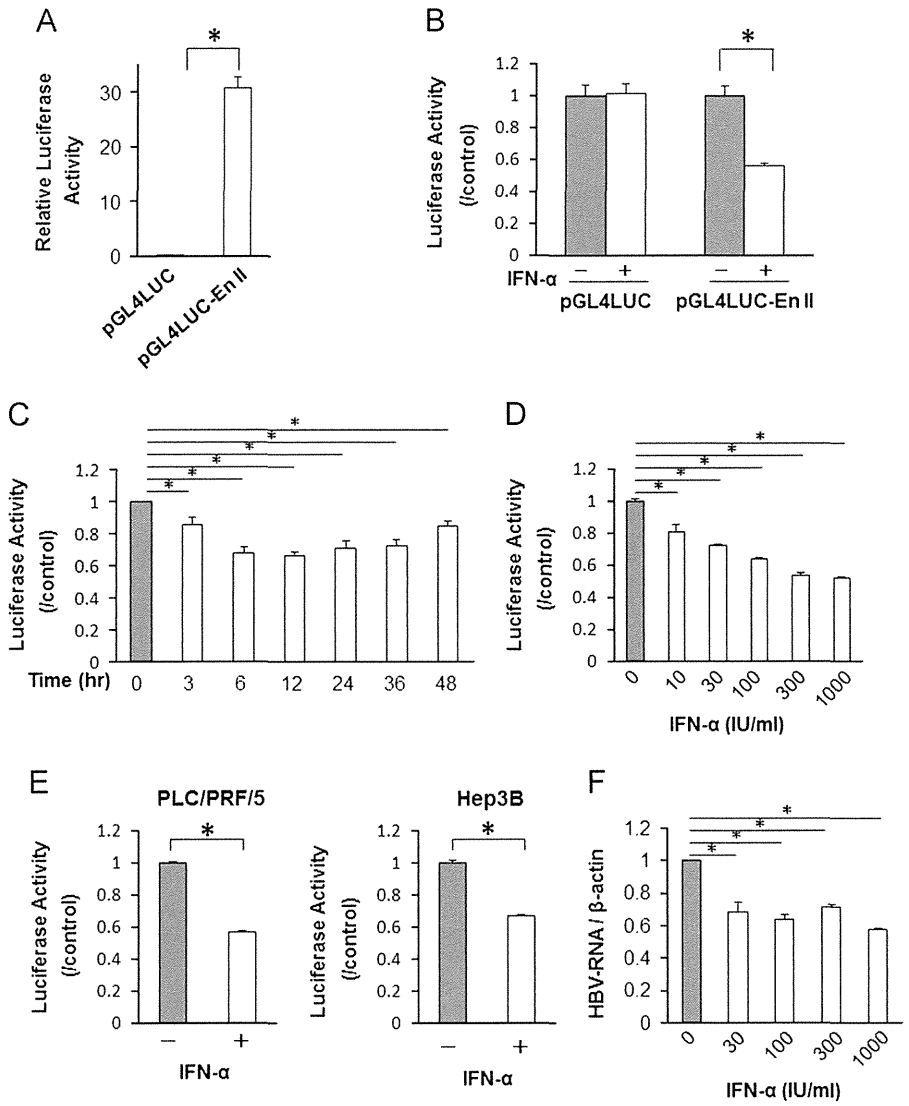


**Fig. 2.** Suppression of HBV En II transcriptional activity and reduction of HBV-RNA by IFN-α. A, B. Huh-7 cells were transfected with pGL4LUC or pGL4LUC-En II or incubated with or without IFN-α (100 IU/ml). After 24 h, the activity of firefly luciferase was evaluated. C. Huh-7 cells were transfected with pGL4LUC-En II, and incubated with IFN-α (100 IU/ml). Luciferase activities were evaluated at the indicated times. D. Huh-7 cells were transfected with various concentrations (0–1000 IU/ml) of IFN-α for 12 h and luciferase activities were evaluated. E. PLC/PRF/5 cells (left panel) and Hep3B (right panel) cells were transfected with pGL4LUC-En II, and incubated with or without IFN-α (300 IU/ml). Luciferase activities were evaluated. F. Huh-7 cells were transfected with pHBV1.5, and treated with IFN-α at various concentrations (0–1000 IU/ml). At 72 h after IFN-α treatment, cells were harvested, and the abundances of HBV-RNA were evaluated by quantitative RT-PCR. The HBV-RNA level of the IFN-α treated cells was normalized with that of non-treated cells. $^*p < 0.05$. "/control" on the vertical axis means the ratio of luciferase activity of IFN-α treated cells normalized with that of non-treated cells.

not. Since staurosporine is a PKC inhibitor showing broad-spectrum activity (Marte et al., 1994), we also examined other inhibitors specific for PKC isoforms. Previous reports demonstrated that IFN-α activated PKC-α/β and PKC-δ (Pfeffer et al., 1990; Uddin et al., 2002). Indeed, activation of PKC-α/β and PKC-δ by IFN-α was confirmed by immunoblot analysis (Fig. 5B). Thus, we examined the PKC inhibitors rottlerin and Gö6976 (Gschwendt et al., 1994; Martiny-Baron et al., 1993). All PKC inhibitors blocked the suppression of En II activity by IFN-α (Fig. 5C). These results suggest that several isoforms of PKC are involved in the IFN-α-mediated suppression of En II activity. We also examined STAT1 activation and ISGs induction by IFN-α in cells pre-treated with these PKC inhibitors using immunoblot analysis (Fig. 5D). Expression levels of phospho-STAT1 and Mx differed among these PKC inhibitors. Staurosporine and Gö6976 slightly diminished the activation of STAT1, but rottlerin did not. This result suggests that PKC isoforms might not strongly regulate activation of STAT1. Rottlerin, a specific inhibitor for PKC-δ, inhibited the induction of Mx, which agreed with previous findings (Kaur et al., 2005). Staurosporine and Gö6976 did not suppress Mx expression. Taken together, all these PKC inhibitors blocked the suppression of En II activity by IFN-α regardless of the expression levels of phospho-STAT1 and Mx. These results suggest that STAT1 activation and ISG induction may be dispensable for the IFN-α-mediated suppression of En II activity. Next, we examined the effect of phorbol 12-myristate 13-acetate (PMA), a PKC activator (Castagna et al., 1982; Griner and Kazanietz, 2007). PMA suppressed En II activity (Fig. 5E), and PMA stimulation did not result in STAT1 phosphorylation and Mx induction (Fig. 5F), suggesting that suppression of En II by PMA is independent of STAT1 activation and ISG induction. On the basis of these findings, we conclude that IFN-α suppresses En II activity via the PKC pathway, which may not involve STAT1 activation and ISG induction.

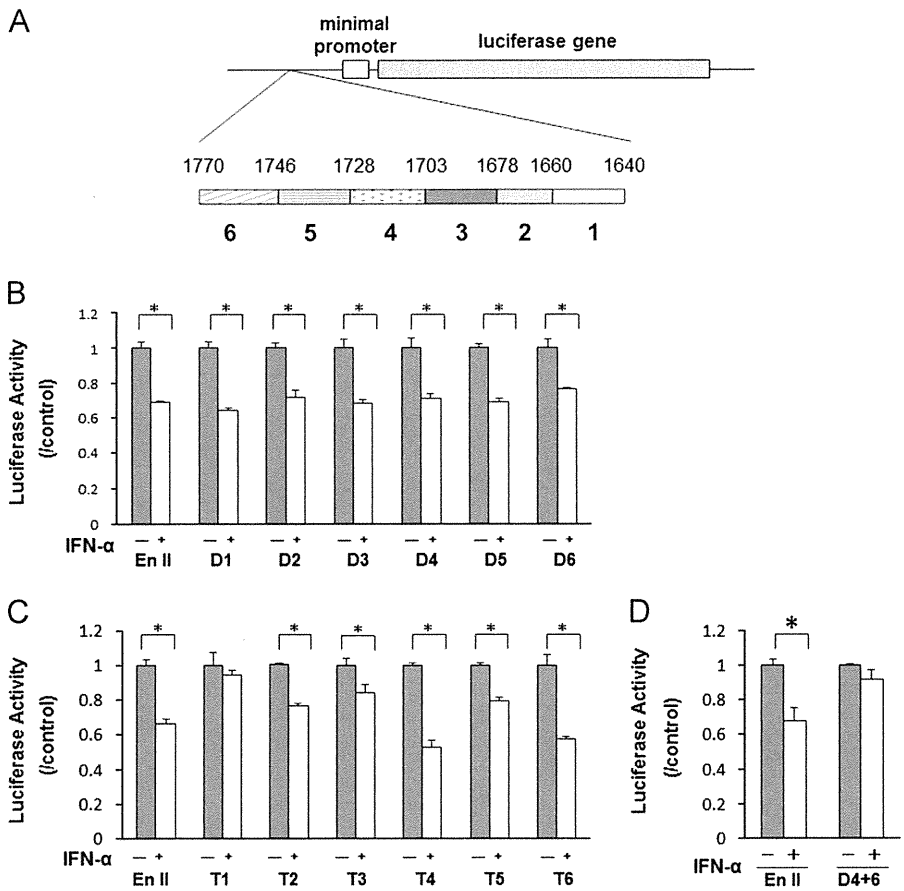


**Fig. 3.** Deletion/mutational analysis to identify the responsive sequence for the suppressive effect of IFN-α on En II. A. Scheme of pGL4LUC-En II and six segments defined within the En II sequence. The En II sequence was integrated just upstream of the minimal promoter of pGL4LUC. B. Huh-7 cells were transfected with the reporter vectors with deletion of each segment (pGL4LUC-En II-D1~6), incubated with 300 IU/ml IFN-α for 12 h, and luciferase activities were evaluated. C. Plasmids containing four iterations of each segment within En II sequence in tandem (pGL4LUC-En II-T1~6) were generated and luciferase activities were evaluated similarly. D. Plasmid with deletion of both nt 1703–1727 and nt 1746–1770 (pGL4LUC-En II-D4+6) was constructed and luciferase activities were evaluated similarly. *$p < 0.05$. "/control" on the vertical axis means the ratio of luciferase activity of IFN-α treated cells normalized with that of non-treated cells.

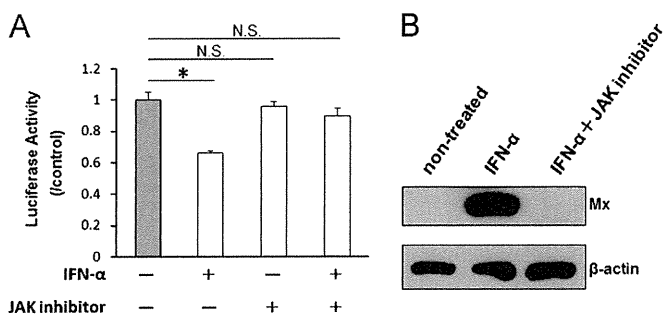


**Fig. 4.** Involvement of JAK activation in the IFN-α-induced suppression of En II activity. A. Huh-7 cells were transfected with pGL4LUC-En II and treated with JAK inhibitor (1 μM) for 1 h. The cells were then incubated with IFN-α(150 IU/ml) for 12 h, followed by luciferase assay. B. Huh-7 cells were pre-treated with JAK inhibitor for 1 h, and then incubated with IFN-α(150 IU/ml) for 12 h, followed by immunoblot analyses to detect Mx protein. *$p < 0.05$. "/control" on the vertical axis means the ratio of luciferase activity of IFN-α treated cells normalized by that of non-treated cells.

**Table 1**
A comment of the inhibitors and its target kinase.

| | |
|---|---|
| PD98059 | MEK inhibitor |
| SB203580 | P38MAPK inhibitor |
| LY294002 | PI3K inhibitor |
| Akt-1-1/2 | Akt inhibitor |
| SP600125 | JNK inhibitor |
| Staurosporine | PKC inhibitor with broad spectrum |
| Rottlerin | Inhibitor specific for PKC-δ |
| Gö6976 | Inhibitor specific for $Ca^{2+}$-dependent PKC isoforms |

*Knockdown of a single transcription factor does not influence IFN-α-induced suppression of En II activity*

We anticipated that IFN-α suppressed En II activity by functional down-regulation of some transcription factor(s) phosphorylated in a PKC-dependent manner. Among transcription factors which bind the En II region, previous reports showed that Specificity Protein 1 (Sp1) (Mahoney et al., 1992; Pal et al., 1998; Rafty and Khachigian, 2001), Retinoid X Receptor α (RXRA) (Delmotte et al., 1999) and C/EBP (Mahoney et al., 1992) were inactivated by PKC. Thus, we examined the En II response to IFN-α after knockdown of these transcription factors. C/EBP, RXR and Sp1 expression was efficiently reduced by siRNA (Fig. 6A). We observed no significant change in the suppression of En II activity compared with control siRNA (Fig. 6B). This result suggests that several transcription factors (including unknown proteins) might be involved in the IFN-α-mediated suppression of En II activity.

## Discussion

In the present study, we demonstrated that IFN-α suppressed HBV En II activity. The inhibition by IFN-α of En II activity could be blocked by pre-treatment with PKC inhibitors, and this

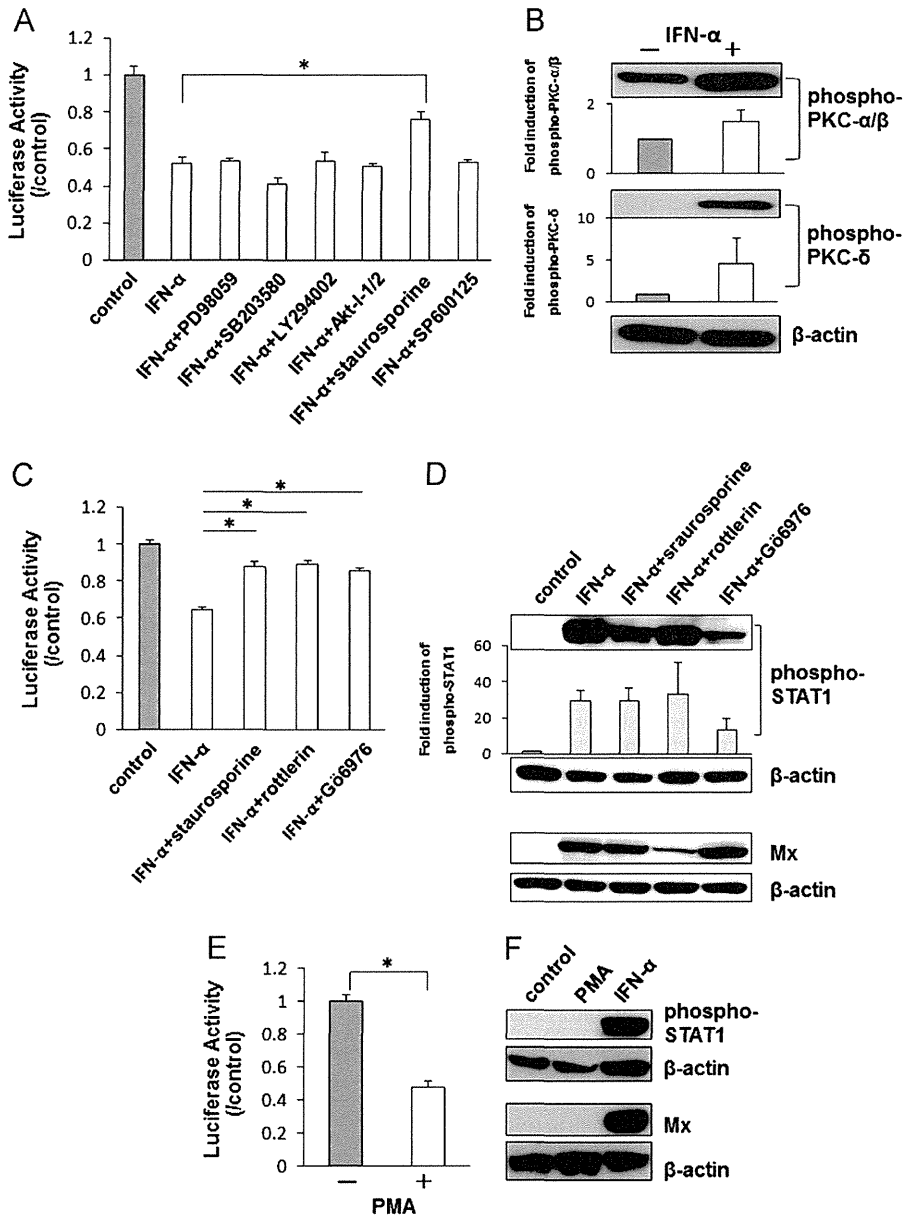


**Fig. 5.** PKC-dependent suppression of En II activity by IFN-α. A and C. Huh-7 cells were transfected with pGL4LUC-En II, treated separately with each kinase inhibitor for 1 h. The cells were then treated with IFN-α(1000 IU/ml) for 12 h, and luciferase activities were evaluated. B. Huh-7 cells were treated with IFN-α(1000 IU/m) for 12 h. Immunoblot analyses were performed to detect phosphorylated PKC-α/β and phosphorylated PKC-δ. Quantitative analysis of the expression level of phopho- PKC-α/β and -δ was performed by using ImageJ. Each level was normalized with that of IFN-α-non-treated cells. D. Huh-7 cells were harvested at 30 min to detect phosphorylated STAT1 and at 12 h to detect the expression of Mx after administration of IFN-α (1000 IU/m), and immunoblot analyses were performed. Quantitative analysis of the expression level of phopho-STAT1 was performed by using ImageJ. Each level was normalized with that of IFN-α-non-treated cells. E. Huh-7 cells were transfected with pGL4LUC-En II, treated with PMA (100 nM) for 12 h, and luciferase activities were evaluated. F. Huh-7 cells were treated with PMA (100 nM) or IFN-α(1000 IU/m). The cells were harvested at 30 min to detect phosphorylated STAT1 and at 12 h to detect the expression of Mx, and immunoblot analyses were performed. *$p < 0.05$. "/control" on the vertical axis means the ratio of luciferase activity of IFN-α/ PMA treated cells normalized with that of non-treated cells.

blocking effect may not involve STAT1 activation and ISG induction. The latter, ISG induction via the JAK-STAT pathway, has been considered to be the main mechanism suppressing viral replication. Our findings suggest a pathway for IFN-α repression of HBV transcription other than ISG induction.

PKCs are involved in a wide variety of cell functions and signal transduction pathways regulating cell migration and polarity, proliferation, differentiation and cell death (Nishizuka, 1988). In the PKC family, there are at least ten isoforms which can be divided into three sub-groups based on their structural characteristics and cofactor requirements. These include the classical PKC (cPKC: α, βI, βII, and γ), the novel PKC (nPKC: δ, ε, η and θ), and the atypical PKC (aPKC: ζ and ι/λ) (Azzi et al., 1992; Breitkreutz et al., 2007; Kikkawa et al., 1989). IFN-α can activate multiple PKC isoforms: not only PKC-δ, but also PKC-α/β (Pfeffer et al., 1990), PKC-ε (Pfeffer et al., 1991), and PKC-θ (Srivastava et al., 2004). Despite the variety of PKC isoforms, most phosphorylate similar sequences (Breitkreutz et al., 2007). Both the PKC-α/β inhibitor (Gö6976) and PKC-δ inhibitor (rottlerin) blocked the inhibitory effect of IFN-α on En II activity. Thus, it was speculated that each PKC isoform might be similarly involved in suppressing of En II activity.

Other studies have examined the role of the PKC pathway in HBV replication. Kang et al. (2008) reported that PKC-mediated phosphorylation increased capsid assembly and stability (von Hahn et al., 2011), and von Hahn et al. (2011) reported that the

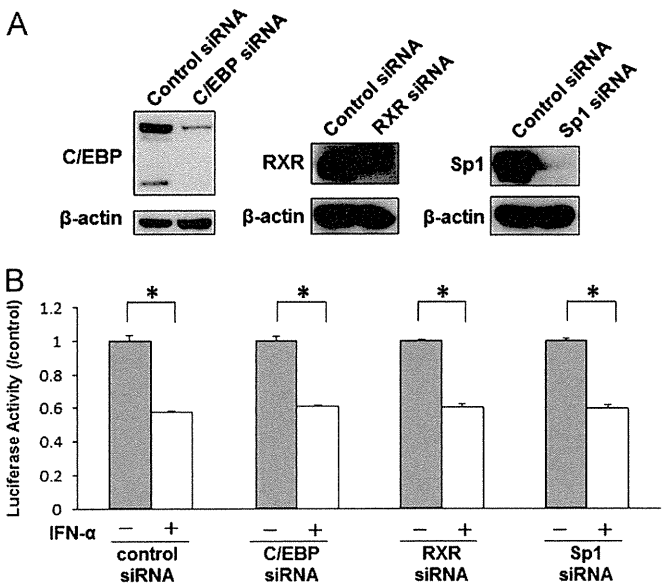


**Fig. 6.** IFN-α-mediated suppression on En II activity with knockdown of C/EBP, RXR and Sp1. A. Huh-7 cells were transfected with 10 nM siRNA (negative control or specific for C/EBP, RXR and Sp1). Immunoblot analyses for expressions of C/EBP, RXR, Sp1 and β-actin were performed at 48 h post siRNA transfection. B. Huh-7 cells were transfected with 10 nM siRNA (negative control or specific for C/EBP, RXR and Sp1). On the next day, si-RNA treated cells were transfected again with pGL4LUC-En II. On the following day, these transfected cells were incubated with IFN-α(1000 IU/ml) for 12 h, and luciferase activities were evaluated. "/control" on the vertical axis means the ratio of luciferase activity of IFN-α treated cells normalized with that of non-treated cells.

pan-PKC inhibitor sotrastaurin did not affect HBV replication. While the role of PKC in the HBV life cycle is still controversial, our findings suggest that PKC isoforms activated by IFN-α play inhibitory roles in HBV transcription by down-regulation of En II activity. As von Hahn et al. reported, sotrastaurin alone did not affect HBV replication. But, based on our present data about another pan-PKC inhibitor, staurosporine, we speculate that sotrastaurin may also block the inhibitory effect of IFN-α on En II activity.

We showed that knockdown of a single transcription factor did not influence the IFN-α-mediated suppression of En II activity, suggesting that several transcription factors might be involved in this suppression. We also showed that both segment 4 (nt 1703–1727) and segment 6 (nt 1746–1770) within the En II region are required for the IFN-α-induced suppression of En II activity. Although these two regions seem to be more important than the others, all the deleted version of reporter constructs showed almost completely similar suppression activities (Fig. 3B). We speculate that there may be some transcription factors which affect both the segment 4 and 6. Even if one of these regions is deleted, some factors may affect the other region, and result in the suppression of En II activity. Further study will be needed to clarify the mechanism.

Indeed, there are no identified transcription factors which could bind both segment 4 and 6. Only two transcription factors (HNF1 and 3) were reported to bind segment 4 (Johnson et al., 1995; Wang et al., 1998), and there have been no reports indicating that IFN-α or PKC inactivates HNF1 or 3. We also examined the expression levels of HNF1 and 3 of the IFN-α treated and the non-treated cells by RT-PCR. There was no significant difference in the expression of these transcription factors between the IFN-α treated and the non-treated cells (Nawa et al., unpublished data). Thus, we speculate that HNF1 or 3 might not be involved in the IFN-α mediated suppression of En II activity. There may be unknown transcription factors in the PKC pathway.

Previous reports showed that IFN-α suppressed En I activity (Nakao et al., 1999; Tur-Kaspa et al., 1990). Nakao et al. (1999) indicated that this occurred due to the binding of ISGF3 to an ISRE-like motif within the En I region. However, Rang et al. (2001) demonstrated that IFN-α reduced HBV-RNA levels derived from both HBV genome wild type and mutated ISRE-like motifs. This result contradicted the Nakao's result that the activity of the En I mutated ISRE-like motif was not suppressed by IFN-α. Schulte-Frohlinde et al. (2002) reported that IFN-α suppressed HBV core promoter regulated transcriptional activity, even when the ISRE-like motif of En I was deleted. The results of Rang et al. and Schulte-Frohlinde et al. suggest that IFN-α might suppress the activity of regions other than En I. In the present study, we demonstrated that IFN-α suppressed En II activity via the PKC pathway. En II might be one of the candidate regions down-regulated by IFN-α within the HBV genome.

Since En II activates viral transcription only in hepatocytes, it is responsible for the hepatocyte-specific gene expression of HBV. There had been no study on the effect of IFN-α on En II activity. Our study clarified that the PKC pathway is involved in the IFN-α-mediated suppression of En II activity, but may not involve ISG induction. Our result should aid in establishing better treatment with IFN-α against HBV infection. As we could not determine the molecule which inhibits En II activity by IFN-α, further study is needed to clarify this molecule and to control hepatitis B by IFN-α treatment.

## Materials and methods

### *Plasmids*

The HBV sequence used in this study was of the *adw2* subtype (GenBank accession no. X02763). Numbering of the HBV sequence started at the unique *Eco*RI site. The En II region in this study was defined as nt 1640–1771 of HBV sequence (Fig. 1) (Ishida et al., 2000). To construct pGL4LUC-En II, a plasmid containing the HBV En II region, the DNA fragment was amplified with PCR and inserted between *Hind* III and *Nhe* I site of pGL4 Luciferase Reporter Vector (pGL4LUC) (Promega, Madison, WI). The PCR primers were as follows: 5′-CCAAGCTTCTGCCCAAGGTC-3′ and 5′-CCCGCTAGCAAAGACCTTTAACCTAATCTCCTCC-3′. The constructs of the En II sequence with various deletions were generated by modifying pGL4LUC-En II using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The constructs containing four tandem repeats of short fragment in En II sequence were generated by inserting duplexes of synthesized oligonucleotides into the multicloning site of pGL4LUC. All of the En II sequences were inserted in the antisense orientation to evaluate their enhancer activity.

Plasmid pHBV1.5 containing a 1.5-fold-overlength genome of HBV-DNA (GenBank accession no. AF305422) has been described previously (Bruss and Ganem, 1991).

### *Cell lines and reagents*

The human hepatocellular carcinoma cell lines Huh-7, PLC/PRF/5, and Hep3B were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, St. Louis, MO) in a humidified incubator at 5% $CO_2$ and 37 °C. Human natural IFN-α was kindly provided by Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan).

The inhibitors/activators and the final concentrations used were: JAK inhibitor I (1 μM), PD98059 (10 μM), SB203580 (10 μM), LY294002 (10 μM), Akt-I-1/2 (5 μM), staurosporine (10 or 20 nM), rottlerin (5 μM), Gö6976 (1 μM), SP600125 (10 μM)

(Calbiochem, San Diego, CA), phorbol 12-myristate 13-acetate (PMA) (100 nM) (Sigma-Aldrich, St. Louis, MO).

*Plasmid transfection and luciferase assay*

Huh-7 cells were co-transfected with the firefly luciferase plasmid and pGL4-RL-tk, an expression vector of renilla luciferase, which was used as an internal control, using FuGENE HD reagent (Roche Applied Science, Indianapolis, IN) according to the manufacturer's protocol. Activities of firefly luciferase and renilla luciferase were measured using the Dual-Glo Luciferase Assay System (Promega, Madison, WI), and then relative luciferase activity was calculated by normalizing firefly luciferase activity to renilla luciferase activity.

*RNA extraction*

Total RNA was isolated from cells using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. The isolated RNA was treated with DNase I (Promega, Madison, WI) to avoid contamination with transfected plasmid, and then purified with a mixture of phenol, chloroform, and isoamylalcohol (pH 7.9), followed by ethanol precipitation.

*Western blot analysis*

Cultured cells were lysed with a lysis buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and protein inhibitor cocktail (Nacalai Tesque), in PBS, pH 7.4). Equal amounts of protein were electrophoretically separated by polyacrylamide gel and transferred onto PVDF membrane. For immunodetection, the following antibodies were used: anti-STAT1 antibody, anti-phospho-STAT1 antibody, anti-phospho-PKC-α/β II (Thr 638/641) antibody, anti-phospho-PKC-δ (Thr 505) antibody, anti-C/EBP antibody, anti-RXR antibody, anti-Sp1 antibody, anti-β-actin antibody from Cell Signaling Technology (Beverly, MA), and anti-Mx antibody from Abcam (Cambridge, UK). The signals of phosphorylated proteins such as phospho-PKC-α/β, -δ and phospho-STAT1 were analyzed quantitatively using image analyzing software (ImageJ; version 1.45).

*Small RNA interference*

Stealth Select RNAi specific for STAT1 (HSS 10273) was purchased from Invitrogen (Carlsbad, CA). Silencer Select siRNA specific for C/EBP (ID: S2890), RXR (ID: S12386) and Sp1 (ID: S13319) were purchased from Ambion (Austin, TX). Stealth RNAi Negative Control Low GC Duplex (Invitrogen, Carlsbad, CA) was used as a control for the off-target effect following Stealth Select RNAi delivery. The transfections were carried out using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) according to the reverse transfection protocol.

*Real-time reverse-transcription PCR*

For cDNA synthesis, 1 μg of total RNA was reverse-transcribed using High Capacity RNA-to-DNA Master Mix (Applied Biosystems, Foster City, CA). cDNA, equivalent to 20 ng RNA, was used as a template for real-time reverse-transcription PCR (RT-PCR) using Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). mRNA expressions of C/EBP, FTF, HNF1, HNF3, and HNF4 were measured using TaqMan Gene Expression Assays and were corrected with the quantified expressions level of β-actin mRNA. Assay IDs for the genes were as follows: C/EBP (Hs00269972_s1), FTF (Hs00187067_m1), HNF1 (Hs00167041_m1), HNF3 (Hs00232754_m1), and HNF4 (Hs01023298_m1).

For the detection of pgRNA and pre-C mRNA, the primers and the probes were designed as follows according to a previous study (Laras et al., 2002): the sense primer was 5′-TCTTGTACATGTCCCACTGTTCAA-3′ (nt 1843–1866); the anti-sense primer was 5′-AATGCCATGCCCCAAAGC-3′ (nt 1890–1909); the probe was 5′-FAM-CTCCAAGCTGTGCCTT -3′ (nt 1869–1884). Since they were within precore/core coding sequence, only the total abundance of pgRNA and pre-C RNA could be detected.

*Statistical analysis*

Data were presented as mean ± SD. Differences between two groups were determined using Student's t-test for unpaired observations. $p < 0.05$ was considered statistically significant.

## Disclosures

All authors have nothing to disclose.

## References


Antonucci, T.K., Rutter, W.J., 1989. Hepatitis B virus (HBV) promoters are regulated by the HBV enhancer in a tissue-specific manner. J. Virol. 63, 579–583.

Azzi, A., Boscoboinik, D., Hensey, C., 1992. The protein kinase C family. Eur. J. Biochem. 208, 547–557.

Beck, J., Nassal, M., 2007. Hepatitis B virus replication. World J. Gastroenterol. 13, 48–64.

Breitkreutz, D., Braiman-Wiksman, L., Daum, N., Denning, M.F., Tennenbaum, T., 2007. Protein kinase C family: on the crossroads of cell signaling in skin and tumor epithelium. J. Cancer Res. Clin. Oncol. 133, 793–808.

Bruss, V., Ganem, D., 1991. The role of envelope proteins in hepatitis B virus assembly. Proc. Nat. Acad. Sci. U.S.A. 88, 1059–1063.

Caraglia, M., Abbruzzese, A., Leardi, A., Pepe, S., Budillon, A., Baldassare, G., Selleri, C., Lorenzo, S.D., Fabbrocini, A., Giuberti, G., Vitale, G., Lupoli, G., Bianco, A.R., Tagliaferri, P., 1999. Interferon-alpha induces apoptosis in human KB cells through a stress-dependent mitogen activated protein kinase pathway that is antagonized by epidermal growth factor. Cell Death Differ. 6, 773–780.

Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U., Nishizuka, Y., 1982. Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. J. Biol. Chem. 257, 7847–7851.

Darnell, J.E., Kerr, I.M., Stark, G.R., 1994. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science 264, 1415–1421.

David, M., Petricoin, E., Benjamin, C., Pine, R., Weber, M.J., Larner, A.C., 1995. Requirement for MAP kinase (ERK2) activity in interferon alpha- and interferon beta-stimulated gene expression through STAT proteins. Science 269, 1721–1723.

Delmotte, M.H., Tahayato, A., Formstecher, P., Lefebvre, P., 1999. Serine 157, a retinoic acid receptor alpha residue phosphorylated by protein kinase C in vitro, is involved in RXR.RARalpha heterodimerization and transcriptional activity. J. Biol. Chem. 274, 38225–38231.

Der, S.D., Zhou, A., Williams, B.R., Silverman, R.H., 1998. Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays. Proc. Nat. Acad. Sci. U.S.A. 95, 15623–15628.

Goh, K.C., Haque, S.J., Williams, B.R., 1999. p38 MAP kinase is required for STAT1 serine phosphorylation and transcriptional activation induced by interferons. EMBO J. 18, 5601–5608.

Griner, E.M., Kazanietz, M.G., 2007. Protein kinase C and other diacylglycerol effectors in cancer. Nat. Rev. Cancer 7, 281–294.

Gschwendt, M., Müller, H.J., Kielbassa, K., Zang, R., Kittstein, W., Rincke, G., Marks, F., 1994. Rottlerin, a novel protein kinase inhibitor. Biochem. Biophys. Res. Commun. 199, 93–98.

Guo, W., Chen, M., Yen, T.S., Ou, J.H., 1993. Hepatocyte-specific expression of the hepatitis B virus core promoter depends on both positive and negative regulation. Mol. Cell. Biol. 13, 443–448.

Ishida, H., Ueda, K., Ohkawa, K., Kanazawa, Y., Hosui, A., Nakanishi, F., Mita, E., Kasahara, A., Sasaki, Y., Hori, M., Hayashi, N., 2000. Identification of multiple transcription factors, HLF, FTF, and E4BP4, controlling hepatitis B virus enhancer II. J. Virol. 74, 1241–1251.

Johnson, J.L., Raney, A.K., McLachlan, A., 1995. Characterization of a functional hepatocyte nuclear factor 3 binding site in the hepatitis B virus nucleocapsid promoter. Virology 208, 147–158.

Jonas, M.M., Block, J.M., Haber, B.A., Karpen, S.J., London, W.T., Murray, K.F., Narkewicz, M.R., Rosenthal, P., Schwarz, K.B., McMahon, B.J., Foundation, H.B., 2010. Treatment of children with chronic hepatitis B virus infection in the United States: patient selection and therapeutic options. Hepatology 52, 2192–2205.

Kang, H., Yu, J., Jung, G., 2008. Phosphorylation of hepatitis B virus core C-terminally truncated protein (Cp149) by PKC increases capsid assembly and stability. Biochem. J. 416, 47–54.
Kaur, S., Parmar, S., Smith, J., Katsoulidis, E., Li, Y., Sassano, A., Majchrzak, B., Uddin, S., Tallman, M.S., Fish, E.N., Platanias, L.C., 2005. Role of protein kinase C-delta (PKC-delta) in the generation of the effects of IFN-alpha in chronic myelogenous leukemia cells. Exp. Hematol. 33, 550–557.
Kikkawa, U., Kishimoto, A., Nishizuka, Y., 1989. The protein kinase C family: heterogeneity and its implications. Annu. Rev. Biochem. 58, 31–44.
Laras, A., Koskinas, J., Hadziyannis, S.J., 2002. In vivo suppression of precore mRNA synthesis is associated with mutations in the hepatitis B virus core promoter. Virology 295, 86–96.
Li, M., Xie, Y., Wu, X., Kong, Y., Wang, Y., 1995. HNF3 binds and activates the second enhancer, ENII, of hepatitis B virus. Virology 214, 371–378.
Li, M., Xie, Y.H., Kong, Y.Y., Wu, X., Zhu, L., Wang, Y., 1998. Cloning and characterization of a novel human hepatocyte transcription factor, hB1F, which binds and activates enhancer II of hepatitis B virus. J. Biol. Chem. 273, 29022–29031.
Liaw, Y.F., 2009. HBeAg seroconversion as an important end point in the treatment of chronic hepatitis B. Hepatol. Int.
Lok, A.S., McMahon, B.J., 2009. Chronic hepatitis B: update 2009. Hepatology 50, 661–662.
López-Cabrera, M., Letovsky, J., Hu, K.Q., Siddiqui, A., 1990. Multiple liver-specific factors bind to the hepatitis B virus core/pregenomic promoter: trans-activation and repression by CCAAT/enhancer binding protein. Proc. Nat. Acad. Sci. U.S.A. 87, 5069–5073.
López-Cabrera, M., Letovsky, J., Hu, K.Q., Siddiqui, A., 1991. Transcriptional factor C/EBP binds to and transactivates the enhancer element II of the hepatitis B virus. Virology 183, 825–829.
Mahoney, C.W., Shuman, J., McKnight, S.L., Chen, H.C., Huang, K.P., 1992. Phosphorylation of CCAAT-enhancer binding protein by protein kinase C attenuates site-selective DNA binding. J. Biol. Chem. 267, 19396–19403.
Marte, B.M., Meyer, T., Stabel, S., Standke, G.J., Jaken, S., Fabbro, D., Hynes, N.E., 1994. Protein kinase C and mammary cell differentiation: involvement of protein kinase C alpha in the induction of beta-casein expression. Cell Growth Differ. 5, 239–247.
Martiny-Baron, G., Kazanietz, M.G., Mischak, H., Blumberg, P.M., Kochs, G., Hug, H., Marmé, D., Schächtele, C., 1993. Selective inhibition of protein kinase C isozymes by the indolocarbazole Gö 6976. J. Biol. Chem. 268, 9194–9197.
Moolla, N., Kew, M., Arbuthnot, P., 2002. Regulatory elements of hepatitis B virus transcription. J. Viral. Hepat. 9, 323–331.
Nakao, K., Nakata, K., Yamashita, M., Tamada, Y., Hamasaki, K., Ishikawa, H., Kato, Y., Eguchi, K., Ishii, N., 1999. p48 (ISGF-3gamma) is involved in interferon-alpha-induced suppression of hepatitis B virus enhancer-1 activity. J. Biol. Chem. 274, 28075–28078.
Nishizuka, Y., 1988. The molecular heterogeneity of protein kinase C and its implications for cellular regulation. Nature 334, 661–665.
Pal, S., Claffey, K.P., Cohen, H.T., Mukhopadhyay, D., 1998. Activation of Sp1-mediated vascular permeability factor/vascular endothelial growth factor transcription requires specific interaction with protein kinase C zeta. J. Biol. Chem. 273, 26277–26280.
Pfeffer, L.M., Eisenkraft, B.L., Reich, N.C., Improta, T., Baxter, G., Daniel-Issakani, S., Strulovici, B., 1991. Transmembrane signaling by interferon alpha involves diacylglycerol production and activation of the epsilon isoform of protein kinase C in Daudi cells. Proc. Nat. Acad. Sci. U.S.A. 88, 7988–7992.
Pfeffer, L.M., Strulovici, B., Saltiel, A.R., 1990. Interferon-alpha selectively activates the beta isoform of protein kinase C through phosphatidylcholine hydrolysis. Proc. Nat. Acad. Sci. U.S.A. 87, 6537–6541.
Rafty, L.A., Khachigian, L.M., 2001. Sp1 phosphorylation regulates inducible expression of platelet-derived growth factor B-chain gene via atypical protein kinase C-zeta. Nucleic Acids Res. 29, 1027–1033.
Raney, A.K., Johnson, J.L., Palmer, C.N., McLachlan, A., 1997. Members of the nuclear receptor superfamily regulate transcription from the hepatitis B virus nucleocapsid promoter. J. Virol. 71, 1058–1071.
Rang, A., Heise, T., Will, H., 2001. Lack of a role of the interferon-stimulated response element-like region in interferon alpha -induced suppression of Hepatitis B virus in vitro. J. Biol. Chem. 276, 3531–3535.
Romero, R., Lavine, J.E., 1996. Cytokine inhibition of the hepatitis B virus core promoter. Hepatology 23, 17–23.
Schulte-Frohlinde, E., Seidler, B., Burkard, I., Freilinger, T., Lersch, C., Erfle, V., Foster, G.R., Classen, M., 2002. Different activities of type I interferons on hepatitis B virus core promoter regulated transcription. Cytokine 17, 214–220.
Srivastava, K.K., Batra, S., Sassano, A., Li, Y., Majchrzak, B., Kiyokawa, H., Altman, A., Fish, E.N., Platanias, L.C., 2004. Engagement of protein kinase C-theta in interferon signaling in T-cells. J. Biol. Chem. 279, 29911–29920.
Su, H., Yee, J.K., 1992. Regulation of hepatitis B virus gene expression by its two enhancers. Proc. Nat. Acad. Sci. U.S.A. 89, 2708–2712.
Sureau, C., Romet-Lemonne, J.L., Mullins, J.I., Essex, M., 1986. Production of hepatitis B virus by a differentiated human hepatoma cell line after transfection with cloned circular HBV DNA. Cell 47, 37–47.
Tur-Kaspa, R., Teicher, L., Laub, O., Itin, A., Dagan, D., Bloom, B.R., Shafritz, D.A., 1990. Alpha interferon suppresses hepatitis B virus enhancer activity and reduces viral gene transcription. J. Virol. 64, 1821–1824.
Uddin, S., Sassano, A., Deb, D.K., Verma, A., Majchrzak, B., Rahman, A., Malik, A.B., Fish, E.N., Platanias, L.C., 2002. Protein kinase C-delta (PKC-delta ) is activated by type I interferons and mediates phosphorylation of Stat1 on serine 727. J. Biol. Chem. 277, 14408–14416.
Uddin, S., Yenush, L., Sun, X.J., Sweet, M.E., White, M.F., Platanias, L.C., 1995. Interferon-alpha engages the insulin receptor substrate-1 to associate with the phosphatidylinositol 3′-kinase. J. Biol. Chem. 270, 15938–15941.
Vannice, J.L., Levinson, A.D., 1988. Properties of the human hepatitis B virus enhancer: position effects and cell-type nonspecificity. J. Virol. 62, 1305–1313.
von Hahn, T., Schulze, A., Chicano Wust, I., Heidrich, B., Becker, T., Steinmann, E., Helfritz, F.A., Rohrmann, K., Urban, S., Manns, M.P., Pietschmann, T., Ciesek, S., 2011. The novel immunosuppressive protein kinase C inhibitor sotrastaurin has no pro-viral effects on the replication cycle of hepatitis B or C virus. PLoS One 6, e24142.
Wang, W.X., Li, M., Wu, X., Wang, Y., Li, Z.P., 1998. HNF1 is critical for the liver-specific function of HBV enhancer II. Res. Virol. 149, 99–108.
Wang, Y., Chen, P., Wu, X., Sun, A.L., Wang, H., Zhu, Y.A., Li, Z.P., 1990. A new enhancer element, ENII, identified in the X gene of hepatitis B virus. J. Virol. 64, 3977–3981.
Yee, J.K., 1989. A liver-specific enhancer in the core promoter region of human hepatitis B virus. Science 246, 658–661.
Yuh, C.H., Ting, L.P., 1990. The genome of hepatitis B virus contains a second enhancer: cooperation of two elements within this enhancer is required for its function. J. Virol. 64, 4281–4287.
Yuh, C.H., Ting, L.P., 1991. C/EBP-like proteins binding to the functional box-alpha and box-beta of the second enhancer of hepatitis B virus. Mol. Cell. Biol. 11, 5044–5052.

Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

# Dual oral therapy with daclatasvir and asunaprevir for patients with HCV genotype 1b infection and limited treatment options

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See Editorial, pages 643–645

**Background & Aims**: Improved therapeutic options for chronic hepatitis C virus (HCV) infection are needed for patients who are poor candidates for treatment with current regimens due to anticipated intolerability or low likelihood of response.

**Methods**: In this open-label, phase 2a study of Japanese patients with chronic HCV genotype 1b infection, 21 null responders (<2 $\log_{10}$ HCV RNA reduction after 12 weeks of peginterferon/ribavirin) and 22 patients intolerant to or medically ineligible for peginterferon/ribavirin therapy received dual oral treatment for 24 weeks with the NS5A replication complex inhibitor daclatasvir (DCV) and the NS3 protease inhibitor asunaprevir (ASV). The primary efficacy end point was sustained virologic response at 12 weeks post-treatment ($SVR_{12}$).

**Results**: Thirty-six of 43 enrolled patients completed 24 weeks of therapy. Serum HCV RNA levels declined rapidly, becoming undetectable in all patients on therapy by week 8. Overall, 76.7% of patients achieved $SVR_{12}$ and $SVR_{24}$, including 90.5% of null responders and 63.6% of ineligible/intolerant patients. There were no virologic failures among null responders. Three ineligible/intolerant patients experienced viral breakthrough and four relapsed post-treatment. Diarrhea, nasopharyngitis, headache, and ALT/AST increases, generally mild, were the most common adverse events; three discontinuations before week 24 were due to adverse events that included hyperbilirubinemia and transaminase elevations (two patients).

**Conclusions**: Dual therapy with daclatasvir and asunaprevir, without peginterferon/ribavirin, was well tolerated and achieved high SVR rates in two groups of difficult-to-treat patients with hepatitis C virus genotype 1b infection.



Keywords: Daclatasvir; Asunaprevir; Hepatitis C; Antiviral.

*Received 15 May 2012; received in revised form 5 September 2012; accepted 30 September 2012; available online 23 November 2012*

☆DOI of original article: http://dx.doi.org/10.1016/j.jhep.2013.01.007.

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*Abbreviations:* HCV, hepatitis C virus; DAA, direct-acting antiviral; PegIFN-α/RBV, peginterferon alfa and ribavirin; SVR, sustained virologic response; HIV, human immunodeficiency virus; NS5A, non-structural protein 5A; NS3, non-structural protein 3; ALT, alanine aminotransferase; ULN, upper limit of the normal reference range; INR, international normalized ratio; CYP3A4, cytochrome P450 3A4.

## Introduction

Therapies for chronic hepatitis C virus (HCV) infection have improved markedly over the past decade. The recent approval of the first direct-acting antivirals (DAAs) was an important milestone in the evolution of HCV therapy, establishing that DAAs can enhance regimen efficacy and provide durable viral clearance. These new agents in combination with peginterferon and ribavirin (PegIFN-α/RBV) achieve overall sustained virologic response (SVR) rates of approximately 70% in treatment-naïve patients with HCV genotype 1 infection [1,2].

Despite these advances, current treatment options remain inadequate for some patients. Patients with prior null response to PegIFN-α/RBV (<2 $\log_{10}$ decline in HCV RNA after 12 weeks) have a particularly acute need for further therapeutic improvements. Null responders generally respond poorly to retreatment with PegIFN-α/RBV; fewer than 10% achieve SVR [3]. Retreatment of null responders with PegIFN-α/RBV combined with telaprevir or boceprevir increases SVR rates to approximately 30–38%, suggesting that addition of a DAA to PegIFN-α/RBV increases efficacy, but that more potent regimens are still urgently needed [4,5]. There are also many patients who cannot be treated with current therapies; this group includes patients with prior intolerance to PegIFN-α/RBV and patients who are ineligible for PegIFN-α/RBV -containing therapy for medical reasons.

There is precedence for use of combination antiviral regimens to treat human immunodeficiency virus (HIV) infections;

evidence is mounting that DAA regimens can also provide durable clearance of HCV infections. Thus, there is a strong rationale for exploration of dual DAA regimens, without PegIFN-α/RBV. In combination, DAAs with different molecular targets can increase regimen potency and raise the barrier to resistance, potentially eliminating the need for PegIFN-α/RBV and providing a viable therapy for patients who are anticipated to be poorly responsive or intolerant to current PegIFN-α/RBV-containing regimens. The improved tolerability and convenience that can be anticipated with dual DAA regimens suggests that they may also benefit treatment-naïve patients and other groups. Previous studies of DAA-only regimens, or DAAs combined with RBV, have demonstrated marked antiviral effects in treatment-naïve and experienced patients, including null responders, supporting the further evaluation of dual DAA therapy reported here [6–10].

Daclatasvir (DCV; BMS-790052) is a first-in-class, highly selective NS5A replication complex inhibitor with picomolar potency and broad genotypic coverage; asunaprevir (ASV; BMS-650032) is a potent NS3 protease inhibitor active against genotypes 1 and 4. Daclatasvir and asunaprevir have different modes of action and resistance-associated variants, and in combination show increased antiviral potency *in vitro* and a high genetic barrier to resistance [11,12]. Daclatasvir and asunaprevir had no clinically meaningful pharmacokinetic interaction in healthy volunteers [13]. Initial efficacy evaluations of daclatasvir and asunaprevir (DUAL therapy) showed potent antiviral effects and SVR rates ⩾90% in Japanese and US/European null responders with HCV genotype 1b infection [7,8].

We present final results of an open-label trial evaluating DUAL oral therapy with daclatasvir and asunaprevir in Japanese patients with chronic HCV genotype 1b infection. Initial results from a sentinel cohort of 10 patients with prior null response to PegIFN-α/RBV have been reported [7]. The present report combines these data with results for 11 additional null responders, together with results for 22 patients with prior intolerance to PegIFN-α/RBV or who were medically ineligible for PegIFN-α/RBV-containing therapy.

## Materials and methods

### *Study design*

This open label, phase 2a study (AI447-017; clinicaltrials.gov identifier NCT01051414) was conducted in two populations of patients with HCV genotype 1 infection, including null responders (<2 $\log_{10}$ decline of serum HCV RNA levels after 12 weeks of prior PegIFN-α/RBV), and PegIFN-α/RBV ineligible/intolerant patients. The latter group discontinued prior therapy with PegIFN-α/RBV due to intolerance after <12 weeks, or patients were treatment-naïve but poor candidates for PegIFN-α/RBV for medical reasons such as advanced age or complications of depression, anemia, myelosuppression, diabetes, or cardiovascular or renal dysfunction.

Patients were enrolled in two cohorts of null responders and two cohorts of PegIFN-α/RBV ineligible/intolerant patients. One cohort of each population included intensive sampling for pharmacokinetic analyses; both cohorts of each population were combined for efficacy and safety assessments. The sentinel cohort of null responders, reported previously, provided 4-week safety data for review by the study Safety Committee, prior to initiation of the other cohorts [7]. The primary efficacy end point was the proportion of patients with undetectable HCV RNA at 12 weeks post-treatment ($SVR_{12}$). Key secondary end points included the proportions of patients with HCV RNA undetectable at week 4, week 12, the end of treatment, and post-treatment week 24 ($SVR_{24}$).

Written informed consent was obtained from all patients. The study was approved by institutional review boards at each site and was conducted in compliance with the Declaration of Helsinki, Good Clinical Practice Guidelines, and local regulatory requirements.

### *Patients*

Eligible patients were men and women aged 20–75 years with HCV genotype 1 infection ⩾6 months and HCV RNA ⩾$10^5$ IU/ml. Women of childbearing potential were using adequate contraception. Patients were excluded if they had evidence of liver cirrhosis within 24 months of screening by laparoscopy, imaging studies, or liver biopsy; a history of hepatocellular carcinoma, other chronic liver disease, variceal bleeding, hepatic encephalopathy, or ascites requiring diuretics or paracentesis; co-infection with hepatitis B virus or HIV; other clinically significant medical conditions; exposure to any investigational drug or placebo within 4 weeks, or any previous exposure to NS5A or NS3 protease inhibitors.

Exclusionary laboratory findings included alanine aminotransferase (ALT) >5× upper limit of normal (ULN), total bilirubin ⩾2 mg/dl, direct bilirubin >1.5× ULN, international normalized ratio (INR) ⩾1.7, albumin ⩽3.5 g/dl, hemoglobin <9.0 g/dl, white blood cells <1500/$mm^3$, absolute neutrophils <750/$mm^3$, platelets <50,000/$mm^3$, and creatinine >1.8× ULN. Prohibited concomitant medications included CYP3A4 inducers or moderate/strong CYP3A4 inhibitors, non-study medications with anti-HCV activity, prescription or herbal products not prescribed for treatment of a specific condition, proton pump inhibitors, and erythropoiesis-stimulating agents. Prescribed H2 receptor antagonists were administered ⩾2 h after and ⩾10 h prior to daclatasvir; other acid modifying agents were administered ⩾2 h prior and ⩾2 h after daclatasvir.

### *Study drug dosing*

Patients received 24 weeks of treatment with daclatasvir 60 mg once daily (two 30 mg tablets), combined with asunaprevir 200 mg twice daily, with 24 weeks of post-treatment follow-up. In the sentinel cohort of null responders, asunaprevir was initially administered as three 200 mg tablets twice daily (600 mg BID), subsequently reduced to 200 mg BID during treatment following reports from another study of greater and more frequent aminotransferase elevations with the higher dose [14].

Patients with HCV RNA <15 IU/ml on or after week 4 continued treatment to week 24; patients discontinued treatment if HCV RNA decreased <2 $\log_{10}$ IU/ml from baseline on or after week 2. Patients with viral breakthrough on or after week 2, or quantifiable HCV RNA (⩾15 IU/ml) on or after week 4, either discontinued treatment or weight-based PegIFN-α/RBV was added (null responders only), for up to 48 additional weeks, at the discretion of the investigator based on anticipated tolerability. Viral breakthrough was defined as confirmed ⩾1 $\log_{10}$ IU/ml increase from nadir of HCV RNA, or HCV RNA ⩾15 IU/ml after confirmed undetectable. Post-treatment relapse was defined as confirmed HCV RNA ⩾15 IU/ml during follow-up in patients with undetectable HCV RNA at the end of treatment.

### *Safety and efficacy assessments*

HCV RNA, physical examinations, adverse events, laboratory parameters, and concomitant medications were assessed at screening, study days 1 (baseline), weeks 1, 2, 3, 4, 6, 8, 10, 12, 16, 20, and 24, and post-treatment weeks 4, 8, 12, and 24. Twelve-lead electrocardiograms were recorded at all visits except weeks 3 and 6.

Serum HCV RNA levels were determined at a central laboratory using the Roche COBAS® TaqMan® HCV Auto assay, (Roche Diagnostics KK, Tokyo, Japan), lower limit of quantitation 15 IU/ml. HCV genotype and subtype and *IL28B* genotype (rs12979860) were determined by PCR amplification and sequencing. Baseline liver fibrosis was assessed by serum blood markers (APRI; AST and Platelet Ratio Index) [15]. HCV resistance-associated polymorphisms were analyzed in stored baseline samples from all patients and post-failure samples from patients with viral breakthrough or post-treatment relapse. Polymorphisms were analyzed by PCR amplification and population sequencing of the HCV NS3 protease and NS5A domains.

### *Statistical analysis*

Categorical variables were summarized using counts and percents; continuous variables were summarized with univariate statistics.

**Table 1. Baseline demographic and disease characteristics.**

| Parameter | | Null responders n = 21 | Ineligible/intolerant n = 22 |
|---|---|---|---|
| Age, median yr (range) | | 61 (31-70) | 68 (47-75) |
| Male, n (%) | | 8 (38.1) | 6 (27.3) |
| HCV genotype 1b, n (%) | | 21 (100) | 22 (100) |
| *IL28B* genotype, n (%) | | | |
| (rs12979860) | CT | 18 (85.7) | 6 (27.3) |
| | CC | 3 (14.3) | 16 (72.7) |
| HCV RNA, mean $\log_{10}$ IU/ml (SD) | | 6.8 (0.47) | 6.6 (0.64) |
| ALT, mean U/L (SD) | | 57.9 (24.86) | 45.7 (25.79) |
| APRI score | | | |
| | Score >2, n (%) | 3 (14.3) | 1 (4.5) |
| | Median (range) | 0.96 (0.24-3.41) | 0.57 (0.40-2.79) |
| PegIFN-α/RBV ineligible, n (%) | | n.a. | 18 (81.8) |
| PegIFN-α/RBV intolerant, n (%) | | n.a. | 4 (18.2) |

n.a., Not available.

## Results

*Patient characteristics and disposition*

Forty-nine patients were screened of which six failed to meet entry criteria; 21 null responders and 22 ineligible/intolerant patients were enrolled and treated (Table 1). The enrolled population was generally older (median 62 years), consistent with HCV epidemiology in Japan, and primarily female (67%); all patients were Japanese. No patient had prior exposure to HCV DAAs. Although any HCV genotype 1 subtype was permitted, all enrolled patients had genotype 1b infection, reflecting the high proportion of this subtype in Japan [16]. Null responders were primarily *IL28B* genotype CT (rs12979860) as expected [17]; ineligible/intolerant patients were primarily genotype CC, consistent with the distribution of *IL28B* genotypes in Japan [18]. Eighteen ineligible/intolerant patients were treatment-naïve and considered ineligible for PegIFN-α/RBV due to anticipated difficulty in completing therapy due to advanced age (⩾70 years) (seven patients), cytopenia (two), depression (two), hypertension (one), or other reasons (six), consistent with common clinical practice in Japan. Four patients had prior PegIFN-α/RBV intolerance due to cytopenia (two patients), depression (one), or other reasons (one). Baseline HCV RNA and ALT levels were similar across patient groups. Although patients with cirrhosis by imaging criteria were excluded, four enrolled patients had APRI scores >2 at baseline, indicating probable cirrhosis [15].

Thirty-six of 43 enrolled patients completed 24 weeks of therapy (Fig. 1). Two null responders discontinued study medication due to hyperbilirubinemia (week 2) and aminotransferase elevation (week 12), respectively. One null responder achieved very low HCV RNA (50 IU/ml) at week 4; however, stringent protocol-defined rules required discontinuation from DAA-only therapy and addition of PegIFN-α/RBV to the dual DAA regimen at week 6. Study drugs were discontinued in four ineligible/intolerant patients due to aminotransferase elevation (week 16), viral breakthrough (week 16), or patient request (weeks 8 and 16); all four patients remained on study for assessment of SVR.

*Virologic response*

High rates of virologic response were seen at all time points in both study populations (Table 2). Overall, 77% of patients achieved $SVR_{12}$ and $SVR_{24}$. HCV RNA was undetectable in more ineligible/intolerant patients than null responders at week 4, suggesting a more rapid initial antiviral effect, but HCV RNA was undetectable in similar proportions of both populations at week 12 and the end of treatment. Rates of $SVR_{24}$ were higher in null responders (91%) than in ineligible/intolerant patients (64%) due to virologic failures in the latter group (3 breakthroughs and 4 relapses). Assessment of virologic response by *IL28B* genotype (rs12979860) showed slightly greater responses at weeks 2, 3, and 4 in patients with genotype CC; however, similar proportions of patients with genotypes CC and CT achieved $SVR_{24}$ (Fig. 2). All four patients with possible cirrhosis based on APRI score achieved $SVR_{24}$.

HCV RNA declined rapidly after initiation of therapy in all patients (Fig. 3). Mean reductions of HCV RNA from baseline at week 4 were 5.6 and 5.4 $\log_{10}$ IU/ml in null responders and ineligible/intolerant patients, respectively; HCV RNA was undetectable by week 8 in all patients on therapy. In the ineligible/intolerant group, initial virologic response in the four intolerant patients was similar to that of the cohort overall; three of these patients subsequently achieved $SVR_{24}$ and one relapsed. The null responder who discontinued at week 2 with hyperbilirubinemia had low-level HCV RNA at discontinuation and undetectable HCV RNA at all post-treatment assessments. The null responder who added PegIFN-α/RBV at week 6 received 46 weeks of quadruple therapy and HCV RNA remained undetectable 24 weeks post-treatment. Among the four ineligible/intolerant patients who discontinued study drugs before week 24, HCV RNA was undetectable at discontinuation (weeks 8 or 16) in three patients and remained undetectable in the two patients who completed post-treatment follow-up.

*Viral breakthrough and relapse*

No null responders experienced virologic breakthrough or relapse (Table 2). Three ineligible/intolerant patients experienced viral breakthrough at weeks 10 or 16 after ⩾4 weeks with undetectable

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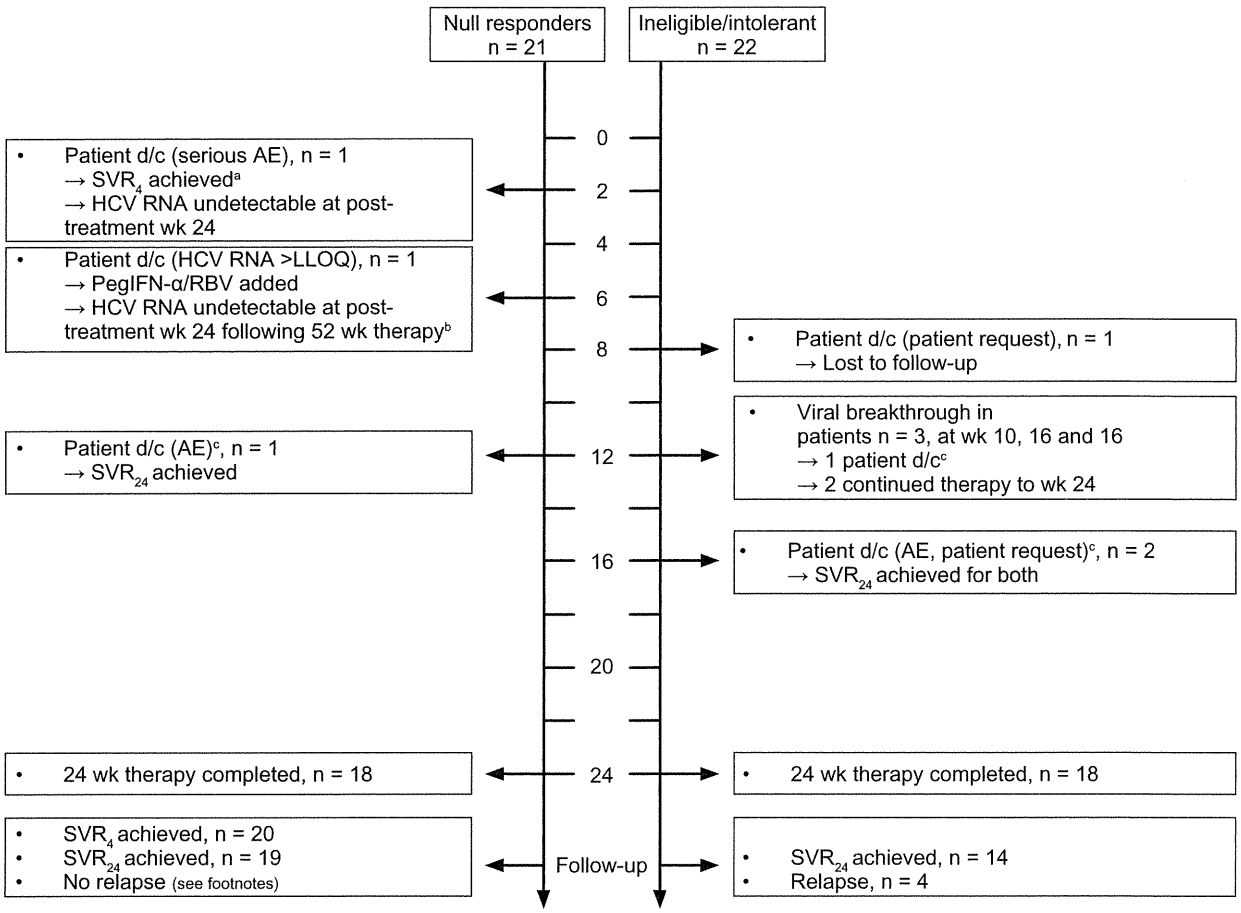


**Fig. 1. Patient disposition**. Patient flow through treatment and follow-up is shown. d/c, Discontinuation of study medication; $SVR_4$, $SVR_{12}$ and $SVR_{24}$, sustained virologic response 4, 12 or 24 weeks post-treatment. [a]On-study follow-up continued to post-treatment week 4; HCV RNA remained undetectable at post-treatment week 24 after study discontinuation, reported as failure for $SVR_{24}$ per statistical protocol requirements; [b]HCV RNA was undetectable at post-treatment week 24 after study discontinuation due to addition of PegIFN-α/RBV, reported as failure for SVR per statistical protocol requirements; [c]on-study follow-up to assess SVR continued after discontinuation of study drugs.

**Table 2. Virologic outcomes.**

| n (%) | Null responders, n = 21 | Ineligible/intolerant, n = 22 |
|---|---|---|
| HCV undetectable | | |
| Wk 4 (RVR) | 11 (52.3) | 19 (86.4) |
| Wk 12 (cEVR) | 19 (90.5) | 20 (90.9) |
| End of treatment | 19 (90.5) | 19 (86.4) |
| $SVR_4$ | 20 (95.2)[1] | 15 (68.2)[2] |
| $SVR_{12}$ | 19 (90.5)[1] | 14 (63.6)[2] |
| $SVR_{24}$ | 19 (90.5)[1] | 14 (63.6)[2] |
| Viral breakthrough | 0 | 3 (13.6) |
| Post-treatment relapse | 0 | 4 (18.2) |

Intention to treat (missing = failure) analysis. End of treatment is week 24 or last on-treatment visit for patients who discontinued early.
RVR, rapid virologic response; cEVR, complete early virologic response; $SVR_4$, $SVR_{12}$, and $SVR_{24}$, sustained virologic response 4, 12 or 24 weeks post-treatment.
[1]Two patients discontinued from the study before completion of follow-up. One patient received added PegIFN-α/RBV per protocol criteria and is counted as failure for $SVR_4$, $SVR_{12}$, and $SVR_{24}$ for DAA only therapy; one patient had missing HCV RNA data for follow-up weeks 12 and 24 and is counted as failure for $SVR_{12}$ and $SVR_{24}$ per statistical protocol.
[2]One patient was lost to follow-up for assessment of $SVR_{12}$ and $SVR_{24}$.

serum HCV RNA, and four patients relapsed at post-treatment week 4 (three patients) or 12 (one patient) after ⩾18 weeks with undetectable HCV RNA. All three patients with viral breakthrough were *IL28B* genotype CT (rs12979860), compared with 6/22 ineligible/intolerant patients overall. Three patients who relapsed were *IL28B* genotype CC; one was genotype CT.

Resistance-associated polymorphisms in NS5A and/or NS3 protease were found pretreatment in 33/43 patients overall, most of whom achieved SVR. Daclatasvir and asunaprevir resistance-associated variants were detected post-failure in all seven patients with virologic failure (Table 3). The NS5A-Y93H variant pre-existed in 10/43 study patients, of which five (50%) experienced virologic failure and five (50%) achieved SVR. NS5A-L31 and NS3-D168 substitutions emerged in all failures, but were not detected pretreatment except for NS5A-L31M in one patient.

In general, patients with virologic failure had concurrent asunaprevir and daclatasvir trough concentrations below median values, but within the expected range (Fig. 4). Notably, most patients with trough concentrations below median values achieved SVR. There were no strong associations between virologic failure and pretreatment parameters that included gender, age, baseline HCV RNA level, *IL28B* genotype, reason for PegIFN-

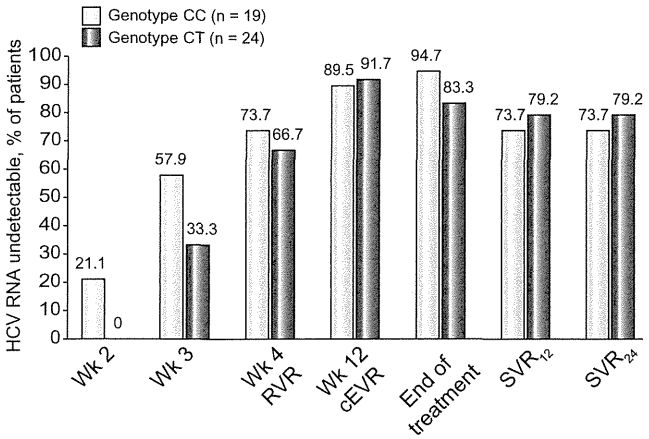


**Fig. 2. Outcomes by *IL28B* genotype.** Virologic outcomes at milestone time points are shown for the overall population by *IL28B* (rs12979860) genotype. End of treatment is week 24 or the last on-treatment visit for patients who discontinued early. RVR, rapid virologic response; cEVR, complete early virologic response; $SVR_{12}$ and $SVR_{24}$, sustained virologic response 12 or 24 weeks post-treatment.

α/RBV ineligibility, and fibrosis stage. Adherence to treatment, assessed by pill counts at study visits, was high in six of the seven patients with virologic failure.

*Safety*

The most frequently reported adverse events were generally mild headache, nasopharyngitis, aminotransferase elevations, and diarrhea (Table 4). The most frequent grade 3 or 4 laboratory abnormalities were serum aminotransferase elevations. There were six serious adverse events in five patients, including grade 2/3 pyrexia (three patients), grade 2 exacerbation of hypochondriasis, and grade 2 gastroenteritis (unrelated to study drugs) with grade 4 hyperbilirubinemia (described in detail previously)

**Table 3. Resistance-associated polymorphisms in patients with virologic failure.**

| | Patient | | NS5A | | | | NS3 | |
|---|---|---|---|---|---|---|---|---|
| | | | L31 | Q54 | P58 | Y93 | Q80 | D168 |
| Viral breathrough | 1 | Baseline | L/M | | | Y/H | | |
| | | Post-VBT | M | | A | H | | A |
| | 2 | Baseline | | Y | | Y/H | L | |
| | | Post-VBT | M | Y | | H | | V |
| | 3 | Baseline | | Y | | H | | |
| | | Post-VBT | M | Y | | H | | V |
| Post-treatment relapse | 4 | Baseline | | | P/S | Y/H | | |
| | | Post-relapse | M | | | H | | A |
| | 5 | Baseline | | | L | | | |
| | | Post-relapse | M | | L | H | | V/D |
| | 6 | Baseline | | | | | | |
| | | Post-relapse | V | | | H | | V |
| | 7 | Baseline | | | | H | | |
| | | Post-relapse | V/M | | | H | | V |

[7]. All three pyrexia events resolved after 4–10 days with continued study treatment; the hypochondriasis persisted for approximately six months and resolved after completion of study treatment. In the patient who discontinued with hyperbilirubinemia, bilirubin normalized four weeks post-treatment [7]. Serum aminotransferases normalized by four weeks post-treatment in the two patients who discontinued for elevations.

## Discussion

High rates of $SVR_{24}$ were achieved after 24 weeks of dual oral DAA therapy in null responders and PegIFN-α/RBV ineligible or

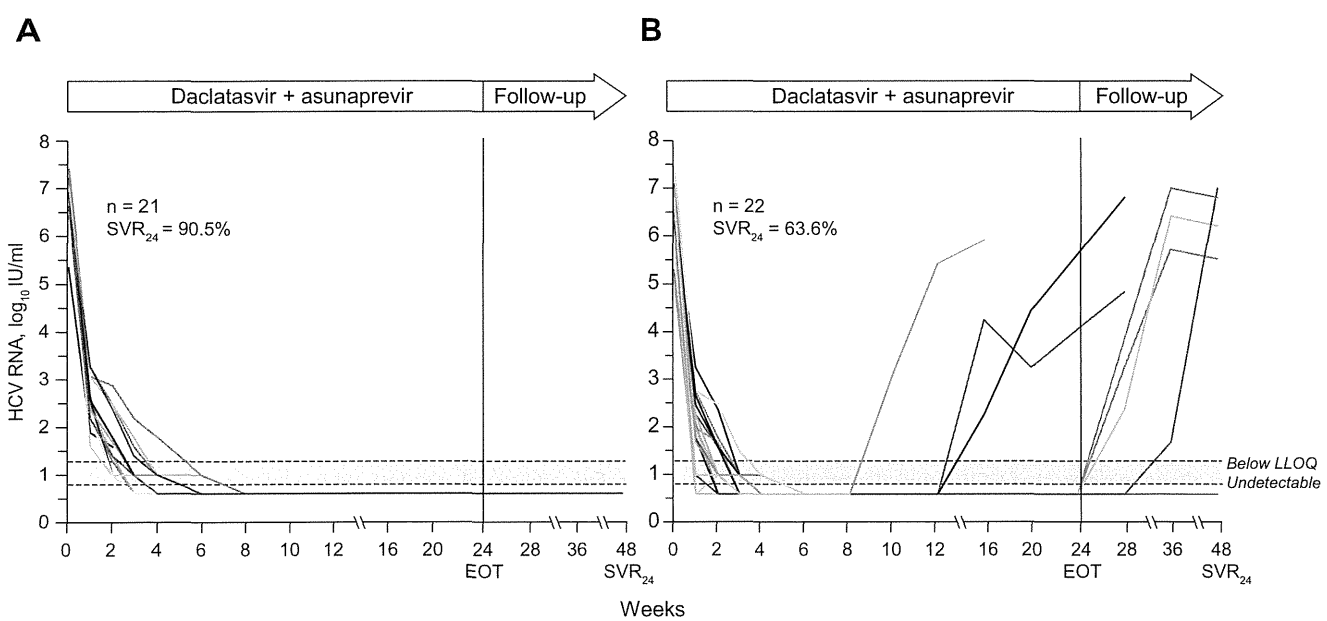


**Fig. 3. HCV RNA levels, individual patients.** Serum HCV RNA levels over time are shown for each patient. (A) Null responders; (B) ineligible/intolerant patients. EOT, end of treatment; $SVR_{24}$, sustained virologic response 24 weeks post-treatment; LLOQ, lower limit of quantitation = 15 IU/ml.

# Research Article

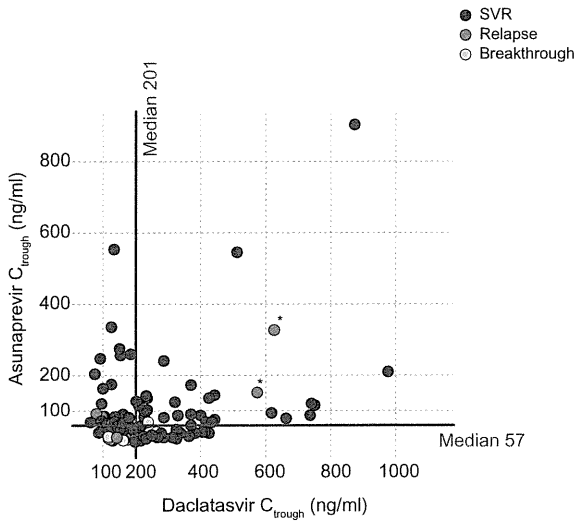


**Fig. 4. Daclatasvir and asunaprevir trough plasma concentrations.** Available trough plasma concentrations of asunaprevir and daclatasvir for individual patients are plotted and color-coded according to each patient's virologic outcome. Multiple determinations are shown for some patients. *Indicates values from a single patient with documented non-compliance.

**Table 4. Most frequent adverse events and laboratory abnormalities.**

| Event, n (%) | | Null responders (n = 21) | Ineligible/ intolerant (n = 22) |
|---|---|---|---|
| Adverse events occurring in ≥3 patients in either group | Headache | 8 (38) | 6 (27) |
| | Nasopharyngitis | 6 (29) | 8 (36) |
| | ALT increase | 6 (29) | 6 (27) |
| | Diarrhea | 9 (43) | 2 (9) |
| | AST increase | 6 (29) | 4 (18) |
| | Pyrexia | 3 (14) | 5 (23) |
| | Eosinophilia | 1 (5) | 4 (18) |
| | Abdominal discomfort | 3 (14) | 2 (9) |
| | Malaise | 2 (10) | 3 (14) |
| | Constipation | 2 (10) | 3 (14) |
| | Back pain | 3 (14) | 1 (5) |
| | Decreased appetite | 0 | 3 (14) |
| Grade 3 or 4 lab abnormalities | ALT | 2 (10) | 2 (9) |
| | AST | 1 (5) | 2 (9) |
| | Lymphocytes | 2 (10) | 1 (5) |
| | Phosphorus | 1 (5) | 1 (5) |
| | Bilirubin, total | 1 (5) | 0 |
| | Leukocytes | 1 (5) | 0 |

intolerant patients, representing two populations that are particularly difficult to treat due to limited therapeutic options. SVR rates were comparable at post-treatment weeks 4, 12, and 24; only one relapse occurred more than 4 weeks post-treatment. The 90.5% SVR rate in null responders is substantially higher than the generally poor response to PegIFN-α/RBV retreatment and the 37% SVR rate reported for genotype 1b null responders treated with PegIFN-α/RBV and telaprevir [4,19]. Therefore, therapy of this population with daclatasvir and asunaprevir appeared to overcome the poor interferon responsiveness, which may be less relevant to the efficacy of this DAA-only regimen. The SVR rate of 63.6% in ineligible/intolerant patients, although lower than results in null responders, is the first demonstration of a potentially effective treatment for these patients who currently have no therapeutic options. High SVR rates in both populations were achieved despite multiple adverse predictors of response to PegIFN-α/RBV therapy, including older age, high viral load, and a high proportion of *IL28B* genotype CT in the null responders.

Detectable HCV RNA was cleared rapidly; viral suppression was greater at all time points compared to reported results with PegIFN-α/RBV combined with telaprevir or TMC435 in genotype 1 null responders [4,20]. The slightly greater early viral suppression in ineligible/intolerant patients may reflect the higher frequency of *IL28B* CC genotype in this group. In the overall population, early virologic response was greater in patients with CC genotype, although this difference disappeared by week 12. Potentially, CC genotype may increase early viral suppression by increasing responsiveness to endogenous interferons that are released as a result of the rapid antiviral activity of the dual DAA therapy, allowing reversal of HCV-induced immunosuppression [21].

These results in patients with HCV genotype 1b differ from those reported for genotype 1a. In a similar study of US/European null responders, 2/9 patients with genotype 1a achieved SVR with daclatasvir + asunaprevir dual therapy, compared with 10/10 patients with genotype 1a who received quadruple therapy combining daclatasvir and asunaprevir with PegIFN-α/RBV [8]. This difference suggests that viral genotype can influence responses to DAA regimens, and outcomes can be optimized by individualized therapy that considers viral genotype.

The two populations included in this study represent substantial numbers of patients worldwide. Approximately 10% of HCV genotype 1-infected patients receiving PegIFN-α/RBV have a null response [22]. The cumulative prevalence of PegIFN-α/RBV null responders and the frequent failure of retreatment with current regimens, together suggest that a large population of null responders is awaiting improved therapies. The population of PegIFN-α/RBV ineligible or intolerant patients has not been extensively studied but may be substantial. In the IDEAL study, 23.2% of the 4469 patients screened were considered ineligible for PegIFN-α/RBV therapy; of these, 30.3% had hematologic or psychiatric conditions that may not preclude DAA-only regimens [23]. In registration trials, 9.7–14% of patients receiving PegIFN-α/RBV discontinued study treatment due to intolerance [24,25]. Moreover, these clinical trial data are likely to underestimate the true size of the ineligible and intolerant populations in community practice.

Virologic failures occurred relatively late in therapy after extended periods with undetectable HCV RNA. All seven patients with virologic failure had emergent NS5A and NS3 mutations that together confer high-level resistance to both daclatasvir and asunaprevir *in vitro* [11,12]. Pretreatment, NS5A-Y93H was detected in five of the seven patients with virologic failure and in five additional patients who achieved SVR, suggesting that pre-existing Y93H is loosely associated with virologic failure but is not an absolute predictor. Pharmacokinetics may also have contributed; nearly all patients with virologic failure had trough plasma concentrations of daclatasvir and asunaprevir below their respective median values. However, SVR was achieved by most patients with trough drug levels below the median, and by

several patients who discontinued study treatment after 2–16 weeks. Thus, the relationship of drug exposure to virologic outcome remains uncertain; further study is needed to define on-treatment predictors of outcome and the optimal duration of therapy.

Current data do not fully explain the observed differences in rates of virologic failure and SVR, between the two study populations. *IL28B* genotype was the primary difference between the two populations pretreatment. All three breakthroughs occurred in ineligible/intolerant patients with the unfavorable *IL28B* CT genotype; however, null responders had no breakthroughs, despite a much higher frequency of this genotype. Differing proportions of patients with concurrent pre-existing resistance-associated polymorphisms and low plasma drug concentrations may have contributed to differing rates of virologic failure between the two populations. Analysis of baseline parameters failed to identify other factors that may have influenced outcomes. However, these analyses were limited by the relatively small study population and may have been confounded by unreported non-adherence or baseline parameters not quantified absolutely, such as the stage of liver fibrosis. This issue requires further study in larger populations to confirm the apparent difference in outcomes and to identify factors predictive of virologic failure.

The adverse event profile of the study regimen was generally more favorable than that typically observed with PegIFN-α/RBV-containing regimens [26]. There were no significant hematologic or psychiatric abnormalities; the most common adverse events were non-specific in nature and generally mild to moderate in intensity. Mild diarrhea was experienced by 26% of study patients, consistent with previous studies of asunaprevir and other drugs of this class [4,6,14]. The four observed grade 3/4 ALT elevations resolved with continued therapy or after discontinuation and were not associated with significant clinical events. A role for study drugs in the reported serious adverse events cannot be ruled out except for gastroenteritis; however, four of the six events resolved spontaneously with continued treatment. The case of hyperbilirubinemia with gastroenteritis was complicated by multiple confounding factors, and the contribution of study drugs is uncertain [7].

In conclusion, dual oral therapy with daclatasvir and asunaprevir elicited rapid clearance of detectable HCV RNA and achieved high rates of SVR in two difficult-to-treat patient populations. These results confirm initial findings that HCV genotype 1b infections can be cured with daclatasvir combined with asunaprevir, without PegIFN-α/RBV [7,8]. Thus, this regimen has potential to offer effective treatment to null responders who have previously shown little or no response to PegIFN-α/RBV, and to PegIFN-α/RBV ineligible/intolerant patients who have no current treatment options. Further research will assess the benefits of this and other DAA combinations in larger and more diverse patient populations, but the promise of all oral and well-tolerated HCV therapy is on the horizon.

## Financial support

This study was funded by Bristol-Myers Squibb.

## Conflicts of interest

K Chayama has received research grants and consulting fees from Bristol-Myers Squibb, Dainippon Sumitomo Pharma, Mitsubishi Tanabe Pharma, Daiichi Sankyo, Toray Industries, Otsuka Pharmaceutical Company, and GlaxoSmithKline KK. Hiroki Ishikawa, Hideaki Watanabe, Wenhua Hu, Timothy Eley, Fiona McPhee, and Eric Hughes are employees of Bristol-Myers Squibb. All other authors have no conflicts to report.

## Acknowledgments

The authors thank the patients and their families, and research staff, investigators and safety committees at all participating sites. Marc Bifano, MS, and Bing He, MS, contributed to analysis and interpretation of pharmacokinetic data. Editorial assistance for preparation of this manuscript was provided by Richard Boehme, PhD, of Articulate Science and was funded by Bristol-Myers Squibb.

## References

[1] Poordad F, McCone Jr J, Bacon BR, Bruno S, Manns MP, Sulkowski MS, et al. Boceprevir for untreated chronic HCV genotype 1 infection. N Engl J Med 2011;364:1195–1206.
[2] Jacobson IM, McHutchison JG, Dusheiko G, Di Bisceglie AM, Reddy KR, Bzowej NH, et al. Telaprevir for previously untreated chronic hepatitis C virus infection. N Engl J Med 2011;364:2405–2416.
[3] Poynard T, Colombo M, Bruix J, Schiff E, Terg R, Flamm S, et al. Peginterferon alfa-2b and ribavirin: effective in patients with hepatitis C who failed interferon alfa/ribavirin therapy. Gastroenterology 2009;136:1618–1628.
[4] Zeuzem S, Andreone P, Pol S, Lawitz E, Diago M, Roberts S, et al. Telaprevir for retreatment of HCV infection. N Engl J Med 2011;364:2417–2428.
[5] Vierling JM, Flamm SL, Gordon SC, Lawitz E, Bronowicki JP, Davis M, et al. Efficacy of boceprevir in prior null responders to peginterferon/ribavirin: the PROVIDE study [abstract]. Hepatology 2011;54:796A–797A.
[6] Gane EJ, Roberts SK, Stedman CA, Angus PW, Ritchie B, Elston R, et al. Oral combination therapy with a nucleoside polymerase inhibitor (RG7128) and danoprevir for chronic hepatitis C genotype 1 infection (INFORM-1): a randomised, double-blind, placebo-controlled, dose-escalation trial. Lancet 2010;376:1467–1475.
[7] Chayama K, Takahashi S, Toyota J, Karino Y, Ikeda K, Ishikawa H, et al. Dual therapy with the NS5A inhibitor BMS-790052 and the NS3 protease inhibitor BMS-650032 in HCV genotype 1b-infected null responders. Hepatology 2012;55:742–748.
[8] Lok AS, Gardiner DF, Lawitz E, Martorell C, Everson GT, Ghalib R, et al. Preliminary study of two antiviral agents for hepatitis C genotype 1. N Engl J Med 2012;366:216–224.
[9] Zeuzem S, Soriano V, Asselah T, Bronowicki JP, Ceausu E, Lohse AW, et al. Virologic response to an interferon-free regimen of BI201335 and BI207127, with and without ribavirin, in treatment-naive patients with chronic genotype-1 HCV infection: week 12 interim results of the SOUND-C2 study [abstract]. Hepatology 2011;54:1436A.
[10] Gane EJ, Stedman CA, Hyland RH, Sorensen RD, Symonds WT, Hindes R, et al. Once daily PSI-7977 plus RBV: pegylated interferon-alfa not required for complete rapid viral response in treatment-naive patients with HCV GT2 or GT3 [abstract]. Hepatology 2011;54:377A.
[11] Fridell RA, Qiu D, Wang C, Valera L, Gao M. Resistance analysis of the hepatitis C virus NS5A inhibitor BMS-790052 in an in vitro replicon system. Antimicrob Agents Chemother 2010;54:3641–3650.
[12] McPhee F, Friborg J, Levine S, Chen C, Falk P, Yu F, et al. Resistance analysis of the hepatitis C virus NS3 protease inhibitor asunaprevir. Antimicrob Agents Chemother 2012. http://dx.doi.org/10.1128/AAC.00308-12.
[13] Bifano M, Sevinsky H, Bedford BR, Coumbis J, Eley T, Huang SP, et al. Coadministration of BMS-790052 and BMS-650032 does not result in a clinically meaningful pharmacokinetic interaction in healthy subjects [abstract]. Hepatology 2010;52:719A.

[14] Bronowicki JP, Pol S, Thuluvath PJ, Larrey D, Martorell CT, Rustgi VK, et al. BMS-650032, an NS3 inhibitor, in combination with peginterferon alfa-2a and ribavirin in treatment-naive subjects with genotype 1 chronic hepatitis C infection [abstract]. J Hepatol 2011;54:S472.
[15] Sebastiani G, Castera L, Halfon P, Pol S, Mangia A, Di Marco V, et al. The impact of liver disease aetiology and the stages of hepatic fibrosis on the performance of non-invasive fibrosis biomarkers: an international study of 2411 cases. Aliment Pharmacol Ther 2011;34:1202–1216.
[16] Sievert W, Altraif I, Razavi HA, Abdo A, Ahmed EA, Alomair A, et al. A systematic review of hepatitis C virus epidemiology in Asia. Australia Egypt Liver Int 2011;31:61–80.
[17] Kurosaki M, Tanaka Y, Nishida N, Sakamoto N, Enomoto N, Honda M, et al. Pre-treatment prediction of response to pegylated-interferon plus ribavirin for chronic hepatitis C using genetic polymorphism in IL28B and viral factors. J Hepatol 2011;54:439–448.
[18] Kobayashi M, Suzuki F, Akuta N, Sezaki H, Suzuki Y, Hosaka T, et al. Association of two polymorphisms of the IL28B gene with viral factors and treatment response in 1,518 patients infected with hepatitis C virus. J Gastroenterol 2012. http://dx.doi.org/10.1007/s00535-012-0531-1.
[19] Ghany MG, Strader DB, Thomas DL, Seeff LB. American Association for the Study of Liver Diseases. Diagnosis, management, and treatment of hepatitis C: an update. Hepatology 2009;49:1335–1374.
[20] Zeuzem S, Foster GR, Fried MW, Hezode C, Hirschfleld GM, Nikitin I, et al. The ASPIRE trial: TMC435 in treatment-experienced patients with genotype-1 HCV infection who have failed previous pegIFN/RBV treatment [abstract]. J Hepatol 2011;54:S546.
[21] Horner SM, Gale Jr M. Intracellular innate immune cascades and interferon defenses that control hepatitis C virus. J Interferon Cytokine Res 2009;29:489–498.
[22] Heathcote J. Retreatment of chronic hepatitis C: who and how? Liver Int 2009;29:49–56.
[23] Melia MT, Muir AJ, McCone J, Shiffman ML, King JW, Herrine SK, et al. Racial differences in hepatitis C treatment eligibility. Hepatology 2011;54:70–78.
[24] Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales Jr FL, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med 2002;347:975–982.
[25] Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, et al. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. Lancet 2001;358:958–965.
[26] McHutchison JG, Everson GT, Gordon SC, Jacobson IM, Sulkowski M, Kauffman R, et al. Telaprevir with peginterferon and ribavirin for chronic HCV genotype 1 infection. N Engl J Med 2009;360:1827–1838.

HEPATOLOGY
Official Journal of the American Association for the Study of Liver Diseases

# Long-Term Entecavir Treatment Reduces Hepatocellular Carcinoma Incidence in Patients With Hepatitis B Virus Infection

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**Chronic hepatitis B virus (HBV) infection leads to cirrhosis and hepatocellular carcinoma (HCC). Antiviral agents are thought to reduce HCC development, but agents such as lamivudine (LAM) have a high rate of drug resistance. We compared the incidence of HCC in 472 entecavir (ETV)-treated patients and 1,143 nontreated HBV patients (control group). Propensity score matching eliminated the baseline differences, resulting in a sample size of 316 patients per cohort. The drug mutation resistance was 0.8% (4/472) in the ETV group. The cumulative HCC incidence rates at 5 years were 3.7% and 13.7% for the ETV and control groups, respectively ($P < 0.001$). Cox proportional hazard regression analysis, adjusted for a number of known HCC risk factors, showed that patients in the ETV group were less likely to develop HCC than those in the control group (hazard ratio: 0.37; 95% confidence interval: 0.15-0.91; $P = 0.030$). Both cohorts were applied in three previously reported risk scales and risk scores were generated based on age, gender, cirrhosis status, levels of alanine aminotransferase, hepatitis B e antigen, baseline HBV DNA, albumin, and bilirubin. The greatest HCC risk reduction occurred in high-risk patients who scored higher on respective risk scales. In sub analyses, we compared treatment effect between nucleos(t)ide analogs, which included matched LAM-treated patients without rescue therapy (n = 182). We found HCC suppression effect greater in ETV-treated ($P < 0.001$) than nonrescued LAM-treated ($P = 0.019$) cirrhosis patients when they were compared with the control group. *Conclusion*: Long-term ETV treatment may reduce the incidence of HCC in HBV-infected patients. The treatment effect was greater in patients at higher risk of HCC.** (HEPATOLOGY 2013;00:000–000)

More than 2 billion people worldwide have been exposed to hepatitis B virus (HBV) and about 350 million people are chronically infected, the majority of whom are in Asia (75%). The prevalence of HBV in Japan is 0.8%, which is lower than other Asian countries such as Taiwan (>10%) and China.[1-3] As chronic HBV infection leads to cirrhosis and hepatocellular carcinoma (HCC), published studies have shown that up to 25% of chronically infected patients eventually die of liver cirrhosis or HCC.[4]

A large-scale longitudinal epidemiologic study has shown that a patient's baseline HBV DNA level is an independent predictor for the development of HCC.[5] Studies have begun to show that treatment to decrease HBV DNA reduces the risk of HCC development in HBV patients with cirrhosis or advanced fibrosis or in chronic HBV patients.[6,7]

Within the past 10 years, new antiviral therapies, including nucleos(t)ide analogs (NAs), have been approved and were successful in suppressing circulating serum viral loads. Studies that have examined the relationship between NA therapy and HCC almost exclusively used older drugs such as lamivudine and/or adefovir. Although results of long-term studies showed the importance of antiviral suppression, HCC risk among patients treated by newer NAs remains inconclusive. Entecavir (ETV) is a relatively new antiviral NA that has proved effective in suppressing HBV

*Abbreviations: ALT, alanine aminotransferase; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; ETV, entecavir; HBeAg, hepatitis B e antigen; HBV DNA, hepatitis B virus deoxyribonucleic acid; HR, hazard ratio; NA, nucleos(t)ide analogs; PS, propensity score; ROC, receiver operating characteristic curve.*

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*Received April 26, 2012; accepted November 15, 2012.*

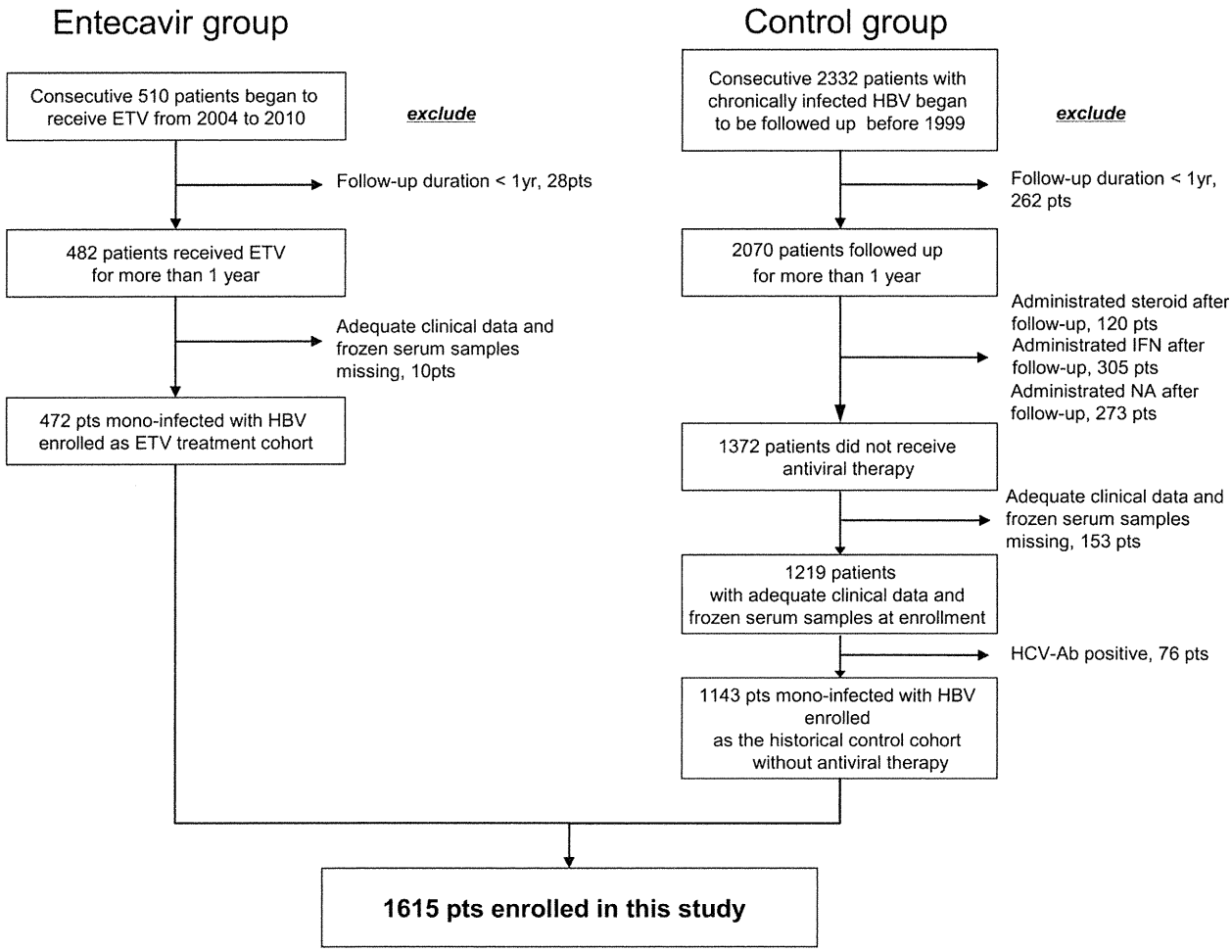


Fig. 1. Entecavir-treated and nontreated cohorts. ETV, entecavir; HBV, hepatitis B virus; IFN, interferon; NA, nucleos(t)ide; HCV-Ab, anti-hepatitis C virus antibody.

DNA replications with minimal drug resistance.[8,9] In this study we examined whether long-term ETV treatment would reduce HCC risk in HBV-infected patients when compared with NA-naïve patients.

## Patients and Methods

***Patients and Design.*** From 2004 to 2010, we consecutively recruited 510 patients treated with 0.5 mg ETV (ETV group); the ETV group was compared with a retrospective cohort of 2,332 NA-naïve, HBV-infected patients (control group).

These patients were chronically monoinfected with HBV and were confirmed as hepatitis B s antigen (HBsAg)-positive for at least 6 months. As a general rule, ETV was initiated in a patient who had both abnormal alanine aminotransferase (ALT) levels (defined as ALT $\geq$45) and elevated HBV DNA levels of $\geq$4 log copies/mL. A patient with advanced fibrosis would be treated with ETV if the ALT level was normal; however, a patient without fibrosis or with a normal HBV DNA/ALT level would not be treated with ETV. Among the treated patients, 38 were excluded from the ETV group either because their follow-up period was less than 1 year (n = 28) or because the clinical data or serum samples were incomplete (n = 10). The remaining 472 ETV-treated patients were included in the analysis (Fig. 1). No patient in the ETV group received other NAs before ETV treatment.

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*View this article online at wileyonlinelibrary.com.*

*DOI 10.1002/hep.26180*

*Potential conflict of interest: Hiromitsu Kumada has received speaker's honoraria from Bristol Myers Squibb. None of the other authors have a conflict of interest to disclose.*

*Additional Supporting Information may be found in the online version of this article.*

The control group patients were recruited from 1973 to 1999. These patients were NA-naïve at baseline, as no NA therapy had yet been approved. Patients were excluded from the control group if (1) their follow-up duration was less than 1 year (n = 262); (2) corticosteroid withdrawal therapy (n = 120), IFN treatment (n = 305) or NA treatment (n = 273) was initiated during follow-up; (3) clinical data or serum samples were incomplete (n = 153); or (4) patients were found to be positive for anti-hepatitis C virus antibodies (HCV-Ab) (n = 76). The remaining 1,143 patients served as the control population (Fig. 1).

We also made subanalyses to examine the difference of HCC suppression effect between NAs. To make this comparison, we recruited a cohort of 949 consecutive patients from our hospital who were treated with lamivudine (LAM) (September 1995 to September 2007). LAM-treated patients who met the same inclusion criteria as the ETV group, who had no rescue therapy (LAM group, n = 492), were used in the comparison.

We received informed consent from each patient at their entry into the study. Informed consent for the clinical data collection and storage of serum samples were obtained from each patient in the historical control group. The study protocol was in accordance with the ethical guidelines of the Declaration of Helsinki and approved by the Toranomon Hospital Ethics Committee.

***Clinical Data Collection and Follow-up.*** All ETV-treated and untreated patients were followed at 1- to 3-month intervals, during which biochemical and HBV virological markers, blood counts, tumor markers (e.g., alpha-fetoprotein and des-$\gamma$-carboxylprothrombin), and cirrhosis and HCC status were monitored. Viral response in the ETV group was defined as a reduction in HBV DNA levels to below 400 copies/mL. Cirrhosis was determined by laparoscopy, liver biopsy, imaging modalities, or portal hypertension. HCC was diagnosed predominantly via imaging, including dynamic computed tomography, magnetic resonance imaging, and/or digital subtraction angiography. When the hepatic nodule did not show typical imaging features, diagnosis was confirmed by fine-needle aspiration biopsy followed by histological examination. Patients were followed until any confirmed HCC diagnosis 1 year after the start of observation (primary outcome) or until the last visit before December 2011. All patients also underwent ultrasonography or helical dynamic computed tomography every 3 to 6 months (cirrhosis patients) or every 6 to 12 months (noncirrhosis patients).

***HBV Infection Markers.*** HBV DNA levels were quantified using the COBAS Amplicor HBV Monitor Test (Roche Diagnostics, Tokyo, Japan), which has a dynamic range of 2.6 to 7.6 log copies/mL, or COBAS TaqMan HBV Test v2.0 (Roche Diagnostics) which has a dynamic range of over 2.1 to 9.0 log copies/mL. HBV DNA of the control group was measured from their stored frozen serum (−80°C) using COBAS TaqMan HBV v.2.0 once at the start of observation. Previous measurements were taken using the old DNA polymerase assay in the control group and thus were not used for comparisons. For the ETV group, drug-resistant mutations were determined from a nested polymerase chain reaction, using a primer specific at the polymerase region in patients who had an HBV DNA relapse of $\geq$1 log copies from nadir. Hepatitis B e antigen; (HBeAg) was determined by enzyme-linked immunosorbent assay with a commercial kit (HBeAg EIA; Institute of Immunology, Tokyo, Japan). A commercial kit (HBV Genotype EIA; Institute of Immunology) was used to serologically determine HBV genotypes using the combination of epitopes expressed on the pre-S2 region product, which is specific for each of the eight major genotypes (A to H).

***HCC Incidence by Risk Scores.*** To examine HCC incidence by risk scores, we applied published HCC risk scales, which are based on the natural course of HCC among HBV-positive patients, to our cohorts. We first searched Medline/PubMed using "hepatitis B," "cancer," and "risk score" as keywords and found four publications in English that used risk-score estimations.[10-13] One article was rejected because we were unable to compute the risk scores with our variables, and therefore we used only the scales indicated by the remaining three publications to generate the risk scores.[13] The risk scales were based on parameters such as age, gender, cirrhosis, levels of ALT, HBeAg, baseline HBV DNA, albumin, and bilirubin. The original risk score formula and the risk score distributions for our two cohorts derived from these formulas are shown in Supporting Table 1. The risk score cutoff points were determined from the following original articles. In Yang et al.'s article,[10] the risk score was derived from 17-point categories. When we applied the scores to our control group, we found that the 12-point scale was at best in detecting a difference in HCC incidence. With that, we examined the HCC suppression treatment effect by dividing the patients into equal halves with 12 points as the cutoff. Yuen et al.[11] divided their cohort in half and found risk scores of 82 as the optimal cutoff point. We also applied the same cutoff point to our cohorts. Wong et al.[12] used their risk scores to categorize their cohort into low-risk, medium-risk, and high-risk groups with respective cutoff points at <4, 4-19, $\geq$20. We also applied the same cutoff points to our cohorts to examine the treatment effect. Cumulative

HCC incidence rates were compared by these risk scores between the ETV and control groups.

***Statistical Analysis.*** Categorical data were compared using chi-square or Fisher's exact tests. Continuous variables with normal distributions were compared using Student's $t$ test, and those without normal distributions were compared using the Mann-Whitney $U$ test. Cumulative HCC incidence rates were analyzed using the Kaplan-Meier method; patients followed beyond 5 years were censored to better compare the two cohorts because the ETV group had a shorter follow-up period when compared with the historical control group. We compared the cumulative incidence of HCC using the log-rank test, and Cox proportional hazard regression analysis, which was used to assess the variables that were significantly associated with the development of HCC. Deaths before HCC development were censored. Significance was defined as $P < 0.05$ for all two-tailed tests.

We used the propensity score (PS) matching method to reduce significant differences in demographics between the ETV and control groups.[14,15] Using multiple logistic regression analysis, a PS was estimated for all patients treated with ETV.[14] Variables used in the model included age, sex, presence of cirrhosis, HBeAg, HBV DNA< aspartate aminotransferase (AST), ALT, $\gamma$-glutamyl transpeptidase; ($\gamma$-GTP), bilirubin, albumin, and platelet counts. We performed caliper matching on the PS (nearest available matching). Pairs (ETV and the control group) on the PS logit were matched to within a range of 0.2 standard deviation (SD).[16,17] The PS logit distributions for each cohort showing the overlaps and SD ranges are shown in Supporting Fig. 1. The balance of covariates was measured by their standardized differences. A difference >10% of the absolute value was considered significantly imbalanced.[17] The cohorts were divided into five PS quintiles (Supporting Table 2). We also made subanalyses to examine the difference of HCC suppression effect between NAs by comparing the HCC incidence between propensity score matched ETV- and lamivudine (LAM)-treated patients without a rescue therapy. The LAM-treated patients were derived from consecutive sampling at our institution and were PS matched with ETV group according to the same method described above. Interaction of the subgroups by preexisting cirrhosis or risk scores and ETV treatment were evaluated. $P < 0.10$ was considered statistically significant. Data analysis was performed using IBM SPSS v. 19.0 software (Armonk, NY) and R software v. 2.13 (R Foundation for Statistical Computing, Vienna, Austria; www.r-project.org).

## Results

***Patient Characteristics.*** The patient characteristics at the baseline, before PS matching are shown in Table 1. The ETV group was followed for an average of 3.2 years (1,561 person-years), whereas the control group was followed for an average of 9.5 years (12,381 person-years). Before matching, patients in the ETV group and the control group differed significantly in age, gender, genotype, baseline HBV DNA level, and other clinical data. In the ETV group, 421 patients (89%) had HBV DNA (<400 copies/mL) at year 1. Not all patients in the control group were tested for HBV DNA level during follow-up. The drug mutation resistance was 0.8% (4/472). The four patients who had drug mutation did not develop HCC. During follow-up, 12 patients (2.54%) in the ETV group and 144 patients (12.60%) in the control group developed HCC. The incidence rates of HCC for the ETV and the control groups were 76/10,000 patient-years and 116/10,000 patient-years, respectively. During this period, 21 patients in the control group developed liver cirrhosis while no patient developed liver cirrhosis in the ETV group. During the same observation period, there were four deaths in the ETV group and 10 deaths in the control group. We took competing risk into account[18,19] and compared incidence of non-HCC deaths between the cohorts and the results were not different. However, because there were only four patients in the non-HCC deaths in the ETV group (two patients in the PS matched cohort) and 10 patients in the control group (six patients in the PS matched cohort), we considered that it was not meaningful to apply competing risk analysis in our cohorts.

***Factors Associated with HCC and Effect of ETV Treatment on HCC Development.*** To allow a common ground for comparison between the two cohorts, we used PS matching with selected key characteristics and compared the two groups within the same time period of 5 years. The PS matching process resulted in a matched sample size that consisted of 316 patients in each group (Table 1). The PS matching reduced the significant variability of the two cohorts. While five (42%) of the 12 covariates varied by >10% before matching, all covariates differed by <10% of the absolute value after matching (Supporting Fig. 2). In the PS score matched cohort, 10 out of the 231 noncirrhosis patients progressed to liver cirrhosis within the 5 years of observation. The cumulative incidence rates of HCC in the matched ETV groups were 0.7% at year 2, 1.2% at year 3, 2.5% at year 4, and 3.7% at year 5. The cumulative incidence rates of HCC in the

**Table 1. Patient Characteristics and Demographics**

| Characteristics | Entire Cohort | | | | Propensity Score Matched Cohort | | |
|---|---|---|---|---|---|---|---|
| | All Patients (n = 1,615) | Entecavir (n = 472) | Control (n = 1,143) | P | Entecavir (n = 316) | Control (n = 316) | P |
| Age (y)† | 42 (13.5) | 47 (12.4) | 39 (13.1) | <0.001 | 46 (12.1) | 46 (13.5) | 0.907 |
| Gender (male:female) | 1,035:580 | 315:157 | 720: 423 | 0.171 | 210:106 | 210:106 | 1.000 |
| Alcohol consumption (>200kg) | 355 (22) | 97 (20.5) | 288 (25.1) | 0.013 | 62 (20) | 105 (33) | <0.001 |
| Cigarette smoking | 443 (27) | 157 (33.2) | 286 (25.0) | 0.005 | 110 (35) | 110 (35) | 1.000 |
| Preexisting cirrhosis | 311 (19) | 116 (25) | 195 (17) | 0.001 | 79 (25) | 85 (29) | 0.324 |
| HBV genotype | – | – | – | <0.001 | – | – | 0.843 |
| A | 53 (3.3) | 12 (2.5) | 41 (3.6) | – | 8 (2.5) | 9 (2.8) | – |
| B | 254 (15.7) | 66 (14.0) | 188 (16.4) | – | 49 (15.5) | 50 (15.8) | – |
| C | 1,135 (70.3) | 344 (72.9) | 791 (69.2) | – | 225 (71.2) | 226 (71.5) | – |
| D | 1 (0.06) | 0 | 1 (0.09) | – | 0 | 0 | – |
| F | 1 (0.06) | 0 | 1 (0.09) | – | 0 | 0 | – |
| H | 2 (0.1) | 2 (0.4) | 0 | – | 0 | 0 | – |
| Unclassified / missing | 169 (10.4) | 48 (10.2) | 121 (10.5) | – | 34 (10.7) | 31 (9.8) | – |
| Baseline HBeAg positive | 617 (38) | 219 (46) | 398 (35) | <0.001 | 135 (43) | 133 (42) | 0.936 |
| Baseline HBV DNA (log copies/mL) | 6.0 (4.3-7.7) | 6.7 (5.3-8.0) | 5.8 (4.0-7.5) | <0.001 | 6.3 (5.2-7.9) | 6.6 (4.5-7.8) | 0.795 |
| Baseline AST level (IU/L) | 35 (22-63) | 53 (35-95) | 28 (20-50) | <0.001 | 45 (32-70) | 49 (27-98) | 0.956 |
| Baseline AST level (x ULN) | 1.1 (0.7-1.9) | 1.6 (1.1-2.9) | 0.8 (0.6-1.5) | <0.001 | 1.4 (1.0-2.1) | 1.5 (0.8-3.0) | 0.989 |
| Baseline ALT level (IU/L) | 42 (22-88) | 70 (42-163) | 33 (20-68) | <0.001 | 61 (39-109) | 60 (28-144) | 0.110 |
| Baseline ALT level (x ULN) | 1.1 (0.7-2.4) | 1.9 (1.2-4.3) | 0.9 (0.6-1.8) | <0.001 | 1.7 (1.0-3.3) | 1.6 (0.8-3.7) | 0.086 |
| Baseline GGTP level (IU/L) | 28 (16-59) | 39 (24-72) | 24 (14-52) | <0.001 | 34 (23-64) | 34 (18-68) | 0.088 |
| Baseline total bilirubin level (mg/dL) | 0.7 (0.5-0.9) | 0.7 (0.5-1.0) | 0.6 (0.5-0.9) | <0.001 | 0.7 (0.5-1.0) | 0.7 (0.5-0.9) | 0.210 |
| Baseline serum albumin level (g/L) | 4.2 (3.9-4.5) | 3.9 (3.6-4.1) | 4.4 (4.1-4.6) | <0.001 | 3.9 (3.7-4.2) | 4.0 (3.8-4.3) | 0.084 |
| †Platelet count ($10^5$/mm$^3$) (SD) | 19.1 (6.3) | 16.9 (5.6) | 20.0 (6.4) | <0.001 | 17.5 (5.2) | 17.2 (6.0) | 0.349 |
| Follow-up duration (yrs) | 5.4 (3.1-13.2) | 3.2 (2.1-4.3) | 9.5 (4.4-16.1) | <0.001 | 3.3 (2.3-4.3) | 7.6 (3.4-13.7) | <0.001 |
| Person-years of follow-up | 13,986 | 1561 | 12381 | – | 1064 | 2978 | – |
| No. of HCC cases | 156 | 12 | 144 | – | 6 | 72 | – |
| Incidence rates per 1000 person-years | 11.15 | 7.69 | 11.63 | – | 5.63 | 24.1 | – |
| Progression of cirrhosis within 5 year | 21 (1.3) | 0 | 21 (1.8) | 0.001 | 0 | 10 (3.2) | 0.001 |
| HBV DNA <400 copies/mL at 1 yearr | – | 421 (89) | NA | – | 288 (90) | NA | – |
| Emergence of drug-resistant mutants during ETV treatment | – | 4 (0.8) | NA | – | 2 (0.6) | NA | – |

HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; AST, aspartate aminotransferase; GGTP, gamma glutamyltransferase (ULN=33 IU/L); ALT, alanine aminotransferase (ULN=42 IU/L for men and 27 IU/L for women); HCC, hepatocellular carcinoma; ETV, entecavir.

*$P$ <0.05.

**$P$ <0.001, comparison of entecavir-treated group and control group.

†Data displayed as mean ± standard deviation. ‡All other values are expressed as median (25th to 75th percentile) or number (percentage of total, %).

matched control group were 4.0% at year 2, 7.2% at year 3, 10.0% at year 4, and 13.7% at year 5. Log-rank test revealed a statistically significant difference between the incidence of HCC in the ETV group and the control group over time ($P < 0.001$) (Fig. 2). We then used Cox proportional regression analysis to estimate the effects of ETV treatment on HCC risk. Factors that were associated with HCC at year 5 in the propensity score matched cohort were age, gender, alcohol consumption (>200 kg), the presence of cirrhosis, HBeAg positivity, baseline viral load, ALT, $\gamma$-GTP, total bilirubin, serum albumin, and platelet counts (Table 2). For ETV treatment effect, we estimated the hazard ratio of HCC development, adjusting for multiple baseline variables (age, gender, alcohol consumption, smoking, preexisting cirrhosis, HBeAg, HBV DNA, ALT, albumin, $\gamma$-GTP, total bilirubin, and platelet count) in the propensity matched cohort. Progression of cirrhosis within 5 years was used as a time-dependent covariate in the proportional hazard regression but it did not show a statistically significant hazard to HCC development.

***Subanalyses Showing HCC Suppression Effect Between ETV and LAM.*** PS matching of the LAM-treated patients without rescue therapy (n = 492) with ETV-treated patients resulted in a matched cohort of 182 patients (Supporting Table 3). The rate of non-rescued LAM-treated group having undetectable HBV DNA at 1 year after treatment was lower when compared with the ETV-treated group. The LAM-treated group also had a higher drug-resistant mutation rate. Comparisons of HCC incidence among the ETV-treated group, nonrescued LAM-treated group, and control showed that the HCC suppression effect was greater in ETV-treated ($P < 0.001$) than nonrescued LAM-treated ($P = 0.019$) when compared with the

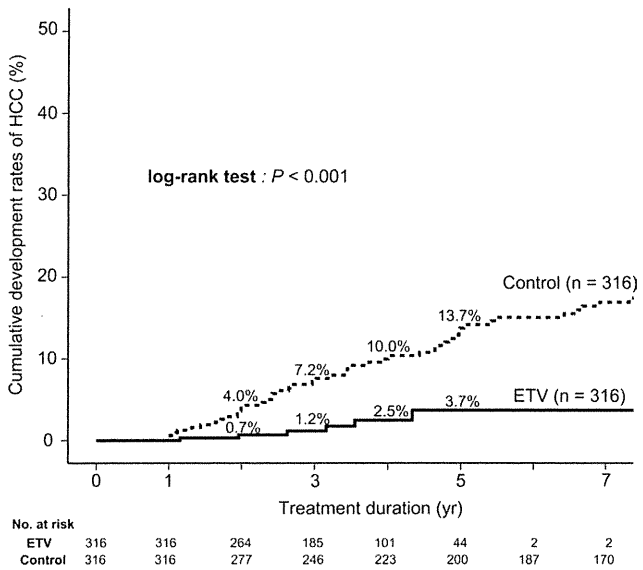


Fig. 2. Comparison of HCC cumulative incidence rates between the entecavir-treated group and the nontreated control group after propensity score matching. The log-rank test revealed a statistically significant difference between the ETV and the control group in the incidence of HCC at 5 years time (log-rank test: $P < 0.001$).

control group (Fig. 3). The difference of effect between ETV and LAM was also significant ($P = 0.043$). The treatment effect was seen in cirrhosis patients but not in noncirrhosis patients. The result showed ETV's superiority to LAM in suppressing HCC.

***Effect of ETV on the Reduction of HCC Development by Preexisting Cirrhosis and Risk Scores.*** To further examine the ETV treatment effect, we compared the ETV and the control groups by preexisting cirrhosis and published risk scores. Viral response rates (HBV DNA < 400 copies/mL) of 1-year post-ETV treatment was 87% in the noncirrhosis patients and 91% in the cirrhosis patients (LC). ALT normalization was 94% and 90% in the chronic hepatitis and cirrhosis patients, respectively. The treatment effect was not inferior by cirrhosis status. Among those who developed HCC, 97 out of 144 patients in the control group and 9 out of 12 patients in the ETV group had cirrhosis. Interactions between preexisting cirrhosis and ETV treatment were not observed ($P = 0.177$).

Cumulative HCC incidence rates by risk scores are compared between the two cohorts in Fig. 4A-G. Figure 4A,B shows the risk scores developed by Yang et al.[10] Figure 4C,D shows the risk scores developed by Yuen et al.[11] Figure 4E-G shows the risk scores developed by Wong et al.[12] All three risk score scales showed that ETV significantly reduced HCC incidence in patients with a higher risk (risk score $\geq 12$, $P = 0.006$; risk score $\geq 82$, $P = 0.002$; medium risk, $P = 0.062$; high risk, $P < 0.001$). Interactions between risk scores and ETV treatment were not observed (Yang et al.: $P = 0.713$, Yuen et al.: $P = 0.267$, Wong et al.: $P = 0.265$).

## Discussion

Our study suggests that long-term ETV therapy would significantly suppress the development of HCC in HBV-infected patients when compared with HBV-infected patients in the control group. The treatment effect was more prominent among patients at high risk of HCC than those at low risk.

**Table 2. Factors Associated with HCC Development as Determined by Cox Proportional Hazard Regression Analysis at 5-Year (Propensity Score Matched Cohort)**

| Variable | Univariate HR (95% CI) | P | Multivariate Adjusted HR (95% CI) | P |
|---|---|---|---|---|
| Age (per year) | 1.05 (1.02-1.07) | <0.001 | 1.06 (1.03-1.09) | <0.001 |
| Gender (M) | 2.81 (1.25-6.32) | 0.012 | | |
| Alcohol consumption (>200kg) | 2.71 (1.49-4.92) | 0.001 | 2.21 (1.18-4.16) | 0.013 |
| Cigarette smoking | 1.53 (0.84-2.80) | 0.164 | | |
| Preexisting cirrhosis | 12.0 (5.57-25.9) | <0.001 | 4.28 (1.88-9.73) | 0.001 |
| HBV genotype (C) | 2.73 (0.98-7.65) | 0.056 | | |
| HBeAg (positive) | 2.64 (1.41-4.94) | 0.002 | 2.26 (1.18-4.34) | 0.014 |
| HBV DNA (≥5.0 log copies/mL) | 4.66 (1.44-15.1) | 0.010 | | |
| ALT (≥45 IU/L) | 2.29 (1.10-4.77) | 0.027 | | |
| GGTP (≥50 IU/L) | 3.79 (2.02-7.09) | <0.001 | | |
| Total bilirubin (≥1.5 mg/dL) | 5.51 (2.87-10.6) | <0.001 | | |
| Serum albumin (<3.8 g/L) | 4.44 (2.42-8.14) | <0.001 | | |
| Platelet count ($<1.5\times10^5/mm^3$) | 14.8 (5.84-37.7) | <0.001 | 5.64 (2.13-15.0) | 0.001 |
| *Progression of cirrhosis within 5 years | 1.80 (0.25-13.2) | 0.562 | | |
| ETV treatment | 0.23 (0.09-0.55) | 0.001 | 0.37 (0.15-0.91) | 0.030 |

Asterisks (*) indicate time-dependent covariates.

†Adjusted for age, gender, alcohol, cigarette, cirrhosis, genotype, HBeAg, HBV DNA, ALT, albumin, GGTP, total bilirubin, and platelet counts

Abbreviations: ETV, entecavir; HR, hazard ratio; CI, confidence interval; HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase; GGTP, gamma glutamyltransferase.