A meta-analysis of the efficacy of anti-viral therapy was reported by Wong *et al.* who analyzed nine cohort studies with a total of 551 patients. The risk of HCC recurrence was reduced by 41% by the anti-viral treatment and they concluded that anti-viral therapy has potentially beneficial effects after the curative treatment of HBV-related HCC in terms of tumor recurrence, liver-related mortality and overall survival.[38]

Antiviral treatment for hepatitis C virus

Regarding the patients with HCV-related HCC, the long-term outcome was investigated including more than 300 patients after surgical resection and survival was shown to have improved in the period from 2000 to 2006 compared with the period from 1990 to 1999. This improvement in survival is attributable to antiviral therapy with interferon.[39] Viral mutations were investigated and postoperative recurrence of HCC was found to be associated with amino acid (aa) substitutions in the HCV core region, such as aa residue 91. The core mutations were shown to be associated with postoperative recurrence or survival in patients infected with HCV genotype 1b and treated by surgical resection.[40]

Interferon was first shown to be effective for the prevention of recurrence by a randomized study using interferon-β (IFN-β).[41] Thereafter, several reports of the preventive effects of interferon on the recurrence of HCC have been published; interferon did not affect overall prevention of HCC recurrence after resection[42] or RFA[42] but, if the HCV infection had been cured, interferon was effective for preventing the development of HCC and improving survival.[43] A meta-analysis has been reported and IFN-α treatment after curative treatment of primary tumors within the Milan criteria may be effective for the prevention of HCC recurrence, and a higher rate of sustained virological response (SVR) may be associated with a better preventive effect of IFN-α treatment on HCC recurrence.[44,45] To improve the SVR rate, peginterferon treatment after curative treatment of HCC was reported to be closely correlated with the prevention of recurrence.[46]

Branched chain amino-acid supplementation

The backgroud liver dysfunction has been shown to correlate with HCC recurrence, and even after curative resection with small HCC less than 2 cm, postoperative hepatic reserve influences HCC recurrence.[47] Recently, supplementation by branched chain amino acid (BCAA)-enrichment for patients with HCC after RFA has been shown to be effective for the improvement of serum albumin and quality of life[48] and a positive effect on serum albumin by BCAA was noted in patients with Child–Pugh B grade.[49]

Whether BCAA supplementation inhibits HCC recurrence or not is an important issue to be investigated, and recently, the mechanism whereby BCAA is effective for the prevention of the development of HCC has been precisely discussed in detail.[50]

Vitamin K2

In 2004, Habu *et al.*[51] reported that the incidence of development of HCC was reduced among cirrhotic women assigned to receive oral vitamin K2. The incidence of HCC recurrence was clearly shown to be lower than the control group in prospective studies by Mizuta *et al.*[52] and Kakizaki *et al.*;[53] however, conflicting results that HCC recurrence was not reduced by administration of vitamin K2 were reported by Hotta *et al.*[54] Therefore, a large scale multicenter prospective randomized study was conducted in Japan to investigate the preventive effects of vitamin K2 on HCC recurrence. The administration of vitamin K2 at 45 mg per day was not effective in preventing HCC recurrence and, moreover, in the patient group treated with a high dose of vitamin K2 of 90 mg per day, the incidence of HCC was rather higher than the control group.[55] Fortunately enough, severe adverse events were not observed.

Acyclic retinoid

Oral polyprenic acid of an acyclic retinoid was shown to inhibit the development of second primary HCC in a prospective randomized study with a median follow-up of 38 months, reported by Muto *et al.*[56] The overall survival of those receiving the acyclic retinoid was shown to be better than the control group.[57] A large scale multicenter, prospective randomized study has been carried out and oral administration of 600 mg per day of acyclic retinoid was shown to be preventive.[58]

Chemotherapeutic agent and molecular targeted agent

Although adjuvant chemotherapy has been considered for other solid malignancies with a high risk of recurrence, this is difficult in the case of HCC because few conventional chemotherapeutic agents are effective and hepatotoxicity can be of critical significance, because liver function often is already impaired. A randomized trial was performed with uracil-tegaful as postoperative adjuvant therapy, but did not improve the recurrence-free survival, and the overall survival appeared to be reduced.[59]

Whether sofarenib is effective for the prevention of recurrence is now under investigation, including in distinguished centers for treating HCC worldwide, in the so-called STORM trial.[60] A phase III study was conducted to determine whether sorafenib is effective for the prolongation of time to progression after transarterial chemoembolization (TACE), and sorafenib did not significantly prolong the time to progression in patients who responded to TACE.[61]

CONCLUSION

TO IMPROVE THE overall survival of patients with HCC, an important issue is to prevent intrahepatic recurrence. Many significant findings regarding gene expression in the liver and adjacent liver tissue, which relate to intrahepatic recurrence have been reported recently. Following such investigations, there is an urgent need for improved methods of prediction and prevention of intrahepatic recurrence of HCC.

REFERENCES

1 Kudo M, Izumi N, Kokudo N *et al.* HCC expert panel of Japan society of hepatology. *Dig Dis* 2011; **29**: 339–64.
2 Poon RT. Prevention of recurrence after resection of hepatocellular carcinoma: a daunting challenge. *Hepatology* 2011; **54**: 757–9.
3 Villanueva A, Hoshida Y, Toffanin S *et al.* New strategies in hepatocellular carcinoma: genomic prognostic markers. *Clin Cancer Res* 2010; **16**: 4688–94.
4 Murakata A, Tanaka S, Mogushi K *et al.* Gene expression signature of the gross morphology in hepatocellular carcinoma. *Ann Surg* 2011; **253**: 94–100.
5 Kimura O, Takahashi T, Ishii N *et al.* Characterization of the epithelial cell adhesion molecule (EpCAM)+ cell population in hepatocellular carcinoma cell lines. *Cancer Sci* 2010; **101**: 2145–55.
6 Yamashita T, Ji J, Brudhu A *et al.* EpCAM-positive hepatocellular carcinoma cells are tumor-initiating cells with stem/progenitor cell features. *Gastroenterology* 2009; **136**: 1012–24.
7 Komuta M, Spee B, Vander Borght S *et al.* Clinicopathological study on cholangiocellular carcinoma suggesting hepatic progenitor cell origin. *Hepatology* 2008; **47**: 1544–56.
8 Ziol M, Nault JC, Aout M *et al.* Intermediate hepatobiliary cells predict an increased risk of hepatocarcinogenesis in patients with hepatitis C virus – related cirrhosis. *Gastroenterology* 2010; **139**: 335–43.
9 Yang XR, Xu Y, Shi GM *et al.* Cytokeratin 10 and cytokeratin 19: predictive markers for poor prognosis in hepatocellular carcinoma patients after curative resection. *Clin Cancer Res* 2008; **14**: 3850–9.
10 Zhuang PY, Zhang JB, Zhu XD *et al.* Two pathologic types of hepatocellular carcinoma with lymph node metastasis with distinct prognosis on the basis of CK 19 expression in tumor. *Cancer* 2008; **112**: 2740–8.
11 Uenishi T, Kubo S, Yamamoto T *et al.* Cytokeratin 19 expression in hepatocellular carcinoma predicts early postoperative recurrence. *Cancer Sci* 2003; **94**: 851–7.
12 Durnez A, Verslype C, Nevens F *et al.* The clinicopathological and prognostic relevance of cytokeratin 7 and 19 expression in hepatocellular carcinoma. A possible progenitor cell origin. *Histopathology* 2006; **49**: 138–51.
13 Tsuchiya K, Komuta M, Yasui Y *et al.* Expression of keratin 19 is related to high recurrence of hepatocellular carcinoma after radiofrequency ablation. *Oncology* 2011; **80**: 278–88.
14 Sasaki A, Kamiyama T, Yokoo H *et al.* Cytoplasmic expression of CD133 is an important risk factor for overall survival in hepatocellular carcinoma. *Oncol Res* 2010; **24**: 537–46.
15 Vasuri F, Golifieri R, Fiorentino M *et al.* OATP 1B1/1B3 expression in hepatocellular carcinomas treated with orthotopic liver transplantation. *Virchows Arch* 2011; **459**: 141–6.
16 Nakamura Y, Mizuguchi T, Kawamoto M *et al.* Cluster analysis of indicators of liver functional and preoperative low branched-chain amino acid tyrosine ration indicate a high risk of early recurrence in analysis of 165 hepatocellular carcinoma patients after initial hepatectomy. *Surgery* 2011; **150**: 250–62.
17 Hoshida Y, Villanueva A, Kobayashi M *et al.* Gene expression in fixed tissues and outcome in hepatocellular carcinoma. *N Engl J Med* 2008; **359**: 1995–2004.
18 Villanueva A, Hoshida Y, Battiston C *et al.* Combining clinical, pathology, and gene expression data predict recurrence of hepatocellular carcinoma. *Gastroenterology* 2011; **140**: 1501–12.
19 Teufel A, Marquardt JU, Galle PR. Novel insight in the genetics of HCC recurrence and advances in transcriptomic data integration. *J Hepatol* 2011 E pub July 23.
20 Amaoka N, Osada S, Kanematsu M *et al.* Clinicopathological features of hepatocellular carcinoma evaluated by vascular endothelial growth factor expression. *J Gastroenterol Hepatol* 2007; **22**: 2202–7.
21 Toyoda H, Kumada T, Kaneoka Y *et al.* Prognostic value of pretreatment levels of tumor markers for hepatocellular carcinoma on survival after curative treatment of patients with HCC. *J Hepatol* 2008; **49**: 223–32.
22 Nobuoka D, Kato Y, Gotohda N *et al.* Postoperative serum alpha-fetoprotein level is a useful predictor of recurrence after hepatectomy for hepatocellular carcinoma. *Oncol Rep* 2010; **24**: 521–8.
23 Yamamoto K, Imamura H, Matsuyama Y *et al.* Significance of alpha-fetoprotein and des-gamma-carboxy prothrombin in patients with hepatocellular carcinoma undergoing hepatectomy *Ann Surg Oncol* 2009; **16**: 2795–804.

24 Zhang XF, Lai EC, Kang XY *et al.* Lens culinaris agglutinin-reactive fraction of alpha-fetoprotein as a marker of prognosis and a monitor of recurrence of hepatocellular carcinoma after curative liver resection. *Ann Surg Oncol* 2011; 18: 2218–23.
25 Beppu T, Sugimoto K, Shiraki K *et al.* Clinical significance of tumor markers in detection of recurrent hepatocellular carcinoma after radiofrequency ablation. *Int J Mol Med* 2010; 26: 425–33.
26 Morimoto M, Numata K, Nozaki A *et al.* Novel lens culinaris agglutinin-reactive fraction of α-fetoprotein: a biomarker of hepatocellular carcinoma recurrence in patients with low α-fetoprotein concentrations. *Int J Clin Oncol* 2011 E pub Aug 26.
27 Kobayashi M, Hosaka T, Ikeda K *et al.* Highly sensitive AFP-L3% assay is useful for predicting recurrence of hepatocellular carcinoma after curative treatment pre-and postoperatively. *Hepatol Res* 2011 E pub Aug 26.
28 Komuta T, Mizukoshi E, Kita Y *et al.* Impact of diabetes on recurrence of hepatocellular carcinoma after surgical treatment in patients with viral hepatitis. *Am J Gastroenterol* 2007; 102: 1939–46.
29 Imai K, Takai K, Nishigaki Y *et al.* Insulin resistance raises the risk for recurrence of stage 1 hepatocellular carcinoma after curative ablation in hepatitis C virus-positive patients: a prospective, case series study. *Hepatol Res* 2010; 40: 376–82.
30 Huo TI, Wu JC, Lui WY *et al.* Differential mechanism and prognostic impact of diabetes mellitus on patients with hepatocellular carcinoma undergoing surgical treatment. *Am J Gastroenterol* 2004; 99: 1479–87.
31 Chen WT, Macatula TC, Lin CC, Lin CJ, Lin SM. Diabetes may not affect outcomes in hepatocellular carcinoma after radio-frequency ablation. *Hepatogastroenterology* 2011; 58: 551–7.
32 Wang WM, Xu Y, Yang XR, Wang YH, Sun HX, Fan J. Prognostic role of diabetes mellitus in hepatocellular carcinoma patients after curative treatments: a meta-analysis. *Hepatobiliary Pancreat Dis Int* 2011; 10: 346–55.
33 Ohki T, Tateishi R, Shiina S *et al.* Visceral fat accumulation is an independent risk factor for hepatocellular carcinoma recurrence after curative treatment in patients with suspected NASH. *Gut* 2009; 58: 839–44.
34 Tokushige K, Hashimoto E, Yatsuji S *et al.* Prospective study of hepatocellular carcinoma in non-alcoholic steatohepatitis in comparison with hepatocellular carcinoma caused by chronic hepatitis C. *J Gastroenterol* 2101; 45: 960–7.
35 Goto T, Yoshida H, Tateishi R *et al.* Influence of serum HBVDNA load on recurrence of hepatocellular carcinoma after treatment with percutaneous radiofrequency ablation. *Hepatol Int* 2011 E pub Jan 25.
36 Yoshida H, Goto E, Sato T *et al.* Safety and efficacy of lamivudine after radiofrequency ablation in patients with hepatitis B virus-related hepatocellular carcinoma. *Hepatol Int* 2008; 2: 89–94.
37 Chan AC, Chok KS, Yuen WK, Chan RT, Lo CM, Fan ST. Impact of antiviral therapy on the survival of patients after major hepatectomy for hepatitis B virus-related hepatocellular carcinoma. *Arch Surg* 2011; 146: 675–81.
38 Wong JS, Wong GL, Tsoi KK *et al.* Meta-analysis: the efficacy of anti-viral therapy in prevention of recurrence after curative treatment of chronic hepatitis B-related hepatocellular carcinoma. *Aliment Pharmacol Ther* 2011; 33: 1104–12.
39 Shirabe K, Takeishi K, Taketomi A, Uchiyama H, Kayashima H, Maehara Y. Improvement of long-term outcomes in hepatitis C virus antibody-positive patients with hepatocellular carcinoma after hepatectomy in the modern era. *World J Surg* 2011; 35: 1072–84.
40 Toyoda H, Kumada T, Kaneoka Y, Maeda A. Amino acid substitutions in the hepatitis C virus core region are associated with postoperative recurrence and survival of patients with genotype 1b-associated hepatocellular carcinoma. *Ann Surg* 2011; 254: 326–32.
41 Ikeda K, Arase Y, Saitoh S *et al.* Interferon beta prevents recurrence of hepatocellular carcinoma after complete resection or ablation of the primary tumor- A prospective randomized study of hepatitis C virus-related cancer. *Hepatology* 2000; 32: 228–32.
42 Mazzaferro V, Romito R, Schiavo M *et al.* HCC Italian Task Force. Prevention of hepatocellular carcinoma recurrence with alpha-interferon after liver resection in HCV cirrhosis. *Hepatology* 2006; 44: 1543–54.
43 Shiratori Y, Ito Y, Yokosuka O *et al.* Tokyo-Chiba Hepatitis Research Group. Antiviral therapy for cirrhotic hepatitis C: association with hepatocellular carcinoma development and improved survival. *Ann Intern Med* 2005; 18: 105–14.
44 Miyake Y, Takaki A, Iwasaki Y, Yamamoto K. Meta-analysis: interferon-alpha prevents the recurrence after curative treatment of hepatitis C virus-related hepatocellular carcinoma. *J Viral Hepat* 2010; 17: 287–92.
45 Shen YC, Hsu C, Chen LT, Cheng CC, Hu FC, Cheng AL. Adjuvant interferon therapy after curative therapy for hepatocellular carcinoma (HCC): a meta-regression approach. *J Hepatol* 2010; 52: 889–94.
46 Hagihara H, Nouso K, Kobayashi Y *et al.* Effect of pegylated interferon therapy on intrahepatic recurrence after curative treatment of hepatitis C virus-related hepatocellular carcinoma. *Int J Clin Oncol* 2011; 16: 210–20.
47 Kaibori M, Ishizaki M, Saito T, Matsui K, Kwon AH, Kamiyama Y. Risk factors and outcome of early recurrence after resection of small hepatocellular carcinomas. *Am J Surg* 2009; 198: 39–45.
48 Kuroda H, Ushio A, Miyamoto Y *et al.* Effects of branched-chain amino acid-enriched nutrient for patients with hepatocellular carcinoma following radiofrequency ablation: a one-year prospective trial. *J Gastroenterol Hepatol* 2010; 25: 1550–5.

49 Ishikawa T, Michitaka I, Kamimura H *et al.* Oral branched-chain amino acids administration improves impaired liver dysfunction after radiofrequency ablation therapy for hepatocellular carcinoma. *Hepatogastroneterology* 2009; **56**: 1491–5.
50 Kawaguchi T, Izumi N, Charlton MR, Sata M. Branched-chain amino acids as pharmacological nutrients in chronic liver disease. *Hepatology* 2011; 54: 1063–70.
51 Habu D, Shiomi S, Tamori A *et al.* Role of vitamin Ks in the development of hepatocellular carcinoma in women with viral cirrhosis of the liver. *JAMA* 2004; **292**: 358–61.
52 Mizuta T, Ozaki I, Eguchi Y *et al.* The effect of menatetteone, a vitamin K2 analogue, on disease recurrence and survival in patients with hepatocellular carcinoma after curative treatment. *Cancer* 2006; **106**: 867–72.
53 Kakizaki S, Sohara N, Sato K *et al.* Preventive effects of vitamin K on recurrence disease in patients with hepatocellular carcinoma arising from hepatitis C viral infection. *J Gastroenterol Hepatol* 2007; **22**: 518–22.
54 Hotta N, Ayada M, Sato K *et al.* Effect of vitamin K2 on the recurrence in patients with hepatocellular carcinoma. *Hepatogastroenterology* 2007; 54: 2073–77.
55 Yoshida H, Shiratori Y, Kudo M *et al.* Effect of vitamin K2 on the recurrence of hepatocellular carcinoma. *Hepatology* 2011; 54: 532–40.
56 Muto Y, Moriwaki H, Nonomiya M *et al.* Prevention of second primary tumors by an acyclic retionid, polyprenoic acid, in patients with hepatocellular carcinoma. Hepatoma Prevention Study Group. *N Engl J Med* 1996; **13**: 1561–7.
57 Muto Y, Moriwaki H, Saito A. Prevention of second primary tumors by an acyclic retinoid in patients with hepatocellular carcinoma. *N Engl J Med* 1999; **340**: 1046–7.
58 Okita K, Matsui O, Kumada H *et al.* Effect of peretinoin on recurrence of hepatocellular carcinoma (HCC): results of a phase II/III randomized placebo-controlled trial. *Am Soc Clin Oncol* 2011. ASCO Annual Meeting. Abstract No. 4024.
59 Hasegawa K, Takayama T, Ijichi M *et al.* Uracil-tegaful as an adjuvant for hepatocellular carcinoma: a randomized trial. *Hepatology* 2006; 44: 891–95.
60 Prints C. Clinical trials of note. Sorafenib as adjuvant treatment for the prevention of disease recurrence in patients with hepatocellular carcinoma (HCC) (STORM). *Cancer* 2009; **115**: 4646.
61 Kudo M, Imanaka K, Chida N *et al.* Phase III study of sorafenib after transarterial chemoembolization in Japanese and Korean patients with unresectable hepatocellular carcinoma. *Eur J Cancer* 2011; 47: 2117–27.

Hepatitis B Virus e Antigen Physically Associates With Receptor-Interacting Serine/Threonine Protein Kinase 2 and Regulates *IL-6* Gene Expression

Shuang Wu,[1] Tatsuo Kanda,[1] Fumio Imazeki,[1] Shingo Nakamoto,[1,2] Takeshi Tanaka,[1,3] Makoto Arai,[1] Thierry Roger,[5] Hiroshi Shirasawa,[2] Fumio Nomura,[4] and Osamu Yokosuka[1]

[1]Department of Medicine and Clinical Oncology, [2]Department of Molecular Virology, [3]Department of Environment Biochemistry, and [4]Department of Molecular Diagnosis, Graduate School of Medicine, Chiba University, Japan; and [5]Infectious Diseases Service, Department of Medicine, Centre Hospitalier Universitaire Vaudois and University of Lausanne, Lausanne, Switzerland

We previously reported that hepatitis B virus (HBV) e antigen (HBeAg) inhibits production of interleukin 6 by suppressing NF-κB activation. NF-κB is known to be activated through receptor-interacting serine/threonine protein kinase 2 (RIPK2), and we examined the mechanisms of interleukin 6 regulation by HBeAg. HBeAg inhibits RIPK2 expression and interacts with RIPK2, which may represent 2 mechanisms through which HBeAg blocks nucleotide-binding oligomerization domain-containing protein 1 ligand–induced NF-κB activation in HepG2 cells. Our findings identified novel molecular mechanisms whereby HBeAg modulates intracellular signaling pathways by targeting RIPK2, supporting the concept that HBeAg could impair both innate and adaptive immune responses to promote chronic HBV infection.

Hepatitis B virus (HBV) nucleoprotein exists in 2 forms [1, 2]. Nucleocapsid, designated HBV core antigen (HBcAg), is an intracellular, 21-kDa protein that self-assembles into particles that encapsidate viral genome and polymerase and is essential for function and maturation of virion. HBV also secretes a nonparticle second form of the nucleoprotein, designated precore or HBV e antigen (HBeAg) [1, 2]. Precore and core proteins are translated from 2 RNA species that have different 5′ initiation sites. Precore messenger RNA (mRNA) encodes a hydrophobic signal sequence that directs precore protein to the endoplasmic reticulum, where it undergoes N- and C-terminal cleavage within the secretory pathway and is secreted as an 18-kDa monomeric protein [3–5].

Nucleotide-binding oligomerization domain–containing protein 1 (NOD1) and NOD2 are cytosolic pattern-recognition receptors involved in the sensing of bacterial peptidoglycan subcomponents [6]. NOD1 and NOD2 stimulation activates NF-κB through receptor-interacting serine/threonine protein kinase 2 (RIPK2; also known as RIP2, RICK, or CARDIAK), a caspase-recruitment domain-containing kinase. RIPK2 is also involved in Toll-like receptor (TLR)–signaling pathway and plays an important role in the production of inflammatory cytokines through NF-κB activation [6, 7].

We previously reported that HBeAg inhibits the production of interleukin 6 (IL-6) through suppression of NF-κB activation [4]. In the present study, we investigated the molecular mechanism of HBeAg functions for the requirement of RIPK2 in NF-κB transcriptional regulation.

METHODS

Cell Culture and Plasmids

HepG2, Huh7, HT1080, COS7, and HEK293T cells were used in the present study. Stable cell lines were obtained as previously described [4]. Briefly, HepG2, Huh7, and HT1080 were transfected with pCXN2-HBeAg(+) or pCXN2-HBeAg(−) in Effectene (Qiagen). After G418 screening, HBeAg-positive and -negative HepG2/Huh7/HT1080 cell lines were collected for further analysis [4]. The plasmid pCXN2-HBeAg(+), which can produce both HBeAg and core peptides, and the plasmid pCXN2-HBeAg(−), which can produce only core peptides, were obtained as described previously [4]. pNF-κB–luc, which expresses luciferase upon promoter activation by NF-κB, was purchased from Stratagene [4]. pGFP–human RIPK2 (kindly provided by Prof John C. Reed, Sanford-Burnham Institute for Medical Research) can express GFP-human $RIP2^{WT}$ [8].

HepG2 cells were transfected with plasmid control–small hairpin RNA (shRNA) or with RIPK2-shRNA (Santa Cruz). After puromycin screening, individual colonies were picked up and examined for expression of endogenous RIPK2, and clones HepG2-shC and HepG2-shRIPK2-3 were selected for subsequent studies.

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Correspondence: Tatsuo Kanda, MD, PhD, Department of Medicine and Clinical Oncology, Chiba University, Graduate School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan (kandat-cib@umin.ac.jp).

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Luciferase Assays and Treatment of Cells With NOD Ligands

Around 1.0×10^5 HepG2 and Huh7 cells were plated in 6-well plates (Iwaki Glass, Tokyo, Japan) for 24 hours and transfected with 0.4 μg of pNF-κB–luc. For luciferase assay of NF-κB activation, cells were treated for 4 hours with or without NOD1 ligand (C12-iEDAP, 2.5 μg/mL) and NOD2 ligand (muramyl dipeptide [MDP], 10 μg/mL) (InvivoGen) at 44 hours after transfection [9]. After 48 hours, cells were lysed with reporter lysis buffer (Promega), and luciferase activity was determined as described previously [4].

RNA Extraction, Complementary DNA (cDNA) Synthesis, Real-Time Polymerase Chain Reaction (PCR) Analysis, and PCR Array

Total RNA was isolated by RNeasy Mini Kit (Qiagen). A total of 5 μg of RNA was reverse transcribed using the First Strand cDNA Synthesis Kit (Qiagen) [4]. Quantitative amplification of cDNA was monitored with SYBR Green by real-time PCR in a 7300 Real-Time PCR system (Applied Biosystems). Gene expression profiling of 84 TLR-related genes was performed using RT^2 profiler PCR arrays (Qiagen) in accordance with the manufacturer's instructions [4].

Gene expression was normalized to 2 internal controls (GAPDH and/or β-actin) to determine the fold-change in gene expression between the test sample (HBeAg-positive HepG2/Huh7/HT1080) and the control sample (HBeAg-negative HepG2/Huh7/HT1080) by the 2^{-ddCT} (comparative cycle threshold) method [4]. Three sets of real-time PCR arrays were performed. Some results of HepG2 cells were previously reported [4].

Coimmunoprecipitation

Cells were cotransfected with 2.5 μg pCXN2-HBeAg(+) or 2.5 μg pCXN2-HBeAg(−), as well as with 2.5 μg pGFP–human RIPK2, and cell lysates were prepared after 48 hours, using lysis buffer containing a cocktail of protease inhibitors. Cell lysates were incubated with anti-GFP rabbit polyclonal antibody (Santa Cruz) or anti-HBe mouse monoclonal antibody (Institute of Immunology, Tokyo, Japan) for 3 hours at 4°C, followed by overnight incubation with protein G–Sepharose beads (Santa Cruz). Immunoprecipitates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electroblotted onto a nitrocellulose membrane. Immunoblotting was performed by incubating the membrane for 1 hour with anti-HBe antibody. Proteins were detected by enhanced chemiluminescence (GE Healthcare), using an image analyzer (LAS-4000, Fuji Film). The membrane was reprobed with a monoclonal antibody to GFP or RIPK2 (Cell Signaling).

Transfection of pGFP–Human RIPK2 and Confocal Microscopy

Formaldehyde (3.7%)–fixed cells were incubated with anti-HBe antibody and stained with fluorochrome-conjugated secondary antibody (Alexa Fluor 555 conjugate, Cell Signaling). Cells were mounted for confocal microscopy (ECLIPSE TE 2000-U, Nikon). Whenever necessary, images were merged digitally to monitor colocalization. Cotransfection of 0.1 μg pCXN2-HBeAg(+) or 0.1 μg pCXN2-HBeAg(−) with 0.3 μg pGFP–human RIPK2 into the cells was performed. After 48 hours, intracellular localization of RIPK2 was visualized by confocal microscopy.

Enzyme-Linked Immunosorbent Assay (ELISA) for IL-6

Cell culture fluid was analyzed for IL-6 by ELISA (KOMA-BIOTECH, Seoul, Korea), in accordance with the manufacturer's protocol [4].

Small Interfering RNA (siRNA) Transfection and Wound-Healing Assay

Control siRNA (siC) and siRNA specific for RIPK2 (siRIPK2) were purchased from Thermo Fisher Scientific. Cells were transfected with siRNA by electroporation. After 48 hours, cells were treated with 10 ng/mL tumor necrosis factor α (TNF-α) (Wako Pure Chemical, Osaka, Japan), while the wound-healing (ie, scratch) assay was performed using a p-200 pipette tip to induce RIPK2 [10]. Up to 12 hours after scratching, the cells were observed by microscopy. Cell migration was measured using Scion Images (SAS). Migration by siC-transfected cells was set at 1.

Statistical Analysis

Results are expressed as mean values ± SD. The Student *t* test was used to determine statistical significance.

RESULTS

HBeAg Downregulates RIPK2 Expression

To explore the effect of HBeAg on TLR-related gene expression, we generated HepG2, Huh7, and HT1080 cell lines that stably expressed HBV core region with or without precore region. HT1080, a primate fibrosarcoma cell line, is useful for the study of interferon signaling. HBeAg and HBV core–related antigen (HBcrAg) levels of these cell lines demonstrated that expression of HBV core region without HBV precore region did not allow HBeAg secretion by cells (data are cited elsewhere [4] or not shown). First, we performed real-time RT-PCR analysis of these cell lines, using focused gene arrays (Figure 1*A*). We observed that, in 3 cell lines, 5 genes (*RIPK2, TLR9, TNF, CD180,* and *IL1A*) were downregulated ≥1.3-fold in HBeAg-positive cells than in HBeAg-negative cells. We chose to focus our investigation on RIPK2 because HBeAg inhibits the production of IL-6 through the suppression of NF-κB activation [4], and NF-κB is known to be activated through RIPK2 [4]. RIPK2 expression was >100-, 1.41-, and 1.45-fold lower in HBeAg-positive HepG2, Huh7, and HT1080 cells, respectively, compared with their HBeAg-negative counterparts

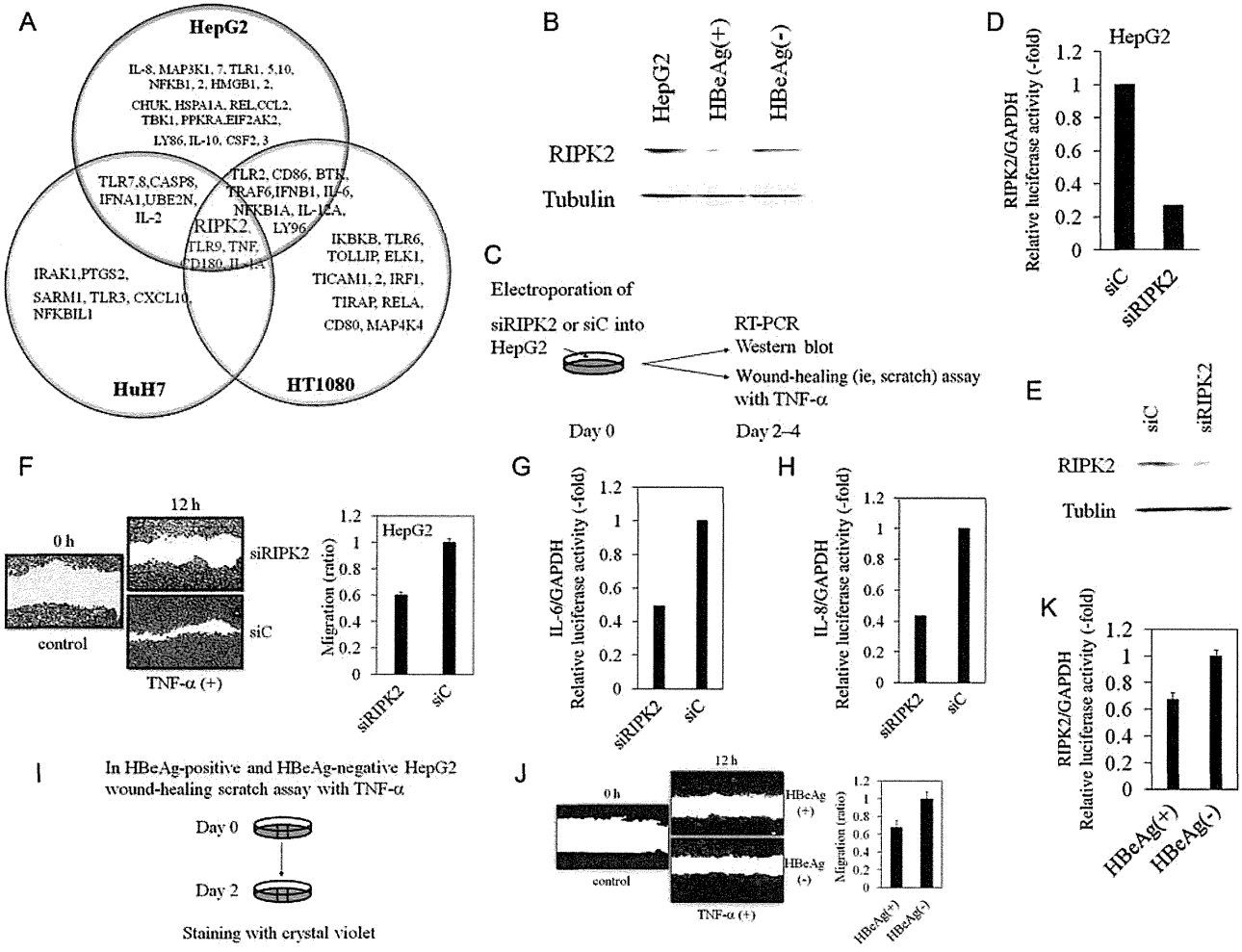

Figure 1. Receptor-interacting serine/threonine protein kinase 2 (RIPK2) expression is downregulated by hepatitis B virus e antigen (HBeAg), and knockdown of RIPK2 and HBeAg impairs hepatic wound repair. *A*, Venn diagram representing Toll-like receptor (TLR)–related genes downregulated ≥1.3-fold in HBeAg-positive HepG2/Huh7/HT1080 cells, compared with HBeAg-negative cells. Cellular RNA was extracted and analyzed with focused array, quantifying 84 genes. Gene expression levels were normalized to actin and GAPDH expression levels. *B*, HBeAg downregulates RIPK2 expression in HepG2 cells. Western blot analysis of RIPK2 and tubulin expression in HepG2, HBeAg(+) HepG2, and HBeAg(−) HepG2. *C*, Experimental protocol of electroporation of control (siC) and RIPK2 (siRIPK2) small interfering RNA (siRNA) into HepG2 cells. *D* and *E*, Real-time polymerase chain reaction (PCR; *D*) and Western blot (*E*) analyses of RIPK2 expression in siC- or siRIPK2-expressing HepG2 cells. RIPK2 messenger RNA (mRNA) levels were normalized to GAPDH levels. *F–H*, siC- and siRIPK2-transfected HepG2 cells were scratch wounded and incubated with 10 ng/mL tumor necrosis factor α (TNF-α), and cell migration was analyzed after 12 hours and quantified using Scion Image (*F*). Interleukin 6 (IL-6; *G*) and interleukin 8 (IL-8; *H*) mRNA expression are quantified by real-time reverse transcription–PCR (RT-PCR) and expressed relative to GAPDH mRNA expression. *I*, Protocol of wound-healing (ie, scratch) assay in HBeAg(+) and HBeAg(−) HepG2 cells. TNF-α was used at 10 ng/mL. *J*, Cell migration was analyzed using Scion Image. *K*, RIPK2 mRNA expression was quantified by real-time RT-PCR and expressed relative to GAPDH mRNA expression. Primers specific for RIPK2 were 5′-AGACAC-TACTGACATCCAAG-3′ (sense) and 5′-CACAAGTATTTCCGGGTAAG-3′ (antisense), and primers for other genes were as described previously [4]. Data are mean values ± SD of 3 independent experiments.

(Figure 1*A*). Western blot analyses confirmed lower levels of RIPK2 in HBeAg-positive HepG2 than in HBe-negative HepG2 or parental HepG2 (Figure 1*B*). The fact that RIPK2 is one of the targets for the ubiquitin proteasome system and uses a ubiquitin-dependent mechanism to achieve NF-κB activation [6] might be a reason for the differences between RIPK2 mRNA and protein expression status. We also observed lower levels of RIPK2 mRNA expression (0.18-fold) in HepG2.2.15 cells, which secrete complete HBV virion and HBeAg, compared with expression in HepG2 cells (data not shown).

Knockdown of RIPK2 and HBeAg Impairs Hepatic Cell Migration

It has recently been reported that RIPK2 expression is induced by TNF-α plus scratch wounding in keratinocytes [10]. Therefore, we next examined whether RIPK2 affected hepatic

wound healing in the presence of TNF-α in vitro (Figure 1*C*). As shown in Figure 1*D* and 1E, RIPK2 mRNA and protein expression were efficiently decreased in HepG2 cells transfected with RIPK2 siRNA (siRIPK2), but not control (siC). RIPK2 silencing reduced hepatic wound closure 1.8-fold, which was associated with a 2-fold decrease in IL-6 production, known to be an important cytokine for the regeneration of liver [11], and a 2.3-fold decrease in interleukin 8 production (Figure 1*F–H*). Importantly, RIPK2 silencing did not affect cell viability (data not shown).

Given that HBeAg downregulates RIPK2 expression (Figure 1*A* and 1*B*), we examined whether HBeAg has an effect on hepatic wound healing in the presence of TNF-α (Figure 1*I*). As expected, we observed that both cell migration

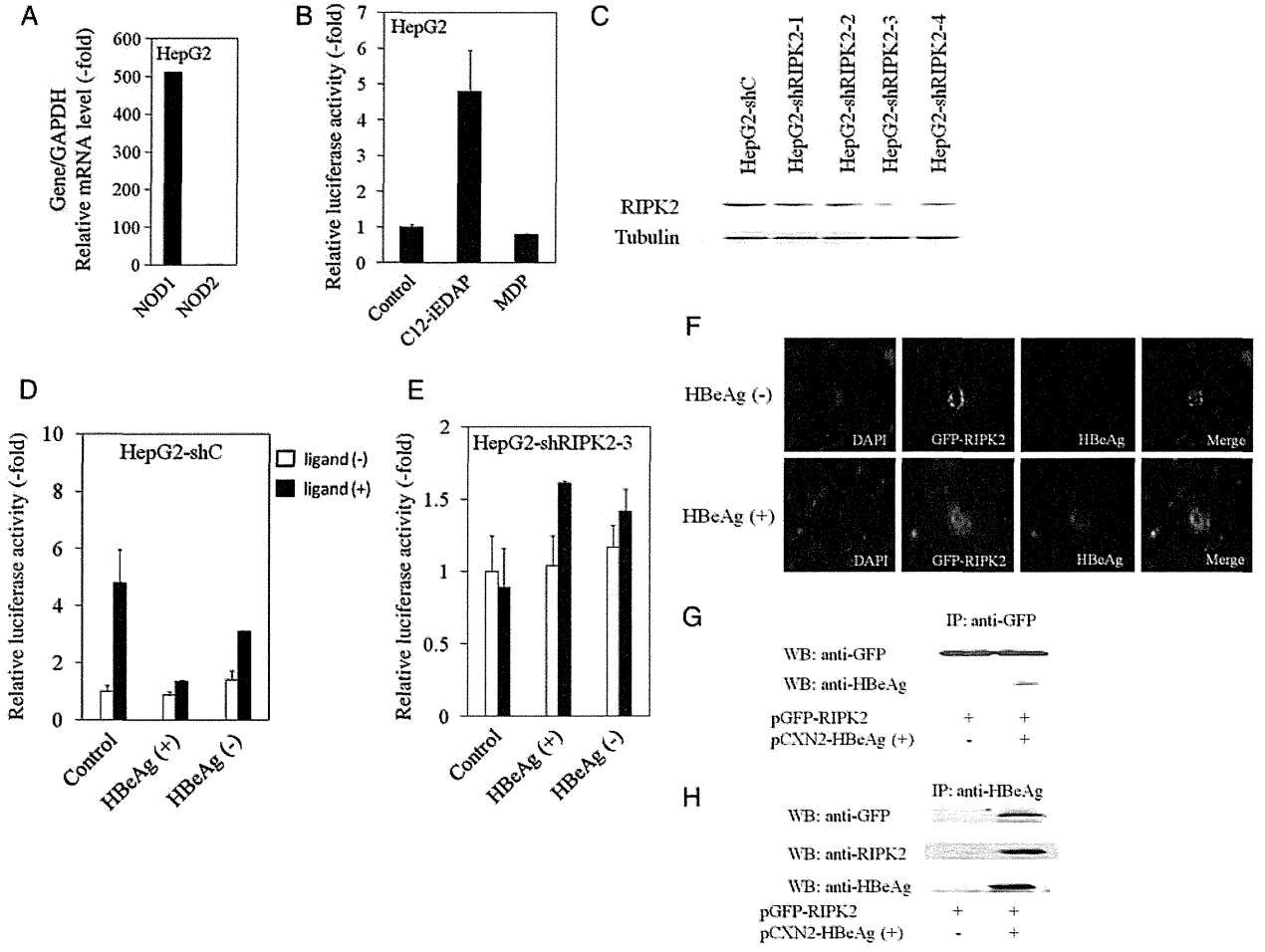

Figure 2. The nucleotide-binding oligomerization domain–containing protein 1 (NOD1) ligand C12-iEDAP induces NF-κB activation, knockdown of receptor-interacting serine/threonine protein kinase 2 (RIPK2) inhibits NOD1 ligand–induced NF-κB activation in HepG2 cells, and hepatitis B virus e antigen (HBeAg) interacts with RIPK2. *A*, Real-time reverse transcription–polymerase chain reaction analysis of NOD1 and NOD2 messenger RNA expression in HepG2. NOD1 and NOD2 expression levels were normalized to GAPDH expression levels. *B*, NF-κB–driven luciferase activity in HepG2 cells stimulated with the NOD1 ligand C12-iEDAP or the NOD2 ligand muramyl dipeptide (MDP) in HepG2. *C*, Western blot analysis of RIPK2 and tubulin expression in HepG2 cells stably transfected with control small hairpin RNA (shRNA; HepG2-shC) or with RIPK2 shRNA (HepG2–shRIPK2-1/2-4) expressing plasmids. *D* and *E*, HepG2-shC (*D*) and HepG2–shRIPK2-3 (*E*) cell lines were transiently transfected with pCXN2, pCXN2-HBeAg(+), or pCXN2-HBeAg(−) plasmids together with pNF-κB–luc. Cells were treated for 4 hours, with or without NOD1 ligand C12-iEDAP (2.5 μg/mL), and luciferase activity was determined. Primers specific for NOD1 (sense primer: 5′-ACTACCTCAAGCTGACCTAC-3′; antisense primer: 5′-CTGGTTTACGCTGAGTCTG-3′), for NOD2 (sense primer: 5′-CCTTGCATGCAGGCAGAAC-3′; antisense primer: 5′-TCTGTTGCCCCAGAATCCC-3′), and for other genes as described previously were purchased from Sigma [4]. *F*, HBeAg specifically colocalizes with RIPK2. COS7 cells were transiently cotransfected with 0.1 μg pCXN2-HBeAg(+) or pCXN2-HBeAg(−) together with 0.3 μg pGFP–human RIPK2. HBeAg was revealed with anti-HBeAg primary antibody and Alexa-Fluor-548 secondary antibody. *G* and *H*, HEK293T cells were transiently transfected with or without GFP-RIPK2 and HBeAg-expressing plasmids. Cellular extracts were precleared with protein G–Sepharose, and interacting complexes were immunoprecipitated (IP) with either anti-GFP (*G*) or anti-HBeAg (*H*) antibodies. Immunocomplexes were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and proteins were visualized by immunoblotting (WB) with indicated antibodies. Results are representative of 3 independent experiments.

and RIPK2 mRNA expression were reduced in HBeAg-positive HepG2 cells, compared with HBeAg-negative cells (1.5-fold decrease; Figure 1*J* and 1*K*). These results suggest that HBeAg impairs hepatic cell migration–dependent RIPK2 expression. Among NF-κB–targeting genes, expression of vimentin mRNA was impaired in HepG2-shRIP2 and in HBeAg-positive HepG2 (data not shown), and vimentin might be one of the candidates for impairment of their migrations [12].

RIPK2 Plays an Important Role in NF-κB Activation Induced by NOD1 Ligand, and HBeAg Blocks This Pathway

HepG2 cells express NOD1 but not NOD2 at the mRNA level (Figure 2*A*). In agreement with this finding, NF-κB was activated in HepG2 cells exposed to NOD1 ligand C12-iEDAP (level of activation, 4.8-fold, compared with untreated control) but not in those exposed to NOD2 ligand MDP (Figure 2*B*). As for Huh7 cells, activation of NF-κB was not detected following exposure to C12-iEDAP or MDP (data not shown). These results suggest that C12-iEDAP triggered NF-κB activation through NOD1 in HepG2 cells, which is consistent with findings from a previous study [9].

We examined whether knockdown of RIPK2 has an effect on NOD1-induced NF-κB activation in HepG2 cells. First, we established HepG2 cell lines that constitutively expressed RIPK2-shRNA (HepG2-shRIPK2-1/2-4) or control-shRNA (HepG2-shC) (Figure 2*C*). The HepG2–shRIPK2-3 cell line, which expresses the lowest levels of RIPK2, and the HepG2-shC cell line were treated for 4 hours, with or without C12-iE-DAP, before measurement by the NF-κB–driven luciferase assay (Figure 2*D* and 2*E*). C12-iEDAP triggered NF-κB activation in HepG2-shC (Figure 2*D*) but not in HepG2–shRIPK2-3 (Figure 2*E*), indicating that RIPK2 plays an important role in NF-κB activation induced through NOD1 triggering.

To assess the influence of HBeAg in that pathway, we measured NOD1-mediated NF-κB activity in HepG2-shC and HepG2–shRIPK2-3 cell lines transiently transfected with HBeAg-expressing plasmids. As shown in Figure 2*D*, HBeAg expression in HepG2-shC abolished C12-iEDAP–induced NF-κB activation.

HBeAg Interacts With RIPK2 and Colocalizes With RIPK2

RIPK2 mediates activation of transcription factors, such as NF-κB, following its activation, which is initiated by membrane-bound or intracytosolic receptors, such as TLR, NOD1, and NOD2 [7, 13, 14]. Confocal microscopy analysis of cells transfected with GFP-RIPK2 revealed subcellular localization of RIPK2 (data not shown). To compare the localization of RIPK2 with that of HBeAg, cells were cotransfected with pGFP–human RIPK2 with pCXN2-HBeAg(+) or pCXN2-HBeAg(−). After 48 hours, cells were stained with mouse monoclonal anti-HBe antibody. Confocal microscopy suggested subcellular colocalization of RIPK2 with HBeAg (Figure 2*F*). Reinforcing this assumption, GFP-RIPK2 coimmunoprecipitated with HBeAg (Figure 2*G*), while HBeAg coimmunoprecipitated with RIPK2 (Figure 2*H*) in transiently transfected cells with RIPK2- and HBeAg-expressing plasmids.

DISCUSSION

In the present study, we have shown the expression of NOD1 and NOD1 ligand–induced NF-κB activation in HepG2 cells and that RIPK2 plays an important role in NOD1 ligand–induced NF-κB activation. NF-κB activation plays an essential role in the production of inflammatory cytokines such as IL-6, which HBeAg could suppress in hepatocytes [4]. We have also shown that HBeAg inhibits RIPK2 expression and interacts with RIPK2, which may represent 2 mechanisms through which HBeAg blocks NOD1 ligand–induced NF-κB activation, thus contributing to the pathogenesis of chronic HBV infection and establishing viral persistence, although further studies including clinical situations might be needed.

HBeAg can be secreted by hepatocytes. Yet, it has been reported that as much as 80% of the precore protein p22 remains localized to the cytoplasm rather than undergoing further cleavage that allows its secretion as mature HBeAg [15]. Our present study showed subcellular colocalization of HBeAg with RIPK2 (Figure 2*F*). In addition to HBeAg protein in cell culture medium, we observed similar inhibition of NF-κB activation (data not shown).

Overall, we provided a novel molecular mechanism whereby HBeAg modulates innate immune signal-transduction pathways through RIPK2. Elsewhere, it was also reported that HBeAg impairs cytotoxic T-lymphocyte activity [2]. HBeAg inhibits RIPK2 expression and interacts with RIPK2, decreasing NF-κB activation and inflammatory cytokine production in hepatocytes. Taken together, HBeAg could impair both innate and adaptive immune responses to promote chronic HBV infection.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Ait-Goughoulte M, Lucifora J, Zoulim F, Durantel D. Innate antiviral immune responses to hepatitis B virus. Viruses **2010**; 2:1394–410.
2. Chen M, Sallberg M, Hughes J, et al. Immune tolerance split between hepatitis B virus precore and core proteins. J Virol **2005**; 79:3016–27.
3. Ou JH, Laub O, Rutter WJ. Hepatitis B virus gene function: the precore region targets the core antigen to cellular membranes and causes the secretion of the e antigen. Proc Natl Acad Sci U S A **1986**; 83:1578–82.
4. Wu S, Kanda T, Imazeki F, et al. Hepatitis B virus e antigen down-regulates cytokine production in human hepatoma cell lines. Viral Immunol **2010**; 23:467–76.
5. Lang T, Lo C, Skinner N, Locarnini S, Visvanathan K, Mansell A. The hepatitis B e antigen (HBeAg) targets and suppresses activation of the Toll-like receptor signaling pathway. J Hepatol **2011**; 55:762–9.
6. Hasegawa M, Fujimoto Y, Lucas PC, et al. A critical role of RICK/RIP2 polyubiquitination in Nod-induced NF-kappaB activation. EMBO J **2008**; 27:373–83.
7. Kobayashi K, Inohara N, Hernandez LD, et al. RICK/Rip2/CARDIAK mediates signalling for receptors of the innate and adaptive immune systems. Nature **2002**; 416:194–9.
8. Krieg A, Correa RG, Garrison JB, et al. XIAP mediates NOD signaling via interaction with RIP2. Proc Natl Acad Sci U S A **2009**; 106:14524–9.
9. Scott MJ, Chen C, Sun Q, Billiar TR. Hepatocytes express functional NOD1 and NOD2 receptors: a role for NOD1 in hepatocyte CC and CXC chemokine production. J Hepatol **2010**; 53:693–701.
10. Adams S, Valchanova RS, Munz B. RIP2: a novel player in the regulation of keratinocyte proliferation and cutaneous wound repair? Exp Cell Res **2010**; 316:728–36.
11. Cressman DE, Greenbaum LE, DeAngelis RA, et al. Liver failure and defective hepatocyte regeneration in interleukin-6-deficient mice. Science **1996**; 274:1379–83.
12. Moura-Neto V, Kryszke MH, Li Z, Vicart P, Lilienbaum A, Paulin D. A 28-bp negative element with multiple factor-binding activity controls expression of the vimentin-encoding gene. Gene **1996**; 168:261–6.
13. Meylan E, Tschopp J. The RIP kinases: crucial integrators of cellular stress. Trends Biochem Sci **2005**; 30:151–9.
14. Chin AI, Dempsey PW, Bruhn K, Miller JF, Xu Y, Cheng G. Involvement of receptor-interacting protein 2 in innate and adaptive immune responses. Nature **2002**; 416:190–4.
15. Garcia PD, Ou JH, Rutter WJ, Walter P. Targeting of the hepatitis B virus precore protein to the endoplasmic reticulum membrane: after signal peptide cleavage translocation can be aborted and the product released into the cytoplasm. J Cell Biol **1988**; 106:1093–104.

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Quantification of hepatitis B surface antigen can help predict spontaneous hepatitis B surface antigen seroclearance

Makoto Arai, Seiko Togo, Tatsuo Kanda, Keiichi Fujiwara, Fumio Imazeki and Osamu Yokosuka

Background and aim **The clinical outcomes of hepatitis B virus (HBV) carriers are favorable following hepatitis B surface antigen (HBsAg) seroclearance. The aim of this study was to investigate the clinical course of spontaneous HBsAg seroclearance and the factors predicting it.**

Methods **A total of 423 patients who tested positive for HBsAg and were referred to Chiba University Hospital between January 1985 and April 2008 were included in the study and the following characteristics were analyzed: age, sex, status of hepatitis B e antigen, alanine aminotransferase level, HBV DNA level, number of platelets, HBV genotype, past treatment with interferon, and HBsAg level. When a nucleotide analog was used for treatment, we stopped follow-up. Measurement of HBsAg was performed using the chemiluminescent enzyme immunoassay method and less than 0.03 IU/ml of HBsAg was designated as HBsAg seroclearance.**

Results **The study group included 239 men and 184 women and their average age was 40.5 ± 13.8 years. Twenty-five patients achieved HBsAg seroclearance during the follow-up period with an incidence rate of 0.97% per year. Multivariate analysis revealed that HBsAg titer (compared with patients with a low HBsAg level: odds ratio = 0.45, 95% confidence interval: 0.29–0.70) at baseline was the only predictive factor for HBsAg seroclearance.**

Conclusion **HBsAg seroclearance occurred at a frequency of 0.97% per year without the use of a nucleotide analog. HBsAg titer at baseline was the only predictive factor for HBsAg seroclearance.** ***Eur J Gastroenterol Hepatol*** **00:000–000 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Keywords: chronic hepatitis B, hepatitis B antigen level, hepatitis B surface antigen seroclearance

Department of Medicine and Clinical Oncology, Graduate School of Medicine, Chiba University, Chiba, Japan

Correspondence to Dr Makoto Arai, MD, PhD, Department of Medicine and Clinical Oncology (K1), Graduate School of Medicine, Chiba University, Inohana 1-8-1, Chiba 260-8670, Japan
Tel: +81 43 226 2083; fax: +81 43 226 2088; e-mail: araim-cib@umin.ac.jp

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Introduction

An estimated 350 million people worldwide are chronically infected with the hepatitis B virus (HBV) [1]. The loss of hepatitis B surface antigen (HBsAg) usually results in normalized serum alanine aminotransferase (ALT) levels and decreased HBV DNA levels, which may lead to improved hepatic necroinflammation, and is thought to indicate clinical healing [2,3]. However, HBsAg seroclearance is a rare event in chronic hepatitis B (CHB) and its incidence is estimated to be approximately 2–3% per year [4]. Because of its rarity, the clinical course during HBsAg seroclearance remains largely unknown, although the clinical course during hepatitis e antigen (HBeAg) seroclearance has been well documented [5,6]. Historically, various factors have been reported to predict HBsAg seroclearance [7] and various studies have been carried out to distinguish the positive and negative prognostic factors for HBV carriers [8,9]. Recently, quantitative serology has been developed for HBsAg and is a promising candidate assay for determining an accurate prognosis for HBV carriers [10]. In this study, on the basis of a cohort of patients with CHB with long-term follow-up, we investigated the clinical course during HBsAg seroclearance.

Materials and methods

Patients

This was a retrospective and hospital-based analysis. Between January 1985 and April 2008, all patients visiting the Chiba University Hospital and who were HBsAg-positive carriers ($n = 676$) were approached for participation in the study. This study was reviewed and approved by the Institutional Review Board of the Chiba University School of Medicine. The patients' consent was obtained for the storage and use of serum. Patients who were positive for the hepatitis C virus antibody and those who had another potential cause for chronic liver disease (autoimmune hepatitis and primary biliary cirrhosis) were excluded from the study. To exclude patients with an acute infection of HBV, we confirmed the persistent infection of HBV before the first visit to our hospital or low titers of the IgM-HBc antibody at entry for all the patients. Those patients who were monitored for less than 1 year or who had been given antiviral drugs (lamivudine or entecavir) before entry were also excluded from the analysis. As a result, 423 patients were selected for further analysis. Study participants were followed up every 6–12 months, and the serum samples obtained from the patients

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each year were stored at −20°C. The earliest sample from each patient was used to define the level of HBsAg at entry. The level of HBsAg in the most recent sample from each of 423 patients was evaluated. When the level of HBsAg was below the cutoff (0.03 IU/ml), we designated this as HBsAg seroclearance. To clarify the relationship between HBsAg seroclearance and other factors, age, sex, HBeAg status, HBV genotype, the use of interferon, HBsAg, HBV DNA, ALT, and the number of platelets were analyzed.

Laboratory assays

Measurement of HBsAg was performed using the chemiluminescent enzyme immunoassay method and the HISCL-2000i (Sysmex Corporation, Kobe, Japan). A positive linear correlation was observed between our method and Architect HBsAg QT (Abbott Laboratories, Abbott Park, Illinois, USA), which is commonly used. A dilution test showed a linear correlation curve in the range from 0.03 to 2360 IU/ml, and the samples that showed a high HBsAg level above this range could be quantified after diluting 40 or 1600 times. In addition, our method can be applied to quantify the HBsAg in serum samples with different HBV genotypes/subgenotypes, as well as in serum-contained HBV vaccine escape mutants (126S, 145R) [11,12]. HBeAg and anti-HBe levels were determined by an enzyme-linked immunosorbent assay (ELISA; Abbott Laboratory). Anti-HCV was detected by ELISA (Ortho Diagnostics, Tokyo, Japan). The serum HBV DNA level was quantified by a polymerase chain reaction assay (Amplicor HBV Monitor; Roche Diagnostics, Basel, Switzerland) with a linear range of quantification of 2.6–7.6 log copies/ml. The six major genotypes of HBV (A–F) were determined by ELISA (HBV Genotype EIA, Institute of Immunology Co. Ltd, Tokyo, Japan).

Serial changes in hepatitis B surface antigen in the patients with hepatitis B surface antigen seroclearance

To monitor the serial changes in HBsAg levels in patients with HBsAg seroclearance, the level of HBsAg was evaluated in all available samples from these patients. Changes in ALT, platelets, and HBsAg were evaluated before and after HBsAg seroclearance.

Statistical analysis

The baseline data are presented as mean ± SD. The difference in the values of the clinical parameters between the two groups was analyzed using a paired *t*-test, an unpaired *t*-test, the Welch *t*-test, and the χ^2-test. All analyses were performed using the statistical program SPSS 16.1 (SPSS Inc., Chicago, Illinois, USA). A *P*-value of less than 0.05 was considered statistically significant.

Results

Characteristics of patients with hepatitis B surface antigen seroclearance

The baseline clinical and virological characteristics of the 423 HBsAg-positive carriers are shown in Table 1. During the follow-up period, monitoring of those patients who received treatment for HBV with nucleotide analogs was discontinued. Twenty-five patients showed HBsAg seroclearance with an incidence rate of 0.97% per year. For these 25 patients, we confirmed the negative results of HBsAg quantification in at least two sequential samples. Two of the 25 patients had received interferon (IFN) therapy before the start of follow-up and HBsAg seroclearance in these patients occurred over 10 years after IFN treatment. First, we investigated the relationship between HBsAg seroclearance and other virological and clinical markers. In terms of HBeAg status, the level of HBV DNA and HBsAg, the number of platelet, and the period of follow-up, there were obvious difference between the patients with and without HBsAg seroclearance (Table 1). No patient suffered from liver failure. Among those with HBsAg seroclearance, hepatocellular carcinoma (HCC) occurred only in one patient (4.0%) after HBsAg seroclearance. This patient underwent a hepatectomy to remove HCC and the degree of liver fibrosis was moderate (F2), not cirrhosis. In the control group, HCC occurred in 20 patients (5.0%).

Table 1 Baseline characteristics of hepatitis B surface antigen-positive patients

Parameters	Total patients	HBsAg seroclearance	No HBsAg seroclearance	*P* value
Number	423	25	398	
Sex (male/female)	239/184	18/7	221/177	NS[a]
Age (years)	40.5±13.8	44.6±9.4	40.2±14.0	NS[d]
HBeAg status (+/−)	183/240	3/22	180/218	0.003[a]
HBV DNA (log copies/ml)	5.6±1.9	4.6±1.8	5.6±1.9	0.007[b]
ALT (IU/l)	72.7±90.4	116.3±206.1	69.9±76.8	NS[d]
Platelet (/μl)	206 000±65 000	178 000±63 000	208 000±65 000	0.026[b]
Genotype A/B/C/D/not determined	5/31/261/1/125	1/2/18/0/4	4/29/243/1/121	NS[c]
Past use of interferon	16/407	2/23	14/384	NS[c]
HBsAg ($\log_{10}$ IU/ml)	3.37±1.21	2.47±1.28	3.44±1.13	<0.001[d]
Follow-up period (days)	2217±1844	3109±2249	2159±1802	0.044[b]

ALT, alanine aminotransferase; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; NS, not significant.
[a]χ^2-test.
[b]Unpaired *t*-test.
[c]Fischer's exact test.
[d]Mann–Whitney *U*-test.

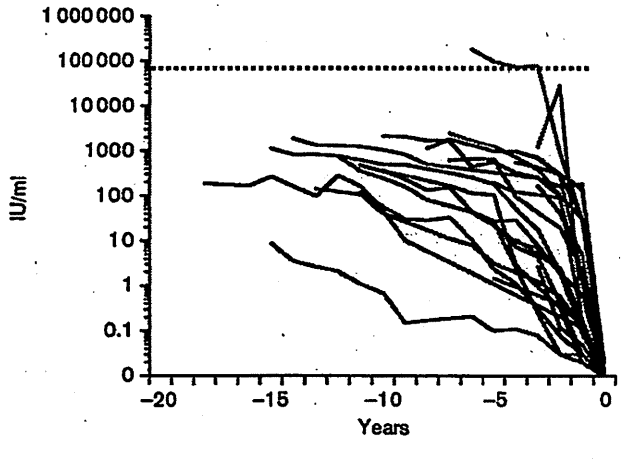
Serial changes in hepatitis B surface antigen, alanine aminotransferase level, and platelets before and after hepatitis B surface antigen seroclearance

The levels of HBsAg, ALT, and platelets in the patients with HBsAg seroclearance were evaluated annually (Figs 1, 2a and b). The average follow-up period after HBsAg seroclearance was 6.5 ± 5.7 years. HBsAg reappeared in three patients at 8, 10, and 11 years after HBsAg seroclearance. Two patients showed HBsAg seroclearance again within 2 and 3 years of the reappearance of HBsAg, but one patient could not be followed up after the reappearance of HBsAg. All 25 patients were negative for HBeAg and HBV DNA and had normal ALT levels. In addition, ALT levels did not fluctuate in these patients after HBsAg seroclearance. Platelets in the patients with HBsAg seroclearance did not show any difference between entry (180 000 ± 44 000/μl) and the end (179 000 ± 55 000/μl) of the follow-up period (paired *t*-test), although three of eight patients with less than 150 000/μl of platelets at HBsAg seroclearance showed an increase in platelets after HBsAg seroclearance.

Factors associated with the future seroclearance of hepatitis B surface antigen

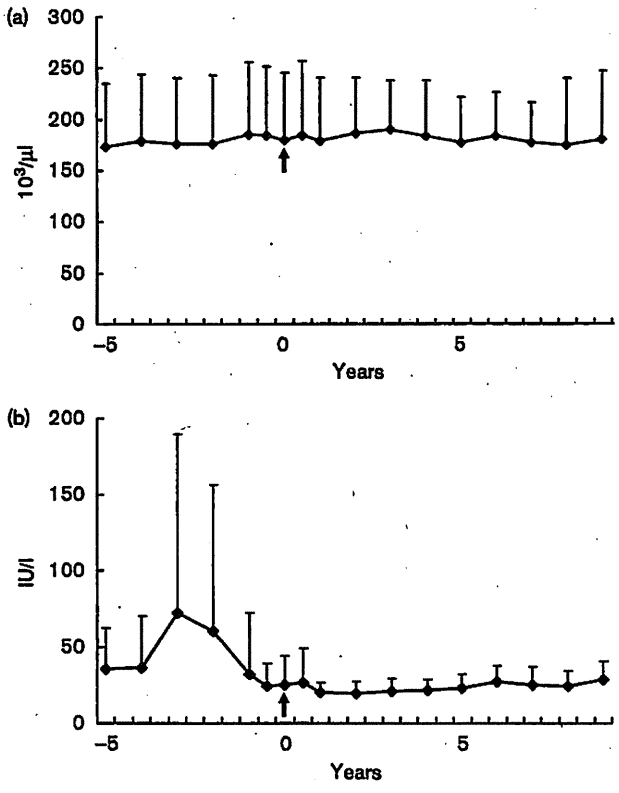
Next, we used the Cox proportional hazards model to investigate the factors associated with the future seroclearance of HBsAg (Table 2). Univariate analysis revealed that age [compared with younger patients: odds ratio (OR) = 1.06, 95% confidence interval (CI): 1.03–1.10], HBeAg negativity (compared with HBeAg positivity: OR = 7.88, 95% CI: 2.34–26.6), HBV DNA level (compared with patients with a low HBV DNA level: OR = 0.58, 95% CI: 0.46–0.75), and HBsAg titer (compared with patients with a low HBsAg level: OR = 0.39, 95% CI: 0.29–0.53) at baseline were predictive factors for HBsAg seroclearance. Multivariate analysis revealed that HBsAg titer (compared with patients with a low HBsAg level: OR = 0.45, 95% CI: 0.29–0.70) at baseline was a predictive factor for HBsAg seroclearance. Thus, these analyses revealed that a low HBsAg level was the most important factor associated with the future seroclearance of HBsAg. We performed the multivariate analysis again, changing the threshold of HBsAg from 1.0 to 5.0 log IU/ml in 1.0 log increments. We determined the threshold when the value of probability was the smallest. As a result, the threshold of HBsAg levels was determined to be 3.0 log IU/ml. The hazard ratio (95% CI) and the *P*-value were 5.32 (1.77–15.9) and 0.003, respectively. When the HBV carriers were divided into two groups, over 1000 IU/ml of HBsAg or not, HBsAg seroclearance occurred in HBV carriers with less than 1000 IU/ml of HBsAg at a higher rate and with a significant difference (log-rank test, $P < 0.01$; Fig. 3).

Fig. 1



Serial changes in hepatitis B surface antigen (HBsAg) levels in patients with HBsAg seroclearance. The average level of HBsAg at entry among all the patients was 16 994 IU/ml (dotted line), although the levels of most patients with HBsAg seroclearance were below the average. Twenty-five patients with HBsAg seroclearance showed a decline in the HBsAg level several years before HBsAg seroclearance.

Fig. 2



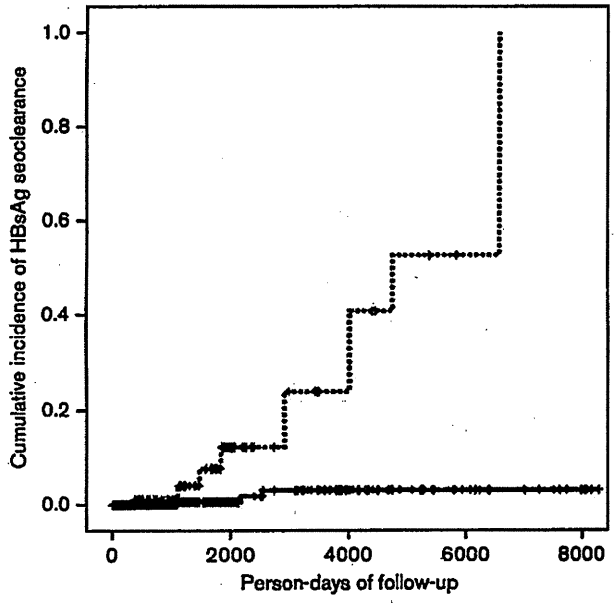
Serial changes in (a) the number of platelets and (b) alanine aminotransferase (ALT) levels before and after hepatitis B surface antigen (HBsAg) seroclearance. Platelets showed no change before and after HBsAg seroclearance. ALT levels fluctuated before HBsAg seroclearance, but did not fluctuate afterward. The arrows indicate the year of HBsAg seroclearance.

Table 2 Cox regression analysis for the predictive factors for hepatitis B surface antigen seroclearance

	Univariate analysis		Multivariate analysis	
	Hazard ratio (95% CI)	*P* value	Hazard ratio (95% CI)	*P* value
Age	1.06 (1.03–1.10)	0.001	1.03 (0.98–1.07)	NS
Sex male	2.35 (0.97–5.68)	NS		
HBeAg negative	7.88 (2.34–26.6)	0.001	2.62 (0.62–11.0)	NS
HBV-DNA	0.58 (0.46–0.75)	<0.001	0.94 (0.66–1.35)	NS
ALT	1.00 (1.00–1.00)	NS		
Platelet	1.00 (0.99–1.00)	NS		
Genotype A	1.92 (0.92–4.00)	NS		
Past use of interferon	1.47 (0.34–6.27)	NS		
HBsAg (log)	0.39 (0.29–0.53)	<0.001	0.45 (0.29–0.70)	<0.001

ALT, alanine aminotransferase; CI, confidence interval; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; NS, not significant.

Fig. 3



Cumulative occurrence of seroclearance of hepatitis B surface antigen (HBsAg) based on the HBsAg levels over 1000 IU/ml of HBsAg or not by the Kaplan–Meier method. A significant difference was observed by the log-rank test ($P<0.01$). The dotted line indicates the group with a low HBsAg level.

Discussion

HBsAg is the fundamental diagnostic marker of HBV infection. HBsAg is a component of the Dane particle, which contains the viral genome, and of subviral particles. But the mechanisms that regulate the production of HBsAg, particularly the subviral particles, are largely unclear [13]. HBsAg seroclearance is a clinical goal for HBV carriers, because, after HBsAg seroclearance, clinical outcomes of HBV carriers are favorable and the incidence of liver failure and HCC in patients with HBsAg seroclearance is much lower than that in HBsAg-positive patients [2,3,14,15]. Individuals who become HBsAg negative can be considered to have resolved CHB. If we can predict the seroclearance of HBsAg among HBV carriers, this can help physicians manage CHB patients.

Spontaneous HBsAg seroclearance has been well documented and predictive factors for the seroclearance of HBsAg were also clarified. Liu *et al.* [4] reported that the level of HBV DNA was an important factor and Kim *et al.* [16] reported that old age and a normal ALT level were factors associated with HBsAg seroclearance. Tai *et al.* [7] reported that male sex, HBeAg negativity, older age, low maximal ALT level, and hepatic steatosis were factors associated with HBsAg seroclearance and that the estimated HBsAg seroclearance rates increased with age and reached a plateau after the age of 50 years. Our study clarified that the level of HBsAg, not the HBV DNA level, is a predictive factor for the clearance of HBsAg. In the previous reports [17,18] and ours [10], the level of HBV DNA showed a good correlation with the level of HBsAg, but there were quite a few outliers. In fact, nine (36.0%) of 25 patients with HBsAg seroclearance showed a high HBV DNA level (over 5.0 log copies/ml) at baseline. In contrast, only three (12.0%) of 25 patients showed a high HBsAg level (over 4.0 $\log_{10}$ IU/ml) at baseline. As far as HBsAg seroclearance is concerned, the HBsAg level is the most reliable predicting factor for it, and future analysis for the outliers between HBsAg and HBV DNA levels might provide a clue toward clarification of the mechanism of HBsAg seroclearance. In this study, the age at HBsAg clearance varied from 27 to 67 years and was scattered and showed no particular trend. This difference was attributed to the difference in the method of HBsAg quantification. Our study involved quantification of the HBsAg level using an assay with higher sensitivity (the cutoff level was 0.03 IU/ml) than traditional and qualitative analysis of HBsAg (the cutoff level was almost 1.0 IU/ml). In addition, most studies had not evaluated the quantitative HBsAg level as a prognostic factor for HBV carriers. In any case, to evaluate the HBsAg seroclearance precisely, HBsAg should be evaluated using a quantitative method.

Nine patients with HBsAg seroclearance showed ALT elevation within 5 years before HBsAg seroclearance. Five of nine patients showed a high HBV DNA level during ALT elevation, which might indicate a severe immune reaction for HBV. These results suggested that there exist two types of progress reaching to HBsAg seroclearance: one with a flare in the ALT level as a severe immune reaction for HBV and the other without it. We should clarify the difference between these two types in the future.

IFN therapy has antiviral and immunomodulatory effects and has been used in the treatment of CHB. In meta-analysis, IFN therapy could induce HBsAg seroclearance at the end of follow-up for at least 3 years [19,20]. In our

study, IFN therapy was not related to HBsAg seroclearance. This difference might be attributable to the difference in the HBV genotype, the small number of patients with IFN treatment, or the past use of IFN.

The average number of platelets in the patients with HBsAg seroclearance did not change after HBsAg seroclearance. In contrast, three of eight patients with less than 150 000/μl of platelets showed an increase in platelets, which was also reported in a previous study [21]. We have reported that the number of platelets is one of the most important factors predicting the prognosis of HBV carriers [22,23]. We do not know the reason for the difference between those with and without an increase in platelets after HBsAg seroclearance; therefore, we should clarify this in the future.

In conclusion, the predictive factor for the seroclearance of HBsAg was a lower level of HBsAg. Therefore, measurement of HBsAg level is one of the most effective means to follow up HBV carriers accurately.

Acknowledgements

Conflicts of interest

The authors thank our staffs for their help. We have no conflict of interest disclosure.

References

1 Lok AS, McMahon BJ. Chronic hepatitis B: AASLD practice guidelines. *Hepatology* 2007; 45:507–539.
2 Arase Y, Ikeda K, Suzuki F, Suzuki Y, Saitoh S, Kobayashi M, *et al.* Long-term outcome after hepatitis B surface antigen seroclearance in patients with chronic hepatitis B. *Am J Med* 2006; 119:e9–e16.
3 Tong MJ, Nguyen MO, Tong LT, Blatt LM. Development of hepatocellular carcinoma after seroclearance of hepatitis B surface antigen. *Clin Gastroenterol Hepatol* 2009; 7:889–893.
4 Liu J, Yang HI, Lee MH, Lu SN, Jen CL, Wang LY, *et al.* Incidence and determinants of spontaneous hepatitis B surface antigen seroclearance: a community-based follow-up study. *Gastroenterology* 2010; 139: 474–482.
5 Hoofnagle JH, Dusheiko GM, Seeff LB, Jones EA, Waggoner JG, Bales ZB. Seroconversion from hepatitis e antigen to antibody in chronic type B hepatitis. *Ann Intern Med* 1981; 94:744–748.
6 Fattovich G, Rugge M, Brollo L, Pontisso P, Noventa F, Guido M, *et al.* Clinical, virologic and histologic outcome following seroconversion from HBeAg to anti-HBe in chronic hepatitis type B. *Hepatology* 1986; 6: 167–172.
7 Tai DI, Tsay PK, Chen WT, Chu CM, Liaw YF. Relative roles of HBsAg seroclearance and mortality in the decline of HBsAg prevalence with increasing age. *Am J Gastroenterol* 2010; 105:1102–1109.
8 Yokosuka O, Kurosaki M, Imazeki F, Arase Y, Tanaka Y, Chayama K, *et al.* Management of hepatitis B: Consensus of the Japan Society of Hepatology 2009. *Hepatol Res* 2011; 41:1–21.
9 Iloeje UH, Yang HI, Su J, Jen CL, You SL, Chen CJ. Risk evaluation of viral load elevation and associated liver disease/cancer-in HBV (the REVEAL-HBV) Study Group: predicting cirrhosis risk based on the level of circulating hepatitis B viral load. *Gastroenterology* 2006; 130:678–686.
10 Togo S, Arai M, Tawada A, Chiba T, Kanda T, Fujiwara K, *et al.* Clinical importance of serum hepatitis B surface antigen levels in chronic hepatitis B. *J Viral Hepat* 2011; 18:e508–e515.
11 Takagi K, Tanaka Y, Naganuma H, Hiramatsu K, Iida T, Takasaka Y, Mizokami M. Clinical evaluation of a novel HBsAg quantitative assay. *Rinsho Byori* 2007; 55:619–625, [Japanese].
12 Takagi K, Tanaka Y, Hiramatu K, Kani S, Tatematsu K, Naganuma H, *et al.* Evaluation of high-sensitivity HBsAg quantitative assay for HBV genotype. *Rinsho Byori* 2009; 57:638–643, [Japanese].
13 Nguyen DH, Hu J. Reverse transcriptase- and RNA packaging signal-dependent incorporation of APOBEC3G into hepatitis B virus nucleocapsids. *J Virol* 2008; 82:6852–6861.
14 Yuen MF, Wong DK, Fung J, Ip P, But D, Hung I, *et al.* HBsAg Seroclearance in chronic hepatitis B in Asian patients: replicative level and risk of hepatocellular carcinoma. *Gastroenterology* 2008; 135:1192–1199.
15 Kim JH, Lee YS, Lee HJ, Yoon E, Jung YK, Jong ES; *et al.* HBsAg seroclearance in chronic hepatitis B: implications for hepatocellular carcinoma. *J Clin Gastroenterol* 2011; 45:64–68.
16 Kim JH, Lee JH, Park SJ, BaeMH, Kim JH, Kim do Y, *et al.* Factors associated with natural seroclearance of hepatitis B surface antigen and prognosis after seroclearance: a prospective follow-up study. *Hepatogastroenterology* 2008; 55:578–581.
17 Deguchi M, Yamashita N, Kagita M, Asari S, Iwatani Y, Tsuchida T, *et al.* Quantitation of hepatitis B surface antigen by an automated chemiluminescent microparticle immunoassay. *J Virol Methods* 2004; 115:217–222.
18 Kohmoto M, Enomoto M, Tamori A, Habu D, Takeda T, Kawada N, *et al.* Quantitative detection of hepatitis B surface antigen by chemiluminescent microparticle immunoassay during lamivudine treatment of chronic hepatitis B virus carriers. *J Med Virol* 2005; 75:235–239.
19 Yang YF, Zhao W, Xia HM, Zhong YD, Huang P, Wen J. Long-term efficacy of interferon alpha therapy on hepatitis B viral replication in patients with chronic hepatitis B: a meta-analysis. *Antiviral Res* 2010; 85:361–365.
20 Sonneveld MJ, Rijckborst V, Boucher CA, Hansen BE, Janssen HL. Prediction of sustained response to peginterferon alfa-2b for hepatitis B e antigen-positive chronic hepatitis B using on-treatment hepatitis B surface antigen decline. *Hepatology* 2010; 52:1251–1257.
21 Okoshi S, Igarashi M, Suda T, Iwamatsu H, Watanabe K, Ishihara K, *et al.* Remote development of hepatocellular carcinoma in patients with liver cirrhosis type B serologically cured for HBs antigenemia with long-standing normalization of ALT values. *Dig Dis Sci* 2002; 47:2002–2006.
22 Ito K, Arai M, Imazeki F, Yonemitsu Y, Kanda T, Fujiwara K, *et al.* Risk of hepatocellular carcinoma in patients with chronic hepatitis B virus infection. *Scand J Gastroenterol* 2010; 45:243–249.
23 Bekku D, Arai M, Imazeki F, Yonemitsu Y, Kanda T, Fujiwara K, *et al.* Long-term follow-up of patients with hepatitis B e antigen negative chronic hepatitis B. *J Gastroenterol Hepatol* 2011; 26:122–128.

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Research Paper

Efficacy of Lamivudine or Entecavir on Acute Exacerbation of Chronic Hepatitis B

Tatsuo Kanda[1*]✉, Masami Shinozaki[2*], Hidehiro Kamezaki[1], Shuang Wu[1], Shingo Nakamoto[1], Makoto Arai[1], Keiichi Fujiwara[1], Nobuaki Goto[2], Fumio Imazeki[1] and Osamu Yokosuka[1]

1. Department of Medicine and Clinical Oncology, Chiba University, Graduate School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan.
2. Department of Medicine, Numazu City Hospital, Numazu, Shizuoka 410-0302, Japan.

* These authors contributed equally to this article.

✉ Corresponding author: Tatsuo Kanda, MD, PhD, Associate Professor, Department of Medicine and Clinical Oncology, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8677, Japan. Tel.: +81-43-226-2086; Fax: +81-43-226-2088; E-mail: kandat-cib@umin.ac.jp

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Abstract

Background/Aims: Spontaneous acute exacerbation of chronic hepatitis B virus (HBV) infection occasionally occurs in its natural history, sometimes leading rapidly to fatal hepatic failure. We compared the effects of lamivudine (LAM) with those of entecavir (ETV) treatments in acute exacerbation of chronic hepatitis B with 500 IU/L or higher alanine aminotransferase (ALT) levels.

Methods: Thirty-four patients with acute exacerbation were consecutively treated with LAM /ETV. Their clinical improvements were compared.

Results: Among LAM-treated and ETV-treated patients, none showed a reduction of <1 log IU/mL in HBV DNA after 1 or 3 months of treatment. Initial virological response, defined as a reduction of 4 log IU/mL in HBV DNA at 6 months, with LAM and ETV, respectively, was 83.3% and 100%. One LAM patient developed hepatic encephalopathy, but all patients in both groups survived. Twelve months after treatment, 41.6% of 24 LAM group patients switched to another drug or added adefovir to their treatment due to the emergence of LAM-resistant mutants. On the other hand, patients receiving ETV did not need to change drugs.

Conclusions: ETV appears to be as effective as LAM in the treatment of patients with acute exacerbation of chronic hepatitis B. Clinicians should carefully start to treat these patients as soon as possible.

Key words: acute exacerbation, ALT, entecavir, HBV, lamivudine

INTRODUCTION

Chronic hepatitis B infection is associated with the development of hepatocellular carcinoma [1]. Infection with hepatitis B virus (HBV) also leads to wide a spectrum of liver injury, including acute, self-limited infection, fulminant hepatitis, and chronic hepatitis with progression to cirrhosis and liver failure, as well as to an asymptomatic chronic carrier state [2, 3].

Reactivation of hepatitis B is a well-characterized syndrome marked by the abrupt reappearance or rise of HBV DNA in the serum of a patient with previously inactivated or resolved HBV infection [4]. Reac-

tivation is often spontaneous, but can also be triggered by cancer chemotherapy and immune suppression. Spontaneous acute exacerbation of chronic hepatitis B infection is seen with a cumulative probability of 15-37% after 4 years of follow-up [5]. Prognosis is generally poor in HBV carriers with spontaneous acute exacerbation together with high alanine aminotransferase (ALT) levels, jaundice, and liver failure [4, 6, 7]. This condition has been defined as acute-on-chronic liver failure according to a recent Asia-Pacific consensus recommendation [8]. Acute exacerbation occasionally leads to a critical scenario, meaning that clinicians need to treat this condition immediately.

Lamivudine (LAM) is a reverse-transcriptase inhibitor of viral DNA polymerase with an excellent profile of safety and tolerability, causing inhibition of viral replication, and it is approved for antiviral treatment of hepatitis B patients [9, 10]. LAM suppresses serum HBV DNA values in up to 98% of patients within a median period of 4 weeks, leading to aminotransferase normalization, increased hepatitis B e antigen (HBeAg) seroconversion rate, and improvement of histological parameters [11, 12]. A study from Taiwan showed that LAM had a survival benefit and was effective for patients with baseline bilirubin levels below 20 mg/dL [7].

Entecavir (ETV), a deoxyguanosine analogue, is a potent and selective inhibitor of HBV replication; its *in vitro* potency is 100- to 1,000-fold greater than that of LAM, and it has a selectivity index (concentration of drug reducing the viable cell number by 50% [CC_{50}]/concentration of drug reducing viral replication by 50% [EC_{50}]) of ~8,000 [13, 14]. At present, the Japanese national health insurance system approves ETV as the first-line therapy for chronic hepatitis B, although some patients are treated with standard interferon-alfa. ETV is a nucleoside analogue (NUC) belonging to a new subgroup, cyclopentane [15], and it has been shown to be highly effective in suppressing HBV replication to an undetectable level and normalizing ALT, although NUCs do not eradicate the virus. ETV develops less resistance than LAM.

We undertook a retrospective study to compare the efficacy of LAM with that of ETV in the reduction of HBV DNA levels and associated improvement in disease severity and biochemical recovery in patients with acute exacerbation together with higher ALT levels due to HBV reactivation.

MATERIALS AND METHODS

Patients

A retrospective analysis of LAM/ETV-treated chronic hepatitis B patients at Chiba University Hospital and Numazu City Hospital, Japan, between May 2003 and December 2009 was performed. The inclusion criteria were: acute exacerbation of chronic hepatitis B characterized by an elevation of ALT level ≥ 500 IU/L along with HBV DNA ≥ 4.5 log IU/mL presenting in a patient with diagnosed chronic liver disease. The exclusion criteria were: acute hepatitis B, superinfection with other viruses (hepatitis E, A, D, or C), other causes of chronic liver failure [16, 17], coexistent hepatocellular carcinoma, portal thrombosis, coexistent renal impairment, pregnancy, coinfection with human immunodeficiency virus (HIV), or patients who had received a previous course of NUC treatment. This retrospective study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in *a priori* approval by the Ethics Committee of Chiba University, Graduate School of Medicine [18].

Baseline assessment of patients

Retrospectively collected data included patient demographics, clinical findings, all laboratory variables including virological tests and abdominal ultrasound. HBsAg, HBeAg, anti-HBe antibody and immunoglobulin M (IgM) anti-HBc antibody were determined by ELISA (Abbott, Chicago, IL, USA) or CLEIA (Fujirebio, Tokyo, Japan) [19]. HBV genotype was determined from patients' sera by ELISA (Institute of Immunology, Tokyo, Japan) as reported by Usuda et al [20]. HBV DNA was measured by Roche Amplicor™ PCR assay (detection limits: 2.6 log IU/mL; Roche Diagnostics, Tokyo, Japan).

Definitions

Primary antiviral treatment failure was defined as a reduction of < 1 log IU/mL in HBV DNA after 3 months of therapy. Initial virological response (IVR) was defined as a reduction of ≥ 4 log IU/mL in HBV DNA after 6 months of therapy [21].

Follow-up

Clinical assessment and routine investigations were done every 15 days or every month for at least 6 months. HBV DNA measurements were repeated monthly.

Statistical analysis

Statistical analyses were performed using Microsoft Excel 2010 for Windows™ 7 and StatView 5 (SAS Institute Inc, Cary, NC). Continuous variables were expressed as mean ± standard deviation and were compared by two-factor analysis of variance (ANOVA) and two-way repeated measures ANOVA. Categorical variables were compared by Chi-square

test. Baseline was taken as the date when the first dose of LAM/ETV was administered. Statistical significance was considered at a *P*-value < 0.05.

RESULTS

Patients

Between May 2003 and December 2009, 34 patients with spontaneous acute exacerbation of chronic hepatitis B, with ALT levels ≥ 500 IU/mL and treated with LAM or ETV, were consecutively enrolled and retrospectively analyzed. 24 (70.5%) were treated with LAM at 100 mg daily and 10 (29.4%) were treated with ETV at 0.5 mg daily. All patients were followed for at least 6 months. Mean follow-up in the LAM and ETV groups was 55.5 ± 25.4 and 16.5 ± 9.9 months, respectively.

Baseline characteristics

Baseline characteristics in the two patient groups were similar **(Table 1)**. Median age was 37 (21-73) years and 79.4% were men. One patient of the LAM group developed hepatic encephalopathy, but recovered. All patients in both groups survived. At admission, the serological profile showed HBsAg positivity in all 34 (100%); 22 (64.7%) were HBeAg positive. The median HBV DNA level was 7.4 log IU/mL in the LAM group and 7.9 log IU/mL in the ETV group **(Table 1)**.

Table 1 Demographic, Clinical, and Laboratory Variables of Patients at Entry.

Parameters	Total Patients (N=34)	LAM (N=24)	ETV (N=10)	*P*-value
Age (years)	37 (21-73)	37 (21-73)	39 (24-67)	NS
Male (%)	27 (79.4)	18 (75)	9 (90)	NS
Cirrhosis (+/-)	2/32	2/22	0/10	NS
ALT (IU/L)	986 (523-2,450)	995 (523-2,450)	1,046 (523-2,140)	NS
T. Bil (mg/dL)	2.0 (0.8-22.0)	2.4 (0.8-20.6)	1.6 (1.9-22.0)	NS
PT (%)	83 (24-121)	81.5 (24-119)	83.6 (35-121)	NS
HBeAg (+/-)	22/12	18/6	4/6	NS
HBV DNA (log IU/mL)	7.6 (4.8-8.7)	7.4 (5.2-8.7)	7.9 (4.8-8.7)	NS

LAM, lamivudine; ETV, entecavir; ALT, alanine aminotransferase; T. BIL, total bilirubin; PT, prothrombin time; NS, statistically not significant.

Reduction in HBV DNA of total patients

LAM significantly reduced HBV DNA levels from baseline 7.24 log IU/mL to 3.27 log IU/mL at 1 month ($P < 0.001$), to 2.21 log IU/mL at 3 months ($P < 0.001$), and to 1.53 log IU/mL at 6 months ($P < 0.001$). ETV also significantly reduced HBV DNA levels from baseline 7.56 log IU/mL to 3.12 log IU/mL at 1 month ($P < 0.001$), to 2.14 log IU/mL at 3 months ($P < 0.001$), and to 1.77 log IU/mL at 6 months ($P < 0.001$). There were no differences in HBV DNA levels from baseline to 6 months between the two groups. None with primary antiviral treatment failure was identified in either group. There were no significant differences in IVR between the two groups **(Figure 1)**.

Reduction in ALT levels of total patients

LAM significantly reduced ALT levels from baseline 1,130 IU/mL to 102 ($P < 0.001$) at 1 month, to 28.6 ($P < 0.001$) at 3 months, and to 23.1 ($P < 0.001$) at 6 months. ETV also significantly reduced ALT levels from baseline 1,210 IU/mL to 117 ($P < 0.001$) at 1 month, to 25 ($P < 0.001$) at 3 months, and to 24.4 ($P < 0.001$) at 6 months. There were no differences in ALT levels from baseline to 6 months between the two groups **(Figure 2)**.

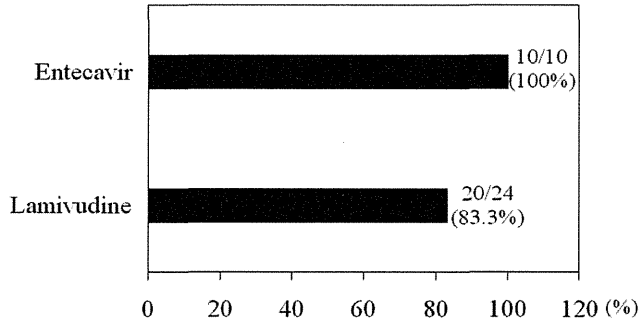

Figure 1 Initial virological response (IVR). IVR was defined as a reduction of ≥ 4 log IU/mL in HBV DNA after 6 months of therapy [21].

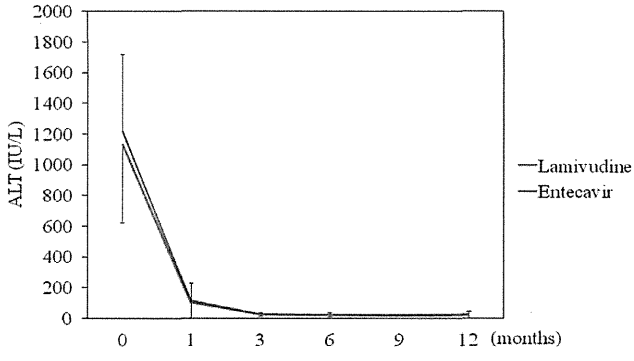

Figure 2 Efficacy of lamivudine and entecavir for ALT levels. Lamivudine (N=24) vs. entecavir (N=10); data are shown as mean ± SD.

Reduction in HBV DNA of HBeAg-positive patients

It has been demonstrated that the levels of HBV DNA in the HBeAg-positive phase were generally higher than those in the ant-HBe-positive phase [19, 22]. HBeAg positivity is also associated with HBV viremia and increased ALT levels in HIV/HBV co-infected patients [23]. Next, we compared the response to LAM or ETV in 18 or 4 HBeAg-positive patients, respectively **(Table 2)**. LAM significantly reduced HBV DNA levels from baseline 7.52 log IU/mL to 3.35 log IU/mL ($P < 0.001$) at 1 month, to 2.38 log IU/mL ($P < 0.001$) at 3 months, and to 1.55 log IU/mL ($P < 0.001$) at 6 months. ETV also significantly reduced HBV DNA levels from baseline 8.42 log IU/mL to 3.87 log IU/mL ($P < 0.001$) at 1 month, to 2.90 log IU/mL ($P < 0.001$) at 3 months, and to 2.22 log IU/mL ($P < 0.001$) at 6 months. There were no differences in HBV DNA levels from baseline to 6 months between the two groups. Primary antiviral treatment failure was not observed in either group. Four patients in the LAM group did not achieve IVR.

Table 2 Demographic, Clinical, and Laboratory Variables of HBeAg-positive Patients at Entry.

Parameters	Total Patients (N=22)	LAM (N=18)	ETV (N=4)	*P*-value
Age (years)	34.5 (21-51)	36.5 (21-51)	30 (24-33)	NS
Male (%)	18 (81.8)	14 (77.7)	4 (100)	NS
Cirrhosis (+/-)	1/21	1/17	0/4	NS
ALT (IU/L)	1,030 (523-2,450)	1,990 (523-2,450)	1,363 (980-1,620)	NS
T. Bil (mg/dL)	1.75 (0.8-20.6)	2.0 (0.8-20.6)	1.5 (1.0-18.7)	NS
PT (%)	77 (24-119)	73.6 (24-119)	95.0 (44.1-113)	NS
HBeAg (+)	22	18	4	
HBV DNA (log IU/mL)	7.6 (5.5- 8.8)	7.6 (5.5- 8.7)	8.6 (7.6- 8.7)	NS

LAM, lamivudine; ETV, entecavir; ALT, alanine aminotransferase; T. BIL, total bilirubin; PT, prothrombin time; NS, statistically not significant.

Reduction in ALT levels of HBeAg-positive patients

LAM significantly reduced ALT levels from baseline 1,150 IU/mL to 84 ($P < 0.001$) at 1 month, to 27.5 ($P < 0.001$) at 3 months, and to 22.0 ($P < 0.001$) at 6 months. ETV also significantly reduced ALT levels from baseline 1,460 IU/mL to 230 ($P = 0.0038$) at 1 month, to 22.2 ($P = 0.0016$) at 3 months, and to 24.0 ($P = 0.0016$) at 6 months. At 1 month after treatment, the ALT levels of the LAM groups were lower than those of the ETV group ($P < 0.0001$) **(Figure 3)**. During follow-up periods, 10 and 1 sero-converters of HBeAg to anti-HBe antibody phase were seen in 18 LAM-treated and in 4 ETV-treated patients, respectively.

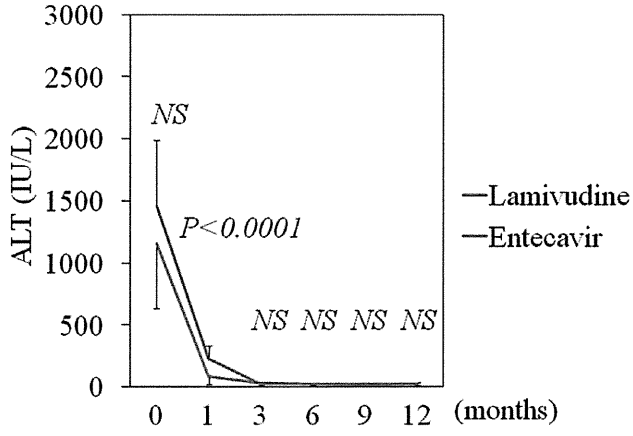

Figure 3 Efficacy of lamivudine and entecavir for ALT levels in HBeAg-positive patients. Lamivudine (N=18) vs. entecavir (N=4); data are shown as mean ± SD.

Safety

No patient stopped taking medications. Twelve months after treatment, 10 of 24 patients (41.6%) in the LAM group switched from LAM to ETV (n=4) or added adefovir (n=6) due to the emergence of LAM-resistant mutants. On the other hand, patients receiving ETV did not need to change their medication.

DISCUSSION

The present study compared the use of NUCs, LAM and ETV, for the treatment of acute exacerbation of chronic hepatitis B. The results clearly showed significant benefits of a rapid reduction of HBV DNA levels, compared with untreated patients in a previous report [4].

It was reported that ETV treatment is associated with increased short-term mortality in patients with severe acute exacerbation of chronic hepatitis B, but that it achieves better virological response in the long run [24]. We used LAM or ETV for patients with acute exacerbation of chronic hepatitis B presenting with ALT ≥ 500 IU/L in the present study. The effects of LAM on HBV DNA levels were the same as those of ETV (Figure 1). But the effects of LAM on ALT levels after 1 month were stronger than those of ETV in HBeAg-positive patients **(Figure 3)**. In spite of the limited number of these patients, the effects were possibly related to immunomodulating activities of LAM [25]. The patients' prognoses were more favorable than in the previous report [4]. This might have

depended on the fact that, in the present study, treatment was begun as soon as possible, and some patients may have had a milder grade of acute exacerbation of chronic hepatitis B than those in the previous report [4]. We believe that patients with acute exacerbation of chronic hepatitis B need to be subjected to treatment as promptly as possible.

The major routes of HBV infection in our country have been mother-to-child transmission and blood transfusion. However, cases with HBV transmitted through sexual contact are increasing, especially among HIV-1-seropositive patients [26]. One should bear in mind that knowledge about interactions between ETV and anti-HIV nucleoside analogues is limited [27]. Because long-term use of LAM induces LAM-resistant mutants [28], we can only use LAM for short-term treatment of patients with acute exacerbation of chronic hepatitis B. On the other hand, the present study also revealed that patients receiving ETV did not need to change drugs.

Recently, there have been several reports that reactivation of HBV is a fatal complication following systemic chemotherapy or other immunosuppressive therapy including rituximab and steroid therapies mainly in HBsAg-positive and -negative lymphoma patients. It is important to enable early diagnosis of HBV reactivation as well as initiation of antiviral therapy [29, 30].

In conclusion, ETV appears to be as effective as LAM in the treatment of patients with acute exacerbation of chronic hepatitis B. Clinicians should start to treat these patients with NUCs as soon as possible.

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ABBREVIATIONS

ETV: Entecavir; HIV: Human immunodeficiency virus; IVR: Initial virological response; LAM: Lamivudine; NUC: Nucleoside analogue.

CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

REFERENCES

1. Di Bisceglie AM. Hepatitis B and Hepatocellular Carcinoma. Hepatology. 2009; 49 (5 Suppl): S56-S60.
2. Omata M, Ehata T, Yokosuka O, et al. Mutations in the precore region of hepatitis B virus DNA in patients with fulminant and severe hepatitis. N Engl J Med. 1991; 324: 1699-1704.
3. Liang TJ, Hasegawa K, Rimon N, et al. A hepatitis B virus mutant associated with an epidemic of fulminant hepatitis. N Engl J Med. 1991; 324: 1705-1709.
4. Garg H, Sarin SK, Kumar M, et al. Tenofovir improves the outcome in patients with spontaneous reactivation of hepatitis B presenting as acute-on-chronic liver failure. Hepatology. 2011; 53: 774-780.
5. Lok AS, Lai CL. Acute exacerbation in Chinese patients with chronic hepatitis B virus (HBV) infection. Incidence, predisposing factors and etiology. J Hepatol. 1990; 10: 29-34.
6. Oketani M, Ido A, Tsubouchi H. Changing etiologies and outcomes of acute liver failure: A perspective from Japan. J Gastroenterol Hepatol. 2011; 26 (Suppl 1): 65-71.
7. Chien RN, Lin CH, Liaw YF. The effect of lamivudine therapy in hepatic decompensation during acute exacerbation of chronic hepatitis B. J Hepatol. 2003; 38: 322-327.
8. Sarin SK, Kumar A, Almeida JA, et al. Acute-on-chronic liver failure: consensus recommendations of the Asian Pacific Association for the study of the liver (APASL). Hepatol Int. 2009; 3: 269-282.
9. Dienstag JL, Perrillo R, Schiff ER, et al. A preliminary trial of lamivudine for chronic hepatitis B infection. N Engl J Med. 1999; 333: 1657-1661.
10. Lai CL, Chien RN, Leung NW, et al. A one-year trial of lamivudine for chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. N Engl J Med. 1998; 339: 61-68.
11. Dienstag JL, Schiff ER, Wright TL, et al. Lamivudine as initial treatment for chronic hepatitis B in the United States. N Engl J Med. 1999; 341: 1256-1263.
12. Potthoff A, Tillmann HL, Bara C, et al. Improved outcome of chronic hepatitis B after heart transplantation by long-term antiviral therapy. J Viral Hepat. 2006; 13: 734-741.
13. Innaimo SF, Seifer M, Bisacchi GS, et al. Identification of BMS-200475 as a potent and selective inhibitor of hepatitis B virus. Antimicrob Agents Chemother. 1997; 41: 1444-1448.
14. Ono SK, Kato N, Shiratori Y, et al. The polymerase L528M mutation cooperates with nucleotide binding-site mutations, increasing hepatitis B virus replication and drug resistance. J Clin Invest. 2001; 107: 449-455.
15. Yuen MF, Lai CL. Treatment of chronic hepatitis B: evolution over two decades. J Gastroenterol Hepatol. 2011; 26 (Suppl 1): S138-S143.
16. Kanda T, Yokosuka O, Ehata T, et al. Detection of GBV-C RNA in patients with non-A-E fulminant hepatitis by reverse-transcription polymerase chain reaction. Hepatology. 1997; 25: 1261-1265.
17. Kanda T, Yokosuka O, Ikeuchi T, et al. The role of TT virus infection in acute viral hepatitis. Hepatology. 1999; 29: 1905-1908.
18. Kamezaki H, Kanda T, Wu S, et al. Emergence of entecavir-resistant mutations in nucleos(t)ide-naïve Japanese patients infected with hepatitis B virus: Virological breakthrough is also dependent on adherence to medication. Scand J Gastroenterol. 2011; 46: 1111-1117.