Table I. *continued*.

Symbol	Name
COL1A2	Collagen, type I, alpha 2
COL3A1	Collagen, type III, alpha 1
COL5A2	Collagen, type V, alpha 2
CTNNB1	Catenin (cadherin-associated protein), beta 1, 88 kDa
DSC2	Desmocollin 2
EGFR	Epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)
ERBB3	V-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)
F11R	F11 receptor
FN1	Fibronectin 1
FOXC2	Forkhead box C2 (MFH-1, mesenchyme forkhead 1)
ILK	Integrin-linked kinase
ITGA5	Integrin, alpha 5 (fibronectin receptor, alpha polypeptide)
ITGAV	Integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)
ITGB1	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)
MMP2	Matrix metallopeptidase 2 (gelatinase A, 72 kDa gelatinase, 72 kDa type IV collagenase)
MMP3	Matrix metallopeptidase 3 (stromelysin 1, progelatinase)
MMP9	Matrix metallopeptidase 9 (gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagenase)
PAI1	Plasminogen activator inhibitor type 1
PTK2	PTK2 protein tyrosin kinase 2
RAC1	RAS-related C3 botulinum toxin substrate 1 (Rho family, small GTP binding protein RAC1)
SPP1	Secreted phosphoprotein 1
TGFB1	Transforming growth factor, beta 1
TGFB2	Transforming growth factor, beta 2
TIMP1	Tissue inhibitor of metalloproteinases-1
VCAN	Versican

Signalling pathways

Symbol	Name
Estrogen Receptor	
CAV2	Caveolin 2
ESR1	Estrogen receptor 1
KRT19	Keratin 19
TGFB3	Transforming growth factor, beta 3
G-Protein Coupled Receptor	
AKT1	V-akt murine thymoma viral oncogene homolog 1
FZD7	Frizzled homolog 7 (*Drosophila*)
GNG11	Guanine nucleotide binding protein (G protein), gamma 11
RAC1	RAS-related C3 botulinum toxin substrate 1 (Rho family, small GTP binding protein RAC1)
RGS2	Regulator of G-protein signalling 2, 24 kDa
Integrin-Mediated	
COL3A1	Collagen, type III, alpha 1
ILK	Integrin-linked kinase
ITGA5	Integrin, alpha 5 (fibronectin receptor, alpha polypeptide)
ITGAV	Integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)
ITGB1	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)
PTK2	PTK2 protein tyrosine kinase 2
Notch	
FOXC2	Forkhead box C2 (MFH-1, mesenchyme forkhead 1)
JAG1	Jagged 1 (Alagille syndrome)
NOTCH1	Notch homolog 1, translocation-associated (*Drosophila*)
Receptor Tyrosine Kinase	
EGFR	Epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)
ERBB3	V-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)
PDGFRB	Platelet-derived growth factor receptor, beta polypeptide
RGS2	Regulator of G-protein signalling 2, 24 kDa
SPARC	Secreted protein, acidic, cysteine-rich (osteonectin)

continued

Table I. *continued.*

Symbol	Name
TGFβ/BMP	
BMP1	Bone morphogenetic protein 1
BMP7	Bone morphogenetic protein 7
COL3A1	Collagen, type III, alpha 1
SMAD2	SMAD family member 2
TGFB1	Transforming growth factor, beta 1
TGFB2	Transforming growth factor, beta 2
TGFB3	Transforming growth factor, beta 3
WNT	
CTNNB1	Catenin (cadherin-associated protein), beta 1, 88 kDa
FZD7	Frizzled homolog 7 (*Drosophila*)
GSK3B	Glycogen synthase kinase 3 beta
WNT11	Wingless-type MMTV integration site family, member 11
WNT5A	Wingless-type MMTV integration site family, member 5A
WNT5B	Wingless-type MMTV integration site family, member 5B

Transcription factors

Symbol	Name
CTNNB1	Catenin (cadherin-associated protein), beta 1, 88 kDa
ESR1	Estrogen receptor 1
FOXC2	Forkhead box C2 (MFH-1, mesenchyme forkhead 1)
GSC	Goosecoid Homeobox
MITF	Microphthalmia-associated transcription factor
NOTCH1	Notch homolog 1, translocation-associated (*Drosophila*)
SIP1	Survival of motor neuron protein interacting protein 1
SMAD2	SMAD family member 2
SNAI2	Snail homolog 2 (*Drosophila*)
SNAI3	Snail homolog 3 (*Drosophila*)
SOX10	SRY (sex-determining region Y)-box 10
STAT3	Signal transducer and activator of transcription 3 (acute-phase response factor)
TCF3	Transcription factor 3 (E2A immunoglobulin enhancer binding factors E12/E47)
TCF4	Transcription factor 4
TWIST1	Twist homolog 1 (*Drosophila*)
ZEB1	Zinc finger E-box binding homeobox 1
ZEB2	Zinc finger E-box binding homeobox 2

EGF: Epidermal growth factor; MMTV: mouse mammary tumor virus.

PAI1 was reported to be associated with treatment response in patients with chronic hepatitis C, treated with pegylated-interferon plus ribavirin (23).

VIM is a member of the intermediate filament family of proteins. VIM provides cellular integrity under mechanical stress *in vivo* with a resilience not related to microtubule or actin filament networks (24). It was reported that VIM interacts with hepatitis B or C viral proteins (25, 26). Our previous study (3) showed that hepatitis B virus e antigen inhibits RIPK2 expression and interacts with RIPK2, which might represent two mechanisms through which hepatitis B virus e antigen blocks NOD1 ligand-induced NF-κB activation in HepG2 cells. We also observed that hepatitis B virus e antigen inhibits cell migration (3).

Our hypothesis is that RIPK2 plays an important role in hepatic cell migration and wound repair in the liver, possibly due to: i) activation of NF-κB through RIPK2; ii) production of inflammatory cytokines IL6 and IL8, which are also important for regeneration of the liver through activation of NF-κB; and iii) up-regulation of EMT-associated NF-κB-dependent genes, such as *PAI1* and *VIM*. In the present study, we also observed up-regulated genes such as *MMPs*, especially important in ECM and EMT. Further studies are required to determine this.

In conclusion, we observed that knockdown of *RIPK2* down-regulated the expression of the NF-κB-dependent genes *PAI1* and *VIM*. RIPK2 might play an important role in hepatic cell migration. These findings could shed new light on carcinogenesis and regeneration of the liver.

Table II. *Genes significantly down-regulated by knockdown of Receptor-Interacting Serine/Threonine Protein Kinase-2 (RIPK2) in HepG2 cells.*

Gene name	Fold change	Biological process
JAG1	−3.49	Differentiation and development, morphogenesis, cell growth and proliferation, migration and motility, Notch singalling
PAI1	−1.92	Genes up-regulated during EMT, extracellular matrix and cell adhesion
RGS2	−1.64	Genes down-regulated during EMT, G-protein coupled receptor, and receptor tyrosine kinase
CDH1	−1.61	Genes down-regulated during EMT
FGFBP1	−1.59	Genes down-regulated during EMT, cell growth and proliferation
SNAI2	−1.55	Genes up-regulated during EMT, differentiation and development
PTP4A1	−1.46	Differentiation and development
KRT19	−1.38	Genes down-regulated during EMT, estrogen receptor
VIM	−1.29	Genes up-regulated during EMT, migration and motility, cytoskeleton
SIP1	−1.24	Transcription factors

Refer to Table I for full gene names. EMT: Epithelial-to-mesenchymal transition.

Table III. *Genes significantly up-regulated by knockdown of Receptor-Interacting Serine/Threonine Protein Kinase-2 (RIPK2) in HepG2 cells.*

Gene name	Fold change	Biological process
WNT11	1.34	Differentiation and development, morphogenesis, WNT signalling
WNT5B	1.38	Genes up-regulated during EMT, differentiation and development, WNT
GSC	1.63	Genes up-regulated during EMT, differentiation and development
MMP2	1.71	Genes up-regulated during EMT, extracellular matrix and cell adhesion
MMP3	2.00	Genes up-regulated during EMT, extracellular matrix and cell adhesion
COL5A2	3.10	Differentiation and development, extracellular matrix and cell adhesion

Refer to Table I for full gene names. EMT: Epithelial-to-mesenchymal transition; WNT, wingless-type mouse mammary tumor virus (MMTV) integration site family.

Declaration of Interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Acknowledgements

This work was supported by the Japan Science and Technology Agency, Ministry of Education, Culture, Sports, Science and Technology, Japan (TK), the Chiba University Young Research-oriented Faculty Member Development Program in Bioscience Areas (TK) and a Research Grant-in-Aid from the Miyakawa Memorial Research Foundation (WS).

References

1 Meylan E and Tschopp J: The RIP kinases: crucial integrators of cellular stress. Trends Biochem Sci *30*: 151-159, 2005.

2 Adams S, Valchanova RS and Munz B: RIP2: A novel player in the regulation of keratinocyte proliferation and cutaneous wound repair? Exp Cell Res *316*: 728-736, 2010.

3 Wu S, Kanda T, Imazeki F, Nakamoto S, Tanaka T, Arai M, Roger T, Shirasawa H, Nomura F and Yokosuka O: Hepatitis B virus e antigen physically associates with receptor-interacting serine/threonine protein kinase-2 and regulates IL-6 gene expression. J Infect Dis *206*: 415-420, 2012.

4 Hasegawa M, Fujimoto Y, Lucas PC, Nakano H, Fukase K, Nunez G and Inohara N: A critical role of RICK/RIP2 polyubiquitination in NOD-induced NF-κB activation. EMBO J *27*: 373-383, 2008.

5 Kobayashi K, Inohara N, Hernandez LD, Galan JE, Nunez G, Janeway CA, Medzhitov R and Flavell RA: RICK/RIP2/CARDIAK mediates signalling for receptors of the innate and adaptive immune systems. Nature *416*: 194-199, 2002.

6 Schuppan D, Ruehl M, Somasundaram R and Hahn EG: Matrix as a modulator of hepatic fibrogenesis. Semin Liver Dis *21*: 351-372, 2001.

7 Schultz GS and Wysocki A: Interactions between extracellular matrix and growth factors in wound healing. Wound Repair Regen *17*: 153-162, 2009.

8 Cox TR and Erler JT: Remodeling and homeostasis of the extracellular matrix: implications for fibrotic diseases and cancer. Dis Model Mech *4*: 165-178, 2011.

9 Lopez-Novoa JM and Nieto MA: Inflammation and EMT: An alliance towards organ fibrosis and cancer progression. EMBO Mol Med *1*: 303-314, 2009.

10 Min C, Eddy SF, Sherr DH and Sonenshein GE: NF-κB and epithelial to mesenchymal transition of cancer. J Cell Biochem *104*: 733-744, 2008.

11 Rhim AD, Mirek ET, Aiello NM, Maitra A, Bailey JM, McAllister F, Reichert M, Beatty GL, Rustgi AK, Vonderheide RH, Leach SD and Stanger BZ: EMT and dissemination precede pancreatic tumor formation. Cell *148*: 349-361, 2012.

12 Wu S, Kanda T, Imazeki F, Arai M, Yonemitsu Y, Nakamoto S, Fujiwara K, Fukai K, Nomura F and Yokosuka O: Hepatitis B virus e antigen down-regulates cytokine production in human hepatoma cell lines. Viral Immunol *23*: 467-476, 2010.

13 Wu S, Kanda T, Imazeki F, Nakamoto S, Shirasawa H and Yokosuka O: Nuclear receptor mRNA expression by HBV in human hepatoblastoma cell lines. Cancer Lett *312*: 33-42, 2011.

14 Porto Neto LR, Jonsson NN, Ingham A, Bunch RJ, Harrison BE, Barendse W and Cooperative Research Center for Beef Genetic Technologies: The RIPK2 gene: a positional candidate for tick burden supported by genetic associations in cattle and immunological response of knockout mouse. Immunogenetics *64*: 379-388, 2012.

15 Nakayama N, Nakamura T, Okada H, Iwaki S, Sobel BE and Fujii S: Modulators of induction of plasminogen activator inhibitor type-1 in HepG2 cells by transforming growth factor–β. Coron Artery Dis *22*: 468-478, 2011.

16 Maier HJ, Schmidt-Strassburger U, Huber MA, Wiedemann EM, Beug H and Wirth T: NF-κB promotes epithelial-mesenchymal transition, migration and invasion of pancreatic carcinoma cells. Cancer Lett *295*: 214-228, 2010.

17 Rossi SW, Jeker LT, Ueno T, Kuse S, Keller MP, Zuklys S, Gudkov AV, Takahama Y, Krenger W, Blazar BR and Hollander GA: Keratinocyte growth factor (KGF) enhances postnatal T-cell development *via* enhancements in proliferation and function of thymic epithelial cells. Blood *109*: 3803-3811, 2007.

18 Han YP, Tuan TL, Wu H, Hughes M and Garner WL: TNF-α stimulates activation of pro-MMP2 in human skin through NF-κB-mediated induction of MT1-MMP. J Cell Sci *114(Pt 1)*: 131-139, 2001.

19 Gao D and Bing C: Macrophage-induced expression and release of matrix metalloproteinase 1 and 3 by human preadipocytes is mediated by IL-1β *via* activation of MAPK signalling. J Cell Physiol *226*: 2869-2880, 2011.

20 Gils A and Declerck PJ: Plasminogen activator inhibitor-1. Curr Med Chem *11*: 2323-2334, 2004.

21 Hillon P, Guiu B, Vincent J and Petit JM: Obesity, type 2 diabetes and risk of digestive cancer. Gastroenterol Clin Biol *34*: 529-533, 2010.

22 Divella R, Lacalamita R, Tommasi S, Coviello M, Daniele A, Garrisi VM, Abbate I, Simone G, Gadaleta C, Paradiso A and Quaranta M: PAI-1, t-PA and circulating hTERT DNA as related to virus infection in liver carcinogenesis. Anticancer Res *32*: 141-145, 2012.

23 Miki D, Ohishi W, Ochi H, Hayes CN, Abe H, Tsuge M, Imamura M, Kamatani N, Nakamura Y and Chayama K: Serum PAI-1 is a novel predictor for response to pegylated interferon-α-2b plus ribavirin therapy in chronic hepatitis C virus infection. J Viral Hepat *19*: e126-e133, 2012.

24 Goldman RD, Khuon S, Chou YH, Opal P and Steinert PM: The function of intermediate filaments in cell shape and cytoskeletal integrity. J Cell Biol *134*: 971-983, 1996.

25 Zhang L, Zhang W, Shao C, Zhang J, Men K, Shao Z, Yan Y and Xu D: Establishment and characterization of a spontaneously immortalized trophoblast cell line (HPT-8) and its hepatitis B virus-expressing clone. Hum Reprod *26*: 2146-2156, 2011.

26 Ghosh S, Ahrens WA, Phatak SU, Hwang S, Schrum LW and Bonkovsky HL: Association of filamin A and vimentin with hepatitis C virus proteins in infected human hepatocytes. J Viral Hepat *18*: e568-e577, 2011.

Received May 10, 2012
Revised July 18, 2012
Accepted July 19, 2012

BRIEF REPORT

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.

Received 1 January 2012; accepted 3 February 2012; electronically published 21 May 2012.
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).

The Journal of Infectious Diseases 2012;206:415–20
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DOI: 10.1093/infdis/jis363

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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

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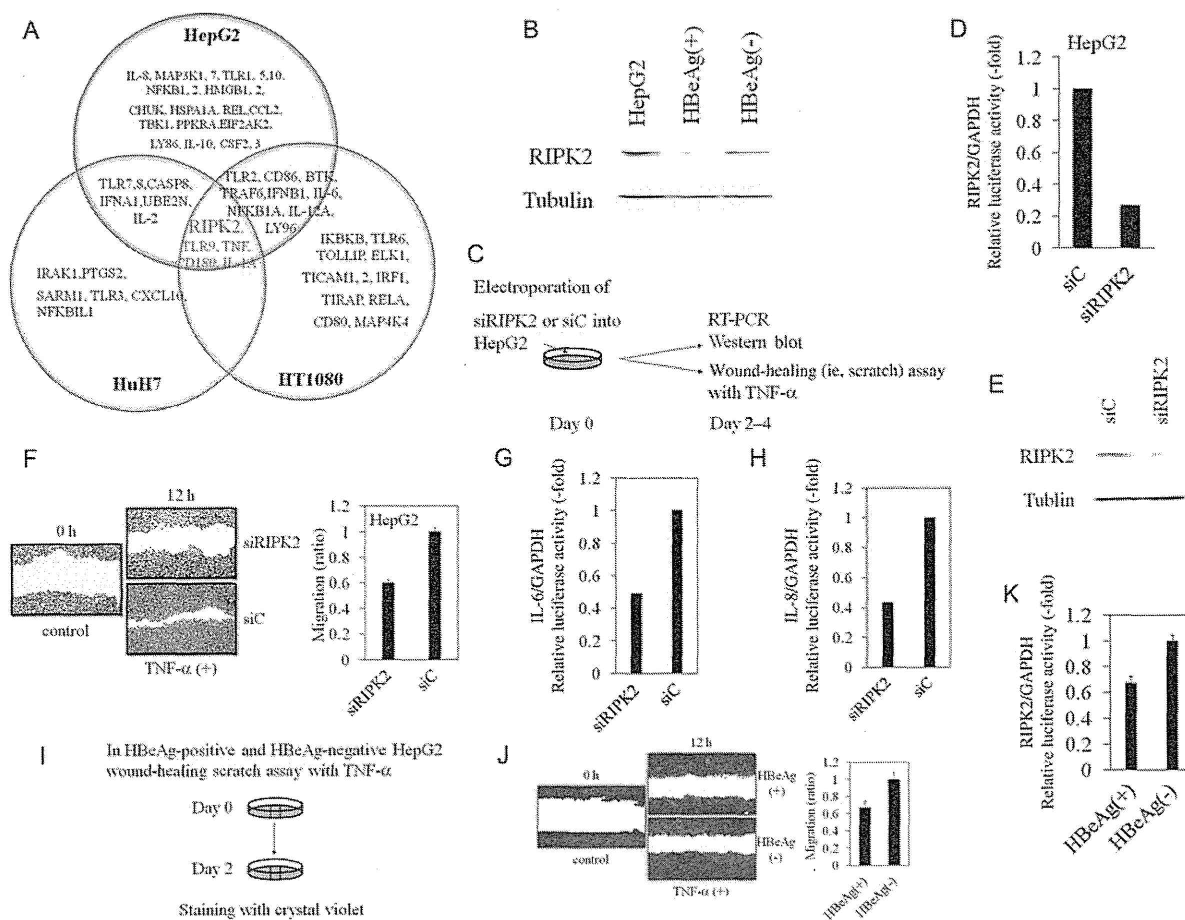

Figure 1. Receptor-interacting serine/threonine protein kinase 2 (RIPK2) expression is downregulated by hepatitis B virus e antigen (HBeAg), and knockdown of RIPK2 and HBeAg impairs hepatic wound repair. *A*, Venn diagram representing Toll-like receptor (TLR)–related genes downregulated ≥1.3-fold in 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′-AGACACTACTGACATCCAAG-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

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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

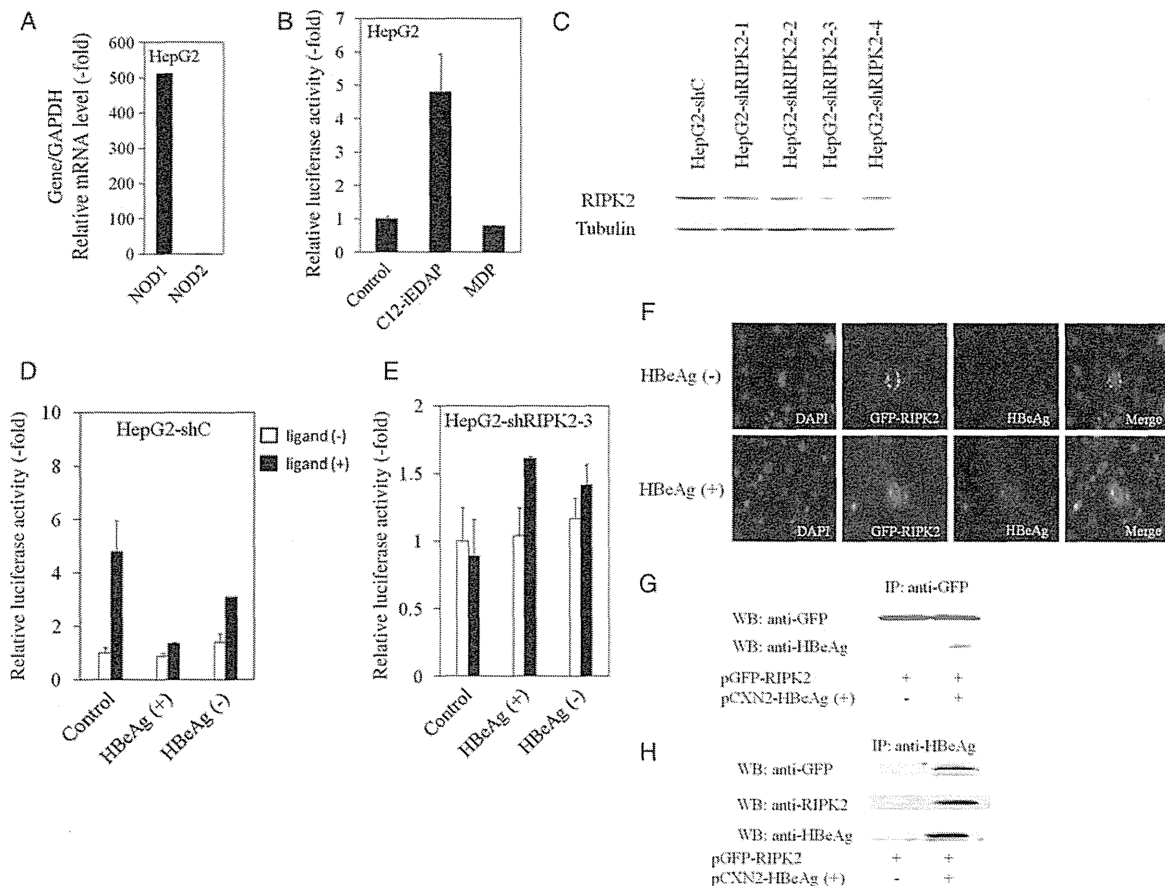

Figure 2. The nucleotide-binding oligomerization domain–containing protein 1 (NOD1) ligand C12-iEDAP induces NF-κB activation, knockdown of receptor-interacting serine/threonine protein kinase 2 (RIPK2) inhibits NOD1 ligand–induced NF-κB activation in HepG2 cells, and hepatitis B virus e antigen (HBeAg) 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.

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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

Acknowledgments. We thank Prof John C. Reed and Prof Junichi Miyazaki, for providing the plasmids, and Ms. Satomi Hasegawa, for providing technical assistance.

Financial support. This work was supported by the Japan Science and Technology Agency, Ministry of Education, Culture, Sports, Science, and Technology, Japan (21590829 to T. K. and 21590828 to F. I.); the Japan Society of Hepatology (T. K.); the Chiba University Young Research-Oriented Faculty Member Development Program in Bioscience Areas (T. K.); and the Research Grant-in-Aid from Miyakawa Memorial Research Foundation (W. S.).

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.

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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 downregulates 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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Hepatology Research

Hepatology Research 2012; 42: 523–542 doi: 10.1111/j.1872-034X.2012.00981.x

Special Report

Guideline on the use of new anticancer drugs for the treatment of Hepatocellular Carcinoma 2010 update

Shuichi Kaneko,[1,2,3] Junji Furuse,[1,2,4] Masatoshi Kudo,[1,2,5] Kenji Ikeda,[1,2,6] Masao Honda,[2,5] Yasunari Nakamoto,[2,7] Morikazu Onchi,[2,8] Goshi Shiota,[2,9] Osamu Yokosuka,[2,10] Isao Sakaida,[2,11] Tetsuo Takehara,[2,12] Yoshiyuki Ueno,[2,13] Kazumasa Hiroishi,[2,14] Shuhei Nishiguchi,[2,15] Hisataka Moriwaki,[2,16] Kazuhide Yamamoto,[2,17] Michio Sata,[2,18] Shuntaro Obi,[2,19] Shiro Miyayama[2,20] and Yukinori Imai[2,21]

[1]*Novel Anticancer Agent Guideline Drafting Committee,* [2]*Study Group on New Liver Cancer Therapies, Departments of* [3]*Gastroenterology, Graduate School of Medicine, Kanazawa University, Kanazawa,* [4]*Medical Oncology, Kyorin University School of Medicine, Mitaka,* [5]*Gastroenterology, Kinki University, Higashiosaka,* [6]*Hepatology, Toranomon Hospital, Tokyo,* [7]*Gastroenterology, Graduate School of Medicine, Fukui University, Fukui,* [8]*Gastroenterology and Metabology, Graduate School of Medicine, Ehime University, Matsuyama,* [9]*Division of Molecular and Genetic Medicine, Tottori University Graduate School of Medicine, Tottori, Departments of* [10]*Medicine and Clinical Oncology, Graduate School of Medicine, Chiba University, Chiba, Japan,* [11]*Gastroenterology and Hepatology, Yamaguchi University Graduate School of Medicine, Yamaguchi,* [12]*Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, Osaka,* [13]*Gastroenterology, Tohoku University Graduate School of Medicine, Sendai,* [14]*Division of Gastroenterology, Department of Medicine, Showa University School of Medicine, Tokyo,* [15]*Division of Hepatobiliary and Pancreatic Diseases, Department of Internal Medicine, Hyogo College of Medicine, Kakogawa, Departments of* [16]*Gastroenterology, Gifu University Graduate School of Medicine, Gifu,* [17]*Gastroenterology and Hepatology, Okayama University Graduate School of Medicine, Okayama,* [18]*Division of Gastroenterology, Department of Medicine, Kurume University School of Medicine, Kurume, Departments of* [19]*Hepatology, Kyoundo Hospital, Tokyo,* [20]*Diagnostic Radiology, Fukuiken Saiseikai Hospital, Fukui, and* [21]*Gastroenterology and Hepatology, Faculty of Medicine, Saitama Medical University, Saitama, Japan*

The "*Guideline on the Use of New Anticancer Drugs for the Treatment of Hepatocellular Carcinoma*" was prepared by the Study Group on New Liver Cancer Therapies established by the "*Research Project on Emergency Measures to Overcome Hepatitis*" under the auspices of the Health and Labour Sciences Research Grant. The Guideline brings together data collected by the Study Group on the use and incidence of adverse events in 264 patients with advanced hepatocellular carcinoma (HCC) treated using sorafenib and in 535 patients with advanced HCC treated using miriplatin at 16 participating institutions up until 22 December 2010, as well as referring to the published studies, academic presentations, and reports from the private sector. The aim of this Guideline is to facilitate understanding and current thinking regarding the proper usage of new anticancer drugs towards actual use in therapy. In terms of the format, the Guideline presents "clinical questions" on issues pertaining to medical care, makes "recommendations" on diagnosis and treatment in response to each of these clinical questions, and provides a rationale for these recommendations in the form of "scientific statements".

Key words: hepatic arterial infusion, hepatocellular carcinoma, miriplatin, molecular targeting therapy, sorafenib

Correspondence: Dr Shuichi Kaneko, 13-1 Kanazawa, Ishikawa, Japan 920-8641. Email: skaneko@m-kanazawa.jp

This article was previously published in Japanese in Kanzo 52: 8, pp. 532–551. June 2011.
Received 10 July 2011; revision 18 January 2012; accepted 29 January 2012.

INTRODUCTION

THE MOLECULAR-TARGETED agent sorafenib has been found to significantly prolong survival in patients with hepatocellular carcinoma (HCC).[1,2] In May 2009, sorafenib was approved in Japan for unresectable

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HCC. Furthermore, miriplatin was approved in Japan for the treatment of HCC in January 2010, and clinical trials are also currently underway on a number of other promising new anticancer agents. Treatment of HCC is thus undergoing a period of major transition, but the role of these anticancer drugs and conventional therapies remains unclear, leading to concerns about the risk of serious adverse events (SAEs).

The Study Group on New Liver Cancer Therapies (the Study Group) was formed as part of the "Research Project on Emergency Measures to Overcome Hepatitis" sponsored by the Health and Labour Sciences Research Grant, with the overall purpose of formulating a guideline to facilitate understanding on the practical usage of new anticancer drugs.

The Study Group collected information on the use of new anticancer drugs, sorafenib and miriplatin at 16 affiliated institutions and compiled current opinions regarding the proper use of these drugs based on published studies, academic conference papers and reports from the private sector. These results have now been compiled in the form of a guideline.

However, of note is that this guideline is provisional and has been prepared to expedite the provision of proper information because information on these new anticancer drugs is constantly being updated.

STUDY METHODS, SUBJECTS AND PARTICIPATING INSTITUTIONS

Basic statistics

THE STUDY GROUP'S "New Liver Cancer Therapies" (NLCT) study was based on data from patients with advanced HCC treated using sorafenib or miriplatin up until 22 December 2010 at the participating institutions. Clinical data were recorded by each institution in case report files (CRFs) created by the Study Group. Of the patients enrolled in this study, 264 were treated with sorafenib and 535 were treated with miriplatin. Any input variables that were unclear were excluded from the analyzed data. After analyzing collecting data on the use of these drugs, the Study Group compiled current opinions on proper use based on published papers, academic conference papers and reports from the private sector. The Study Group proposed a series of "clinical questions" (CQ) on issues pertaining to practical medical care and summarized the current evidence in response to each of these CQ in the form of "scientific statements", as well as making "recommendations".

Participating institutions

The 16 institutions that participated in this study were: Kinki University; Chiba University; Yamaguchi University; Kurume University; Kyorin University; Showa University; Ehime University; Okayama University; Kyoundo Hospital; Tohoku University; Osaka University; Gifu University; Hyogo College of Medicine; Toranomon Hospital; Saitama Medical University; and Kanazawa University.

RESULTS

Sorafenib therapy

Indications

CQ1-1 For which patients with HCC is sorafenib therapy indicated?

Recommendation Sorafenib therapy is indicated in HCC patients with good performance status (PS) and Child–Pugh class A for whom surgical resection, local ablation therapy (LAT), and transcatheter arterial chemoembolization (TACE) are not possible or not indicated.

The safety and efficacy of sorafenib has not been established in Child–Pugh class B/C patients.

Furthermore, the usefulness of sorafenib as adjuvant chemotherapy after resection, LAT, or TACE of HCC has not been demonstrated.

Scientific statement Two randomized, placebo-controlled trials demonstrating the usefulness of sorafenib were conducted on patients in whom surgical resection, LAT and TACE were not indicated or who were unresponsive to TACE.[1,2]

The Japan Society of Hepatology provides the following definitions for impossible and refractory cases to TACE.[3]

Definition of "Impossible cases to TACE"

1 Deterioration of treated vessel resulting in inability to select catheter for insertion into the nutrient vessel;
2 Deterioration in hepatic function to Child–Pugh class C due to repeated treatment;
3 Patients with tumor thrombus in main trunk or first branch of portal vein;
4 Patients with large arterio-portal shunts.

Definition of "Refractory cases to TACE"

(1) Intrahepatic lesion(s)
 (i) Poor Lipiodol deposits (≤50%) observed on at least two consecutive occasions in computed tomography (CT) assessment of therapeutic response immediately after (>1 month) correctly performing TACE;

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(ii) Multiple new lesions observed on at least two consecutive occasions in CT assessment of therapeutic response immediately after (>1 month) TACE;

(2) Appearance of vascular invasion;
(3) Appearance of distant metastasis;
(4) Tumor markers.
  (i) Continued increase in tumor markers with transient decrease only immediately after TACE procedure.

In the present NLCT study, as many as 91% of patients underwent prior treatment, in whom 29% received hepatic arterial infusion chemotherapy (HAIC). Comparison of the characteristics of the remaining NLCT study patients with those of previous clinical trials[1,2,4–6] is presented in Table 1.

An adverse event (AE) report on all-patient special drug use surveillance (SDUS) conducted in Japan[7] contains analysis and reporting of AEs for 777 patients for whom CRFs were collected up until 19 December 2009.

That report compared the clinical characteristics for 51 of these 777 patients who died within 30 days of treatment ("early death group") and the 382 patients who survived for ≥61 days ("control survival group"). The data indicate that the prevalence of Eastern Cooperative Oncology Group (ECOG) PS grades ≥2 tended to be high among patients in the "early death group" at 5.9% compared with those in the "control survival group" at 0.5%, suggesting the need to carefully follow the course of patients with poor PS. In the NLCT study, 98% of patients had a PS score of 0–1.

In terms of hepatic function, two randomized, placebo-controlled trials demonstrating the usefulness of sorafenib were conducted on Child–Pugh class A patients.[1,2]

Meanwhile, in the NLCT study, 81% of evaluable patients were Child–Pugh class A, and 94% had a Child–Pugh score of ≤7. Comparison of treatment results of Child–Pugh class A and B patients did not reveal any difference in tumor control rates (46% vs. 50%; $P = 0.52$), but overall survival (OS) was inferior in Child–Pugh class B patients (median OS: 11.5 months vs. 5.2 months; $P < 0.01$).

In a Phase I trial conducted in Japan, no clear increase in toxicity was observed in Child–Pugh class B patients compared with Child–Pugh class A patients.[8] On the other hand, the aforementioned SDUS found that hepatic functional reserve was poor in the "early death group" compared to the "control survival group".[4]

A Phase II study of sorafenib therapy in HCC patients including those with Child–Pugh class B is currently underway in Japan (UMIN [University Hospital Medical Information Network] 000002972). Another study currently being conducted worldwide is the Global Investigation of therapeutic decisions in HCC and of its treatment with sorafenib (GIDEON); a large-scale prospective study on actual sorafenib therapy of patients with unresectable HCC. The GIDEON study is recruiting 3000 patients from over 400 sites in more than 40 countries in the Asia-Pacific region, Europe, USA, Latin America, and Japan.[9] The study's first interim analysis has been released and the findings of 511 recruited patients including those in Child–Pugh class B have been examined. No significant difference in grade 3 or 4 AEs was found to exist between Child–Pugh class A and B patients, at 31% and 38%, respectively.[10] Future GIDEON study analyses are expected to provide crucial information concerning the safety of sorafenib for Child–Pugh class B patients.

A Phase III study of post-TACE adjuvant sorafenib chemotherapy versus placebo conducted in Japan and South Korea failed to demonstrate the usefulness of sorafenib administration.[11] In addition, a Phase III placebo-controlled trial of adjuvant sorafenib chemotherapy following radical treatment (either surgical resection or LAT) of HCC (STORM Trial) is currently underway.[12]

The NLCT study did not include any patients treated with sorafenib as adjuvant chemotherapy.

Method of administration

CQ1-2 What is the optimal dosage regimen for sorafenib therapy?

Recommendation The standard dosage regimen for sorafenib therapy is 400 mg administered twice daily (800 mg/day).

The safety and efficacy of sorafenib therapy in combination with other anti-neoplastic agents or TACE have not been established.

Scientific statement In the two aforementioned randomized, placebo-controlled trials demonstrating the usefulness of sorafenib, a single 400 mg dose of sorafenib was administered twice daily (800 mg/day),[1,2] and usefulness was not observed at a reduced dosage. A high-fat diet reportedly lowers the plasma concentration of sorafenib so administration should be avoided from 1 h before to 2 h after meals.

Reduced dose regimen due to AEs was conducted in the abovementioned studies as follows:
Step-down dose (step 1): 400 mg once a day
Step-down dose (step 2): 400 mg every another day

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Table 1 Characteristics of patients receiving sorafenib therapy

	NLCT Study (*n* = 264) %	SDUS[4,6] (*n* = 777) %	SHARP Trial[1] (*n* = 299) %	Asia-Pacific Trial[2] (*n* = 150) %	Sorafenib phase II[5] (*n* = 137) %
Age (years)					
Median	70		64.9 ± 11.2	51	69
Range	33–87		(mean ± SD)	23–86	28–86
Gender					
Male	79		87	84.7	71
PS					
0	83	69.5	54	25.3	50
1	15	26.5	38	69.3	50
Child–Pugh class					
A	81	88.2	95	97.3	72
B	19	9.9	5	2.7	28
HBs antigen					
Positive	20	24.6	19	70.7	17
HCV antibody					
Positive	62	52.2	29	10.7	48
Prior treatment					
Yes	91	91.2	49		
Resection	31		19		
LAT	47		15		
TACE	78		29		
HAIC	29				
Advanced vascular invasion					
Yes	18		36	36.0	
Extrapulmonary lesion(s)					
Yes	51	54.4	53	68.7	–
Lymph node(s)	22	15.4	30	52	–
Lung(s)	26	30.6	22	30.7	–
Maximum tumor size (mm)	34				
Range	7–170				
≥30 mm	59				
Stage	†	‡	§	§	‡
I	1	1.2			0
II	9	4.8			3
III	30	20.7	B: 18	B: NE	31
IV A	17	23	C: 82	C: 95.3	66
IV B	43	47.6			
T-Bil (mg/dL)					
Median	0.8		0.7		
Range	0–7.7		0.1–16.4		
Alb (g/dL)					
Median	3.5		3.9		
Range	1.7–4.8		2.7–5.3		
AFP (ng/mL)					
Median	218		44.3		
Range	0.8–252150		0–2080000		
≥10	84			77.3	76

†Japanese Classification of Liver Cancer.
‡UICC classification.
§BCLC classification.
AFP, α fetoprotein; Alb, albumin; HAIC, hepatic arterial infusion chemotherapy; HBs, Hepatitis B surface antigen; HCV, hepatitis C virus; LAT, local ablation therapy; NLCT, New Liver Cancer Therapies; PS, performance status; SD, standard deviation; SDUS, special drug use surveillance; SHARP, sorafenib hepatocellular carcinoma assessment randomized protocol; TACE, transcatheter arterial chemoembolization; T-Bil, total bilirubin.

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In the NLCT study, 77% of patients received the standard dosage regimen of 400 mg twice daily, while 21% were started on a reduced dose.

Comparison of the group started on the standard dose of 800 mg/day and the group started on a reduced dose did not reveal any significant differences in either duration of treatment (117 days vs. 81 days; $P = 0.05$) or number of dosing days (107 days vs. 78 days; $P = 0.10$). Furthermore, dosage was subsequently increased in 22% of the reduced initial dose group. Daily dosage intensity (DI) was 615 mg in the standard-dose group and 387 mg in the reduced-dose group.

It is conceivable to start sorafenib therapy at a reduced dose according to the condition of the patient or prevention of AEs. Because efficacy at reduced doses has not been demonstrated, as long as no AEs are encountered in the course of treatment, consideration should be given to increasing the dose to the standard dosage regimen.

With regard to sorafenib combination therapies, Phase I and Phase II studies on systemic chemotherapy in combination with sorafenib therapy have been published for radiotherapy,[13,14] doxorubicin,[15] tegafur/uracil,[16] and octreotide.[17] Several Japanese clinical trials are also being conducted on combination therapy, specifically low-dose cisplatin/fluorouracil HAIC (UMIN000004315), cisplatin HAIC (UMIN000001496), and S-1 chemotherapy (UMIN000002418, UMIN000002590). Therapies combining sorafenib with other anti-neoplastic agents are therefore still in the research stage, and their efficacy is yet to be demonstrated.

In terms of sorafenib combined with LAT, a Phase III placebo-controlled trial of adjuvant sorafenib chemotherapy following radical treatment (surgical resection or LAT) of HCC (STORM Trial) is presently underway.[12] Meanwhile, sorafenib combined with TACE has been investigated in a Phase III study of post-TACE adjuvant sorafenib chemotherapy versus placebo conducted in Japan and South Korea, but the study failed to demonstrate the usefulness of sorafenib administration.[11] Another Phase II trial on TACE in combination with sorafenib is presently being carried out in Japan (TACTICS; UMIN 000004316).

Discontinuation criteria

CQ1-3 How and when should sorafenib therapy be discontinued?

Recommendation Administration of sorafenib should be discontinued immediately in the event of SAEs.

Discontinuation should also be considered when disease progression is confirmed by radiological imaging or on the basis of patient symptoms.

Scientific statement In the two randomized, placebo-controlled trials demonstrating the usefulness of sorafenib therapy, administration was discontinued upon confirmation of radiologic or symptomatic progression or in the event of SAEs.[1,2]

In the NLCT study, sorafenib therapy was discontinued in 185 patients with 63% due to disease progression and 22% due to AEs. Moreover, 60% of discontinued patients did not undergo post-treatment.

No data are currently available on the efficacy/safety of continued administration of sorafenib after disease progression.

Adverse events

CQ1-4 What are the adverse events associated with sorafenib therapy?

Recommendation Some form of AE has appeared in almost all patients treated with sorafenib.

These AEs vary, and have even included serious adverse events (SAEs) resulting in death. Familiarity with these AEs is therefore essential, to carefully monitor patient progress while taking the necessary precautions, and to respond rapidly when an AE occurs.

The following AEs are known to occur frequently in patients treated with sorafenib.

1 Hand-foot skin reaction (HFSR);
2 Rash/desquamation;
3 Diarrhea;
4 Anorexia;
5 Hypertension;
6 Fatigue;
7 Alopecia;
8 Nausea.

While infrequent, life-threatening SAEs include hepatic failure, interstitial pneumonia, and gastrointestinal hemorrhage.

In addition, the following blood test abnormalities are known to occur frequently in patients treated with sorafenib.

1 Leukopenia;
2 Neutropenia;
3 Anemia;
4 Thrombocytopenia;
5 Hepatic impairment (elevated AST [aspartate aminotransferase], ALT [alanine aminotransferase], ALP [alkaline phosphatase], γ-GTP [γ-glutamyltransferase], T-Bil [total bilirubin]);

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6 T-Bil elevation;
7 Amylase elevation;
8 Electrolyte abnormality (hyponatremia, hypokalemia, hypocalcemia, hypophosphatemia);
9 Hypoalbuminemia.

Scientific statement The incidence of sorafenib-related AEs was 80% in the Sorafenib Hepatocellular Carcinoma Assessment Randomized Protocol (SHARP) trial and 81.9% in the Asia-Pacific trial. Frequently occurring AEs were HFSR, rash/desquamation, diarrhea, anorexia, hypertension, fatigue, alopecia, and nausea.[1,2]

Sorafenib-related AE incidence in the NLCT study was 87%, of which 36% were ≥grade 3 AEs. While incidences of HFSR, diarrhea and alopecia in the NLCT study were similar to those of the Asia-Pacific trial[2] and SDUS,[6] incidences of rash/desquamation, anorexia, hypertension and fatigue were slightly higher in the present study (Table 2).

Evaluation of changes in clinical laboratory data was achieved by examining the CRFs to find the largest variations during sorafenib therapy, as well as the test date on which variations occurred. Consequently, the frequency of abnormal values in the NLCT study differed from those of the SHARP trial[1] and SDUS[6] (Table 3).

Changes in laboratory values were seen in 96% of the sorafenib group, with 64% showing an AE ≥ grade 3. Incidence of diminished blood cell counts was high compared with previous studies, with thrombocytopenia, leukopenia, neutropenia, and anemia seen in 56%, 43%, 37%, and 34% of the sorafenib group, respectively.

Hepatic impairment was also frequent, with elevated AST and ALT occurring in ≥50% of sorafenib-treated patients (70% and 55%, respectively), of whom a further 25% and 15% had AST and ALT readings ≥grade 3, indicating levels exceeding 200 IU/L after commencement of treatment. Similar results were observed for ALP and γ-GTP. Elevated T-Bil was seen in 53% of the sorafenib group, of whom 11% had readings that were ≥grade 3, which is more than three times the upper limit of normal (ULN).

Increased amylase was seen in 49% of the sorafenib group, of whom 12% had levels ≥grade 3, which is more than twice the ULN. In terms of electrolyte abnormalities, hyponatremia and hypokalemia were observed in 50% and 25% of the sorafenib group, respectively. Hypocalcemia and hypophosphatemia were also seen in ≥50% of the sorafenib group, but the valid response rate was low for these variables.

Hypoalbuminemia was seen in 48% of the sorafenib group, of whom only 5% had readings <2.0 g/dL.

No significant difference was seen in AE incidences for Child–Pugh class A and B patients, at 88% and 83%, respectively ($P = 0.53$). The incidence of AEs ≥grade 3 was also insignificant between Child–Pugh class A and B patients (35% vs. 39%, $P = 0.76$).

Similar comparisons for sorafenib group patients with Child–Pugh class A scoring 5 and 6 also did not reveal any significant differences in either total incidence of AEs at 89% and 88%, respectively ($P > 0.99$), or in the incidence of AEs ≥ grade 3, at 35% each ($P > 0.99$).

Incidence of abnormal laboratory data also did not vary significantly among Child–Pugh class A and B patients, at 96% and 95%, respectively ($P > 0.99$). Similarly, no significant difference was observed in the incidence of abnormal laboratory data ≥grade 3, at 63% and

Table 2 Incidence of drug-related adverse events with sorafenib therapy

AE	NLCT Study ($n = 264$)		SDUS[6] ($n = 777$)		SHARP Trial[1,6] ($n = 267$)		Asia-Pacific Trial[2] ($n = 149$)	
	Total (%)	G3/4 (%)	Total (%)	SAEs (%)	Total (%)	G3/4 (%)	Total (%)	G3/4 (%)
HFSR	44	10	47.9	2.8	21.2	7.7	45.0	10.7
Rash/desquamation	31	5	20.7	3.1	15.8	1.08	21.1	0.7
Diarrhea	32	5	21.9	1.4	39.1	8.4	25.5	6.0
Anorexia	27	4	13.8	1.9	13.8	0.3	12.8	0
Hypertension	26	8	19.2	0.6	5.1	1.7	18.8	2.0
Fatigue	24	2	4.6	0.6	–	–	20.1	3.4
Alopecia	15	0	11.4	–	13.8	–	24.8	–
Nausea	10	1	4.0	0.3	11.1	0.3	11.4	0.7

Common Terminology Criteria for Adverse Events (CTC-AE) v3.0
HFSR, hand-foot skin reaction; NLCT, New Liver Cancer Therapies; SDUS, special drug use surveillance; SHARP, sorafenib hepatocellular carcinoma assessment randomized protocol.

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Table 3 Abnormal clinical laboratory values with sorafenib therapy

Clinical laboratory data	NLCT Study ($n = 264$)		SDUS[6] ($n = 777$)		SHARP Trial[1,6] ($n = 297$)	
	AE incidence					
	Total (%)	G3/4 (%)	Total (%)	SAEs (%)	Total (%)	G3/4 (%)
Leukopenia	43	8	1.9	0.3	0.3	0.3
Neutropenia	37	6	0.9	0.2	–	–
Anemia	34	11	0.8	0.2	4.4	1.3
Thrombocytopenia	56	12	8.5	0.9	1.7	0.7
PT-INR	25	2	–	–	–	–
Elevated AST	70	25	1.4	–	1.7	1.7
Elevated ALT	55	15	0.9	0.2	0.7	0.7
Elevated ALP	35	5	0.3	–	–	–
Elevated γ-GTP	36	19	0.2	–	–	–
Elevated T.Bil	53	11	2.6	0.2	0.7	–
Elevated amylase	49	12	4.2	–	–	–
Elevated lipase	78	37	3.7	–	1.3	–
Elevated Cre	23	2	–	–	–	–
Hyponatremia	50	14	–	–	–	–
Hypokalemia	25	6	–	–	–	–
Hypocalcemia	55	1	–	–	–	–
Hypophosphatemia	66	29	3.6	0.5	34.9	10.5
Hypoalbuminemia	48	5	1.1	–	–	–

Common Terminology Criteria for Adverse Events (CTC-AE) v3.0.
ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GTP, γ-glutamyltransferase; NLCT, New Liver Cancer Therapies; SAEs, severe adverse events; SDUS, special drug use surveillance; SHARP, sorafenib hepatocellular carcinoma assessment randomized protocol; T-Bil, total bilirubin.

66% of class A and B patients, respectively. Performing the same comparisons for sorafenib group patients with Child–Pugh class A scoring 5 and 6 also failed to reveal any significant differences either in total incidence of abnormal laboratory values (97% and 95%, respectively; $P > 0.80$) or in the incidence of abnormal laboratory data ≥ grade 3 (58% and 68%, respectively; $P > 0.26$), despite a higher percentage for patients with Child–Pugh score 6.

AE management

CQ1-5 What measures should be taken in management to sorafenib-related AEs?

Recommendation Preventative measures and careful monitoring of the patient are required for frequently occurring AEs such as HFSR, hypertension, and hepatic impairment.

Patients undergoing sorafenib therapy often experience AEs soon after beginning of treatment. Careful monitoring of the patient by carrying out blood test and medical examinations etc. at least once a week for 4 weeks after initiating therapy is therefore preferable.

Scientific statement The NLCT study investigated measures taken in management to sorafenib-related AEs (Table 4). Management to HFSR was common, with topical application of emollients performed most frequently (69%), and followed by topical application of steroids (38%) and consultation to a dermatologist

Table 4 Incidence of drug-related adverse events with sorafenib therapy

Response to AE	Valid responses %	Prevention for AE %
Consultation to dermatologist	89	24
Steroid ointment	89	38
Emollient	91	69
Hypotensive drug dose increased	90	21
Intestinal drug	90	19
Anti-diarrheal drug	89	16
Antiemetic drug	89	5

AE, adverse event.

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(24%). An increased dose of hypotensive drugs was prescribed in 21% of patients, while diarrhea was treated with antiflatulent and anti-diarrheal drugs in 19% and 16% of patients, respectively. Antiemetic agents were administered in 5% of patients.

Most AEs observed in the NLCT study, including abnormal laboratory values, occurred early at up to 8 weeks after initiating sorafenib therapy. For this reason, careful, early monitoring of the patient is essential. Bayer Yakuhin's "Nexavar Proper Use Guidelines"[7] recommends that a battery of tests be performed regularly or as required during sorafenib therapy (Table 5). Educating patients to withhold taking the drug and consult their doctors immediately if they begin to feel unwell early in the treatment is another important way to prevent AEs from becoming severe.

Serious adverse events (SAEs) should generally be handled by immediately withholding administration or reducing the dose, and reinstitution of treatment or dose increase can be considered if the patient recovers.

Provided below is a summary of management to prevent and respond to major sorafenib AEs.

- Hand-foot skin reaction (HFSR)

Prevention: HFSR occurs most frequently in areas affected by hyperkeratosis and induration. Risk factors for HSFR include physical stimulation of the skin such as compression, heat or friction, so the patient's hands and feet should always be inspected before treatment. Any thickening of the stratum corneum should be removed and the patient instructed to cover and bathe the affected areas to prevent physical stimulation. An emollient containing urea or salicylic acid should be applied to the hands from 1–2 weeks before commencing therapy.[7]

Management: Minor, painless skin changes such as erythema can be treated with steroid ointment without reducing or discontinuing sorafenib therapy. If further deterioration such as formation of blisters occurs, the dosage should be reduced. If the condition interferes with the patient's activities of daily living due to ulcers, cracking or pain etc., the therapy should be withheld and the patient consulted to a dermatologist as necessary. If the condition improves after withholding the sorafenib, therapy can be resumed at a reduced dose, and can subsequently be increased on the basis of the AE condition.

- Hepatic impairment, hepatic failure and hepatic encephalopathy

Prevention: Sorafenib therapy should be avoided in patients with severe liver impairment; particularly those with AST and ALT levels exceeding 200 IU/L.

Management: The patient should be carefully monitored by performing medical examinations and hepatic function tests once weekly for the first month of treatment, once fortnightly for the next 3 months, and once monthly thereafter. Reducing, withholding, or discontinuing sorafenib therapy should be considered if the patient exhibits symptoms of hepatic failure including hepatic encephalopathy and ascites or a sudden increase in AST and ALT levels. Immediate suspension of therapy and careful in- or outpatient monitoring is recommended if the patient's AST and ALT levels increase beyond 200 IU/L or if T-Bil exceeds 3.0 mg/dL.[7] Treatment can be resumed after the patient recovers and increased on the basis of the AE condition.

- Diarrhea

Prevention: Patients should refrain from eating foods and beverages that contain a lot of spices, fat, or caffeine. Laxatives and dietary fiber supplements should also be avoided.

Management: If frequency of defecation increases to 3 times/day, intestinal drugs such as bifidobacterium powders and albumin tannate, and anti-diarrheal drugs such as loperamide and cholestyramine should be administered.[18] In addition, the patient should be instructed to drink fluids to prevent dehydration. Reducing, withholding, or discontinuing sorafenib therapy should be considered if the frequency of defecation increases to ≥4 times/day and the patient exhibits symptoms of dehydration. Dehydration symptoms should be managed systemically with fluid replacement, etc. Treatment can be resumed after the patient recovers and subsequently increased on the basis of the AE conditions.

- **Hypertension**

Prevention: If hypertension is observed prior to sorafenib therapy, systolic blood pressure (SBP) and diastolic blood pressure (DBP) should be controlled to ≤140 mmHg and ≤90 mmHg, respectively.

Management: Patients should be instructed to measure home blood pressure during the early treatment period. If elevated blood pressure (BP) is observed, hypotensive drugs should be administered or the dosage increased. Calcium antagonists and angiotensin receptor blockers (ARBs) are commonly used as hypotensive agents. A single drug is typically administered to begin with, and other types of hypotensive drugs may be co-administered if the reduction in BP is insufficient. Regardless of therapy, administration of sorafenib should be withheld if SBP is ≥180 mmHg or DBP is ≥110 mmHg. Treatment can be resumed after the patient recovers and then increased on the basis of the AE conditions.

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Table 5 Clinical laboratory tests recommended in proper use guidelines for sorafenib therapy[7]

Test/Test variable	Cautionary AEs etc.	Subjects	Frequency/Duration												
			Baseline	1 week	2 weeks	3 weeks	4 weeks	6 weeks	8 weeks	10 weeks	12 weeks	16 weeks	20 weeks	–	Post-therapy
Hepatic function	Hepatic impairment	All patients	○	○	○	○	○	○	○	○	○	○	○		○
Pancreatic function	Increased pancreatic function, pancreatitis	All patients	○		○		○		○		○	○	○		○
Blood count	Neutropenia, thrombocytopenia, etc.	All patients	○		○		○		○		○	○	○		○
Serum phosphate	Hypophosphatemia	All patients	○		○		○		○		○	○	○		○
Blood pressure	Hypertension, hypertensive crisis, reversible leukoencephalopathy	All patients	At hospital visit (simple HBP measurement once weekly [daily if possible])												
Abdominal imaging	GI perforation, pancreatitis	Patients complaining of abdominal pain	As appropriate												
Coagulation parameters	Hemorrhage	Patients on concomitant vitamin K antagonists	As appropriate												
Thyroid function (thyroid hormone, thyroid-stimulating hormone, etc.)	Reduced thyroid function	Patients with specific symptoms suggestive of reduced thyroid function	As appropriate												
Thoracic imaging (Chest x-ray, chest CT, KL-6)	Interstitial pneumonia	Patients with symptoms suggestive of interstitial pneumonia	As appropriate												

AEs, adverse events; CT, computed tomography; GI, gastrointestinal; HBP, home blood pressure.

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- **Amylase elevation**

Management: Increases in amylase are usually transient and gradually subside even when sorafenib therapy is continued. However, some cases of pancreatitis has previously been reported in patients treated with sorafenib, so if the patient has abdominal pain or other symptoms suggestive of pancreatitis, or elevated amylase levels are sustained, sorafenib therapy should be withheld and imaging procedures such as dynamic CT performed to determine whether pancreatitis is present.[7]

- **Interstitial pneumonia**

Management: Interstitial pneumonia should be suspected and sorafenib therapy discontinued immediately in patients exhibiting clinical symptoms such as dyspnea, dry cough and fever, and lung crepitation or reduced SpO_2 (percutaneous oxygen saturation) on physical examination. In addition, diagnosis and proper treatment should be carried out based on prompt diagnostic imaging such as chest X-ray or high-resolution chest CT (HRCT) and blood tests such as KL-6 after consulting with a respiratory specialist.[7]

Evaluation of therapeutic response

CQ1-6 How and when should therapeutic response of sorafenib be evaluated?

Recommendation The antitumor effects of sorafenib therapy are normally evaluated by diagnostic imaging with dynamic CT or dynamic magnetic resonance imaging (MRI) and subsequent measurement of tumor size based on a single cycle of 4–6 weeks of sorafenib administration.

Changes in intra-tumoral blood flow are often seen following sorafenib therapy, so evaluation can also be performed by measuring the area of tumor staining in addition to tumor size.

α-fetoprotein (AFP) and PIVKA-II (DCP) (protein induced by vitamin K absence or abnormality, des-γ-carboxyprothrombin) tumor markers are also typically evaluated in conjunction with tumor images at cycles of 4–6 weeks.

Elevated PIVKA-II (DCP) concentrations during sorafenib therapy may not always be due to disease progression. Consideration should also be given to evaluation of tumors in patients for whom treatment was interrupted due to AEs.

Scientific statement In the two randomized, placebo-controlled trials demonstrating the usefulness of sorafenib therapy,[1,2] therapeutic response to sorafenib was evaluated every 6 weeks on the basis of diagnostic imaging.

In the NLCT study, median overall survival (OS) was 10.8 months, 6-month survival rate was 65%, 1-year survival rate was 45%, and median progression-free survival (PFS) was 2.1 months (Fig. 1). Comparison of efficacy evaluation findings with those of previous clinical trials[1,2,5] are presented in Table 6.

Reductions in intra-tumoral blood flow are often observed with sorafenib therapy, so instead of simply evaluating tumor size based on the conventional Response Evaluation Criteria in Solid Tumors (RECIST), the use of therapeutic response criteria for evaluating intra-tumoral necrotic regions such as modified RECIST[19] or the Response Evaluation Criteria in Cancer of the Liver (RECICL)[20] has recently been advocated.[21,22] Even if the size of the tumor has slightly increased, therapy may be deemed effective and subsequently continued if the area of reduced intra-tumoral blood flow has increased.

Previous studies have reported that PIVKA-II (DCP) expression is induced in hypoxic HCC cells following sorafenib therapy[23] and that elevated PIVKA-II (DCP) concentrations may act as surrogate markers for HCC tissue ischemia.[24] However, elevated PIVKA-II levels are also seen in disease progression, so care should be taken during assessment of therapeutic response.

According to the NLCT study data, therapeutic response was not evaluated in 20% of sorafenib group patients. However, short-term administration of sorafenib was found to inhibit tumors in some patients on whom therapy was interrupted due to AEs, suggesting that regular tumor assessment should also be considered for patients with interrupted treatment.

Continuation of therapy

CQ1-7 How long should sorafenib therapy be continued?

Recommendation Sorafenib therapy should preferably be maintained until clear disease progression is determined on evaluation of therapeutic response.

If clear disease progression is not identified in diagnostic imaging, therapy may be continued after considering the risks and benefits.

No data are currently available on the efficacy/safety of continued sorafenib administration after disease progression has been confirmed.

Scientific statement In the NLCT study, 31% of patients in the sorafenib group underwent some form of additional treatment after completion of the therapy. Specifically, 12% underwent TACE, 8% underwent systemic chemotherapy, 7% underwent HAIC, 4% underwent radiotherapy, and 2% underwent hepatectomy/LAT.

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