原 著

新しい超高感度 HBs 抗原定量試薬の基礎的・臨床的有用性

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Evaluation and Application of a Newly Developed Highly Sensitive HBsAg Chemiluminescent Enzyme Immunoassay for Chronic Hepatitis B Patients

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Aim: A high sensitive chemiluminescent enzyme immunoassay (CLEIA) was developed for quantitative hepatitis B surface antigen (HBsAg) detection by a combination of monoclonal antibodies, each one for a specific epitope of HBsAg, and by improving the conjugation technique (Matsubara, et al. Transfusion 2009). We modified and automated Matsubara's techniques. Our aim is to evaluate the fundamental performance of our assay (prototype) and to investigate the clinical significance of prototype in patients with hepatitis B virus (HBV) infection.

Methods: We used 226 HBsAg-negative samples and 59 HBsAg-positive samples for evaluation of prototype's accuracy, reproducibility, specificity and sensitivity. We examine correlation between the prototype assay and commercial quantitative HBsAg detection assay (the Abbott ARCHITECT). Performance of prototype was compared with the Abbott ARCHITECT in one chronic hepatitis B patient and one patient with HBsAg seroconversion sequentially.

Results: The prototype assay had good accuracy, reproducibility, specificity and sensitivity. There is positive correlation between the prototype and the Abbott ARCHITECT. The sensitivity of the prototype (5mIU/mL) was approximately 10 fold higher than the Abbott ARCHITECT (50mIU/ml). The prototype could detect HBsAg at the HBsAg-negative point by Abbott ARCHITECT in these patients.

Conclusions: Automatic highly sensitive HBsAg CLEIA prototype is convenient and precise assay for HBV monitoring.

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【Key Words】 hepatitis B virus (B 型肝炎ウイルス), highly sensitive HBsAg chemiluminescent enzyme immunoassay (高感度 HBs 抗原測定), CLEIA (化学発光酵素免疫測定), Lumipulse G1200 (ルミパルス G1200)

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世界の4億2,000万人以上の人にB型肝炎ウイルス(以下，HBV)は持続感染している[1)]。現在使用されているB型慢性肝炎患者のHBVのウイルスのモニタリングにはHBV DNA(リアルタイムPCR)，HBe抗原，HBs抗原，HBコア関連抗原がある。

現在，PCR測定をベースにしたHBV DNAが臨床の場においては特に使用頻度が高いが，最近導入された高感度リアルタイムPCRを用いてもHBV DNA検出には限界があり，特にHBV治療薬として核酸アナログを投与されている患者においては速やかに感度以下となるため，ウイルス複製のモニタリングには必ずしも十分とはいえない。

HBVの持続感染の間，HBs抗原は血中に分泌されるエンベロープ蛋白である。HBs抗原検出は持続感染の指標とされ，ウイルスの転写のテンプレートとなる肝内のcccDNAと関連がある[2)]。また，ペグインターフェロンαの治療をうけているB型慢性患者において，HBs抗原定量がウイルス複製の代用マーカーとなりうる可能性が出てきている[3)~5)]。

現在，本邦ではHBs抗原定量測定系としては，アーキテクトHBsAg-QT(Abbott Japan)(測定範囲50～250,000mIU/mL)，HISCL HBsAg(Sysmex)(測定範囲30～2,500,000mIU/mL)が存在し，両社の測定系は相関が良好であり，ともに高感度で広い測定域を有する。最近，松原ら[6)]により新しい高感度のHBs抗原定量系が報告された。これはHBV感染初期のHBV検出においてPCR法と同等の感度であるとされている。今回，松原らの検査系を元に新たに開発された超高感度HBs抗原定量試薬(富士レビオ株式会社)(以下，「本試薬」とする)の性能(自動化)，特異度，従来のHBs抗原定量測定法との相関，臨床経過のモニタリングにおける有用性について評価した。

I. 方法と試料(または材料)

A. 対　象

対象となるサンプルはHBs抗原陰性の3検体，HBs抗原量の異なるHBs抗原陽性の5検体を正確性，同時再現性，日差再現性に用いた。また，226サンプルのHBs抗原陰性検体(うち118サンプルが抗HCV抗体陽性検体)を特異性試験に用いた。アーキテクトHBsAg-QT(Abbott Japan)と本試薬との相関を確認するためにB型慢性肝炎患者からの59検体を用いた。また，経時的にHBs抗原を追跡したB型慢性肝炎1患者から4ポイント，経過中にHBs抗原が消失したB型慢性肝炎1患者から7ポイントのサンプルを使用した。なお，検体は名古屋市立大学大学院医学研究科倫理委員会の承認を得た上で患者の同意のもと採取した。

B. 本試薬によるHBs抗原定量の測定原理

松原らの報告[6)]に準ずるが，変性剤を主成分とする検体処理液で検体を前処理することにより，検体中のHBVエンベロープを破壊する。HBs抗体が存在する場合にはHBs抗原/HBs抗体複合体を乖離させることによりHBs抗原を遊離させる。さらに遊離したHBs抗原の立体構造は変性によりリニアエピトープ化する。これらを特異的に捕捉するモノクローナル抗体を用いて高感度に定量する2ステップサンドイッチ法である。抗体に関しては固相に2種類(外側構造認識：1種類，内側リニア認識1種類)，標識に2種類のモノクローナル抗体を使用しており，それぞれが理論上1対1に対応するように設計されている(**Fig. 1**)。一次抗体に，従来の外側構造認識"a" determinantのみではなく，内側リニア認識抗体も使用している。

C. 本試薬によるHBs抗原定量の測定方法

測定は全自動化学発光酵素免疫測定装置であるルミパルスG1200(富士レビオ株式会社)を用いた。試薬は1テストごとのカートリッジタイプで，検体処理液と磁性フェライト粒子に結合した前述の抗HBsモノクローナル抗体液(固相抗体液)およびアルカリホスファターゼ標識抗HBsモノクローナル抗体液(標識抗体液)で構成されている。抗体の性状に関しては上記のとおりである。この固相抗体と標識抗体によりHBs抗原をサンドイッチした免疫複合物を形成させ，標識された酵素と化学発光基質(AMPPD)の反応による発光強度を測定する。すなわち，第一反応で検体100μLと検体処理液20μLを固相抗体に加えて10分間反応させ，磁石を用いたB/F分離後，第二反応で標識抗体250μLを固相抗体に加えて10分間反応させる。再度，磁石を用いたB/F分離後，AMPPDを加えて5分間酵素反応が行われる。その後，AMPPDの分解に伴う発光量をルミノメーターで測定し，予め作成された検量線より検体中のHBs抗原濃度が出力される。これらの操作は全て装置内で自動的に行われる。測定範囲は5～150,000mIU/mLであり，判定は5mIU/mL以上を陽性とする。なお，レンジオーバーした検体については200倍希釈による希釈測定が可能である。

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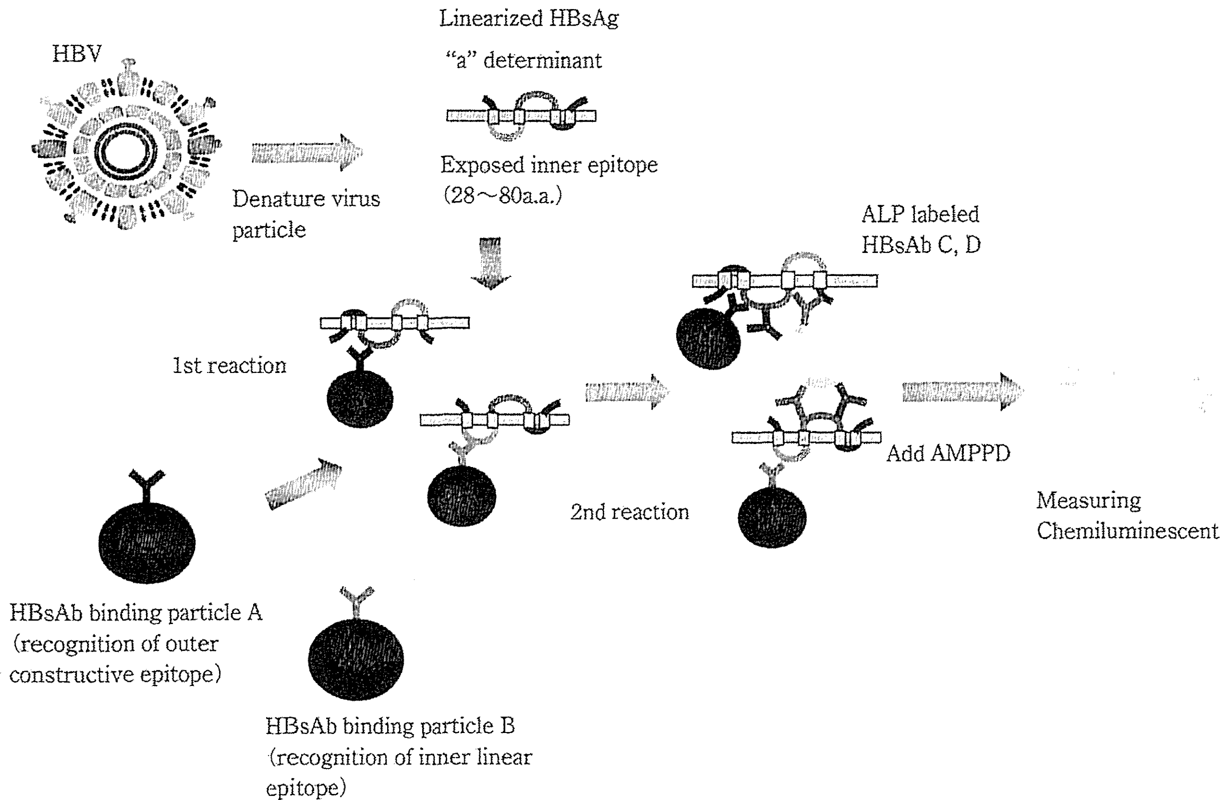

Figure 1 A principle of a newly developed highly sensitive HBsAg chemiluminescent enzyme immunoassay (prototype).

Table 1 Acuracy of highly sensitive HBsAg chemiluminescent enzyme immunoassay (prototype)

Samples	actual value measured by prototype (mIU/ml)	per control value (%)
N1	1.8	−
N2	0.4	−
N3	0.1	−
LL	47.7	96.7
L	538.5	101.8
M	6,413.3	102.5
H	56,872.0	101.6
HH	342,672.5	99.9

II. 結　　果

本試薬を①正確性，同時再現性，日差再現性，②特異性試験，③従来のHBs抗原定量測定法としてアーキテクトHBsAg-QT(アボット ジャパン株式会社)(the Abbott ARCHITECT HBsAg-QT.)との相関について評価した。

A. 正 確 性

①正確性を検討するために陰性検体3例(N1〜N3)とHBs抗原量の異なるHBV陽性検体5例(LL, L, M, H, HH)の8検体を測定した。HBV陽性検体の管理値(control value)は本試薬3ロットを用いて，各6重測定を行い，その平均値として設定された。測定値(actual value)は，管理値と比較してほぼ100%の結果が得られた(**Table 1**)。②同時再現性についても，前述のHBV陽性検体5例(LL, L, M, H, HH)を用いてそれぞれ6重測定したところ変動係数は5%以下と良好であった(**Table 2**)。③日差再現性に関しても，前述のHBV陽性検体5例(LL, L, M, H, HH)を用いて6日測定を施行した。変動係数は

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Table 2 Simultaneous repeatability of highly sensitive HbsAg chemiluminescent enzyme immunoassay (prototype) (6 times examination)

Samples	Coefficient of variation (%)
LL	3.2
L	2.8
M	1.7
H	1.9
HH	5.0

Table 4 Specificity of highly sensitive HBsAg chemiluminescent enzyme immunoassay (prototype)

Value (mIU/ml)	n
0	217
1	5
2	2
3	0
4	2
5.0≦	0
total	226

Table 3 Daily repeatability of highly sensitive HBsAg chemiluminescent enzyme immunoassay (prototype) (6 days measurement)

Samples	date						Coefficient of variation (%)
	Sep 14th 2009	Sep 15th 2009	Sep 16th 2009	Sep 17th 2009	Sep 18th 2009	Sep 24th 2009	
LL	47.7	53.7	54.0	55.4	51.2	48.9	5.9
L	538.5	524.9	566.1	580.3	546.4	500.7	5.3
M	6,413.3	6,481.9	6,490.8	6,457.4	6,579.8	5,936.9	3.6
H	56,872.0	57,030.6	56,762.1	59,069.5	57,932.6	55,353.7	2.2
HH	342,672.5	358,378.4	391,019.9	373,374.9	380,694.8	372,547.0	4.6

Table 5 The comparison of highly sensitive HBsAg chemiluminescent enzyme immunoassay (prototype) with Abbott ARCHITECT

		Highly sensitive HBsAg chemiluminescent enzyme immunoassay		
		positive	negative	total
Abbott ARCHITECT	positive	47	0	47
	negative	2	10	12
	total	49	10	59

最大5.9%と良好な再現性であった(**Table 3**)。

B.　特異性試験

226例のHBs抗原陰性検体(うち118例が抗HCV抗体陽性検体)を使用して特異性試験を行ったところ測定値はすべて5mIU/ml未満であり特異度は100%であった(**Table 4**)。

C.　従来法との比較

以前にB型肝炎と診断された患者血清59検体を用いて，従来のHBs抗原定量測定法としてアーキテクトHBsAg-QTとの相関を確認した。本試薬とアーキテクトHBsAg-QTとの両者の判定一致率は96.6%であった。うち2検体においてアーキテクトHBsAg-QTにて陰性で，本試薬にて陽性であった。その2検体に関しては本試薬にて10.2mIU/ml，14.8mIU/mlとアーキテクトHBsAg-QTの検出限界である50mIU/ml未満であり，HBs抗原定量試薬の高感度化により検出可能となった(**Table 5**)。本試薬とアーキテクトHBsAg-QTとの相関を検討した。アーキテクトHBsAg-QTにてHBs抗原陽性であった47サンプルにて検討したところ，相関傾きは若干高かったものの，相関係数は良好であった(y=1.4911x−1,435,268，r=0.953)(**Fig. 2a**)。なお，低値域41サンプルについて検討したところ，相関係数はより良好であった(y=1.0895x−38,242，r=0.977)(**Fig. 2b**)。

D.　HBV DNA TaqMan PCRとの比較

以前にB型肝炎と診断された患者血清58検体を用いて本試薬によるHBs測定とHBV DNA TaqMan

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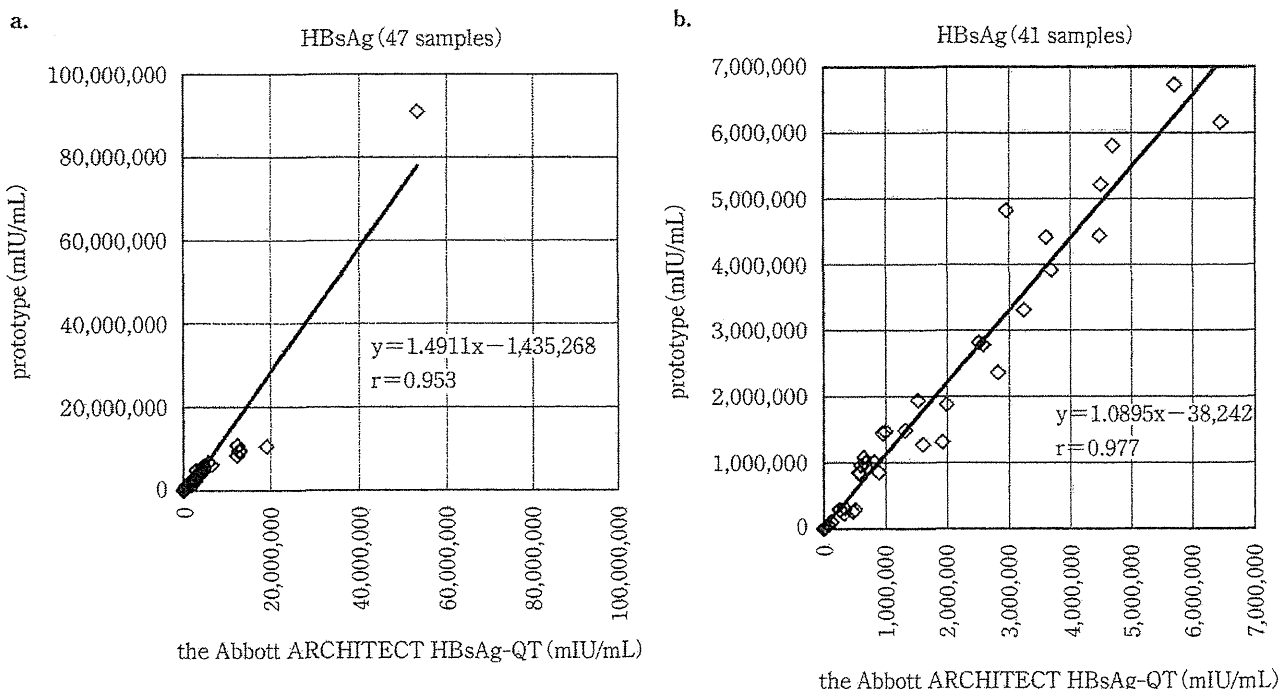

Figure 2

a. Correlation for HBsAg of serum samples between prototype and the Abbott ARCHITECT HBsAg-QT.
b. Correlation for HBsAg of serum samples between prototype and the Abbott ARCHITECT HBsAg-QT (low value).

Table 6 The comparison of highly sensitive HBsAg chemiluminescent enzyme immunoassay (prototype) with HBV DNA Taqman PCR

		highly sensitive HBsAg chemiluminescent enzyme immunoassay (prototype)		
		positive	negative	total
HBV DNA TaqMan PCR	positive	24	1	25
	under detectable limit	6	0	6
	negative	18	9	27
	total	48	10	58

PCR(コバス TaqMan HBV「オート」(ロシュ・ダイアグノスティックス株式会社))それぞれにおいて相関性を比較した。判定一致率は67.2%であった。HBV DNA Taqman PCR で陰性のもの27サンプル中18サンプルで本試薬において陽性であった(**Table 6**)。

実際の症例を提示する：症例1：55歳男性，ALTは23 IU/L，HBV-DNAはTMA法で3.7 LGE/ml未満，HBe抗原陰性，HBe抗体陽性であった。経過観察中に核酸アナログの投与はしていない。ALTは経過中正常値のままであった。HBV-DNAは最終的にTaqMan法にても陰性化した。経時的にHBs抗原を経過観察したところ，本試薬とアーキテクトHBsAg-QTの両者において類似の推移を示した(**Fig. 3a**)。症例2：60歳男性，ALTは55 IU/Lと軽度上昇，HBV-DNAはTMA法で3.7 LGE/ml未満，HBe抗原陰性，HBe抗体陽性であった。経過観察中に核酸アナログの投与はしていない。経過観察中にHBV DNAはアンプリコア法でも測定したが検出感度未満であった。経過中にALTは一時122 IU/mlまで上昇したが，その後は改善した。HBs抗原は持続的に減少し，アーキテクトHBsAg-QTの検出感度以下になったが，その時点において本試薬ではHBs抗原の定量をすることが可能であった(**Fig. 3b**)。

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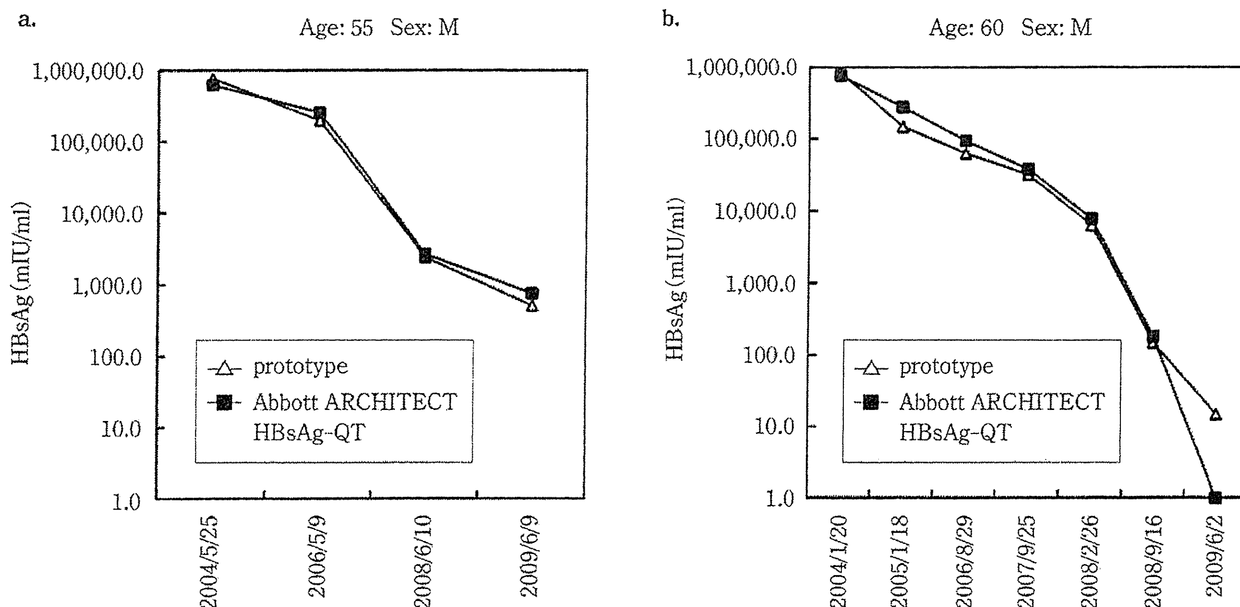

Figure 3

a. Prototype and the Abbott ARCHITECT HBsAg-QT changes over time in one chronic hepatitis B patient.

b. Prototype and the Abbott ARCHITECT HBsAg-QT changes over time in one chronic hepatitis B patient with HBsAg seroconversion.

III. 考　　察

本試薬は，HBV エンベロープを破壊し，HBs 抗原を遊離させせることで検出感度を向上させている。しかしながらそれによって本来 HBV 粒子(Dane 粒子)上の HBs 抗原のみならず Dane 粒子の 10,000～1,000,000 倍以上存在すると言われている，ウイルス DNA の存在しない小型球状粒子上の small S 蛋白を検出することで，従来法と本試薬との間でデータが大きく乖離する懸念があったが，**Fig. 2a, b** に示したようにアーキテクト HBsAg-QT との相関は良好であり，かつ，従来法よりも感度が 10 倍向上し検出限界が 5mIU/ml になったことで **Fig. 3a, b** に示したように HBs 抗原をより低値まで追跡可能であった。また，自動化することにより PCR 法を用いた HBV-DNA 定量より簡便で，しかも安価な検査であることから実際の臨床の場で汎用されることが期待される。高感度で迅速検査にも対応可能となり，HBV 感染を疑う重症肝炎患者など緊急を要する場合においても有用であると考えられる。

最近，HBc 抗体あるいは HBs 抗体陽性の既往感染者からの HBV 再活性化に伴う劇症肝炎の報告が散見されている[7)~9)]。特に，リツキシマブ＋ステロイドを含む化学療法後に肝炎を発症する頻度は比較的高く，ガイドラインでは HBV-DNA のモニタリングが推奨されている[10)]。確かに HBV-DNA 定量は非常に有用な手段であるが，高価かつ煩雑であり，今回提示した本試薬の感度がさらに向上すれば，HBV-DNA 定量に代わる検査となり得るかもしれない。また，従来の HBs 抗原検出系においては一次抗体が HBs 抗原の外側エピトープ認識抗体を使用していた。近年，“a” determinant におけるワクチンエスケープ変異が報告されており[11)~13)]，従来の HBs 抗原検出系では検出感度の低下が指摘されている[14)]。今回の本試薬は内側リニア認識抗体も一次抗体に使用することで，そうした懸念を解消するかもしれない。

IV. 結　　語

新しい検査試薬である超高感度 HBs 抗原定量試薬はその高感度と即時性において臨床応用が期待される。

文　　献

1) Lok AS. Chronic hepatitis B. New Engl J Med 2002; 346: 1682-3.

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2) Wursthorn K, Lutgehetmann M, Dandri M, et al. Peginterferon alpha-2b plus adefovir induce strong cccDNA decline and HBsAg reduction in patients with chronic hepatitis B. Hepatology 2006; 44: 675-84.

3) Brunetto MR, Moriconi F, Bonino F, et al. Hepatitis B virus surface antigen levels: a guide to sustained response to peginterferon alfa-2a in HBeAg-negative chronic hepatitis B. Hepatology 2009; 49: 1141-50.

4) Chen CH, Lee CM, Wang JH, et al. Correlation of quantitative assay of hepatitis B surface antigen and HBV DNA levels in asymptomatic hepatitis B virus carriers. Eur J Gastroenterol Hepatol 2004; 16: 1213-8.

5) Deguchi M, Yamashita N, Kagita M, et al. Quantitation of hepatitis B surface antigen by an automated chemiluminescent microparticle immunoassay. J Virol Methods 2004; 115: 217-22.

6) Matsubara N, Kusano O, Sugamata Y, et al. A novel hepatitis B virus surface antigen immunoassay as sensitive as hepatitis B virus nucleic acid testing in detecting early infection. Transfusion 2009; 49: 585-95.

7) Law JK, Ho JK, Hoskins PJ, et al. Fatal reactivation of hepatitis B post-chemotherapy for lymphoma in a hepatitis B surface antigen-negative, hepatitis B core antibody-positive patient: potential implications for future prophylaxis recommendations. Leuk Lymphoma 2005; 46: 1085-9.

8) Fukushima N, Mizuta T, Tanaka M, et al. Retrospective and prospective studies of hepatitis B virus reactivation in malignant lymphoma with occult HBV carrier. Ann Oncol 2009; 20: 2013-7.

9) Wu JM, Huang YH, Lee PC, et al. Fatal reactivation of hepatitis B virus in a patient who was hepatitis B surface antigen negative and core antibody positive before receiving chemotherapy for non-Hodgkin lymphoma. J Clin Gastroenterol 2009; 43: 496-8.

10) 坪内 博, 熊田 博, 清澤 研, 他. 免疫抑制・化学療法により発症するB型肝炎対策 厚生労働省「難治性の肝・胆道疾患に関する調査研究」班劇症肝炎分科会および「肝硬変を含めたウイルス性肝疾患の治療の標準化に関する研究」班合同報告. 肝臓 2009; 50: 38-42.

11) Ly TD, Servant-Delmas A, Bagot S, et al. Sensitivities of four new commercial hepatitis B virus surface antigen (HBsAg) assays in detection of HBsAg mutant forms. J Clin Microbiol 2006; 44: 2321-6.

12) Carman WF. The clinical significance of surface antigen variants of hepatitis B virus (Suppl 1). J Viral Hepat 1997; 4: 11-20.

13) Gerlich WH. Diagnostic problems caused by HBsAg mutants-a consensus report of an expert meeting. Intervirology 2004; 47: 310-3.

14) Coleman PF. Detecting hepatitis B surface antigen mutants. Emerg Infect Dis 2006; 12: 198-203.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Long-term effect of lamivudine treatment on the incidence of hepatocellular carcinoma in patients with hepatitis B virus infection

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Abstract

Background Nucleotide analogues have recently been approved for the treatment of patients with hepatitis B virus (HBV) infection. However, it is still controversial whether the decrease of HBV-DNA amount induced by treatment with nucleotide analogues can reduce the risk of hepatocellular carcinoma (HCC) development in HBV patients.

Methods A total of 293 HBV patients without HCC who were treated with lamivudine (LAM) were enrolled in a multicenter trial. The incidence of HCC was examined after the start of LAM therapy, and the risk factors for liver carcinogenesis were analyzed. The mean follow-up period was 67.6 ± 27.4 months.

Results On multivariate analysis for HCC development in all patients, age ≥50 years, platelet count $<14.0 \times 10^4/mm^3$, cirrhosis, and median HBV-DNA levels of ≥4.0 log copies/ml during LAM treatment were significant risk factors. The cumulative carcinogenesis rate at 5 years was

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3% in patients with chronic hepatitis and 30% in those with cirrhosis. For the chronic hepatitis patients, the log-rank test showed the significant risk factors related to HCC development to be age $\geq$50 years, platelet count $<14.0 \times 10^4/\text{mm}^3$, and hepatitis B e antigen negativity, but median HBV-DNA levels of <4.0 log copies/ml (maintained viral response, MVR) did not significantly suppress the development of HCC. In cirrhosis patients, however, the attainment of MVR during LAM treatment was revealed to reduce the risk of HCC development.

Conclusions These results suggest that the incidence of HCC in HBV patients with cirrhosis can be reduced in those with an MVR induced by consecutive LAM treatment.

Keywords Lamivudine · Chronic hepatitis B · Cirrhosis · Hepatocellular carcinoma · HBV-DNA level

Abbreviations

HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
LAM	Lamivudine
ADV	Adefovir
ETV	Entecavir
Hbs Ag	Hepatitis B surface antigen
PCR	Polymerase chain reaction
TMA	Transcription-mediated amplification
IVR	Initial viral response
MVR	Maintained viral response
HBe Ag	Hepatitis B e antigen
CT	Computed tomography
MRI	Magnetic resonance imaging
ALT	Alanine aminotransferase

Introduction

More than 350 million people worldwide suffer from chronic infection with hepatitis B virus (HBV) [1–3]. Chronic HBV infection eventually leads to the development of cirrhosis and hepatocellular carcinoma (HCC), and raises the risk of hepatic disease-related death [4–6]. In Japan, up to 15% of HCC patients are diagnosed with HBV-related liver disease [7].

HCC is one of the most common malignancies in Japan and its incidence has been increasing over the past 30 years. Recently, various treatments such as transcatheter arterial embolization/chemoembolization, radio-frequency ablation, and hepatic resection have been reported to yield significant improvements in overall patient survival [8–11]. However, HCC relapse has thus far been observed in a majority of treated patients due to its highly malignant potential. In this regard, successful treatment of chronic HBV infection should prevent the patient's liver from progressing to cirrhosis and reduce the risk of HCC development. In recent years, the treatment of chronic hepatitis has changed greatly with the development of various antiviral therapies with nucleoside/nucleotide analogues such lamivudine (LAM), adefovir (ADV), and entecavir (ETV) [12–15]. LAM has long been used against chronic hepatitis, and many reports have demonstrated that LAM is effective in stabilizing inflammatory activity, suppressing HBV-DNA replication, and improving liver histological findings in chronic hepatitis patients [16, 17] and in HBV-related cirrhosis patients [18]. Furthermore, LAM has been reported to reduce the incidence of HCC in patients with chronic hepatitis B [19]. However, it is still controversial whether or not treatment using nucleotide analogues can reduce the risk of HCC development in HBV-infected patients [20, 21], and the relationship between the effect of HBV suppression and HCC development during LAM treatment has not yet been discussed in detail. Also, the risk factors for HCC development in HBV-infected patients who have been treated with LAM have not been sufficiently evaluated. In this study, we aimed to clarify whether the decrease of HBV-DNA amount induced by LAM therapy could reduce the incidence of HCC in HBV-infected patients.

Patients and methods

Patient selection and study design

This study was conducted at Osaka University Hospital and other institutions participating in the Osaka Liver Forum in Japan. The subjects were 293 consecutive patients with HBV infection who underwent continuous LAM therapy for more than 24 weeks from September 2000 to September 2006. All patients tested positive for hepatitis B surface antigen (HBs Ag) or had detectable levels of HBV DNA in their sera according to findings from a polymerase chain reaction (PCR)-based method or a transcription-mediated amplification (TMA) method. Exclusion criteria were patients with anti-hepatitis C antibody, anti-human immunodeficiency virus antibody, and other liver diseases (alcoholic liver disease, drug-induced liver disease, and autoimmune hepatitis). Also excluded were patients with a history of HCC and those who developed HCC within the first 24 weeks of the follow-up period after the initiation of LAM therapy (because of the possibility that microscopic HCC had been present before the initiation of treatment).

All patients were treated with 100 mg of LAM daily. Of the 293 patients, 129 underwent ADV (10 mg/day) therapy in addition to receiving ongoing LAM treatment. For 43 patients who started ETV administration in lieu of LAM, the observation period was terminated when they started

ETV. LAM resistance was confirmed by virological breakthrough and was defined as an increase in serum HBV-DNA by >1 $\log_{10}$ greater than the nadir [22]. If virological breakthrough developed and alanine aminotransferase (ALT) was elevated over the upper normal limit, the patients received add-on ADV at 10 mg/day.

In this study, all patients were examined for serum HBV-DNA level just before therapy initiation and every 6 months during treatment. The initial viral response (IVR) was defined as HBV-DNA <4.0 log copies/ml in the first 24 weeks of the follow-up period after the initiation of LAM therapy, and the maintained viral response (MVR) was defined as median HBV-DNA levels of less than 4.0 log copies/ml measured every 6 months during therapy.

This study protocol followed the ethical guidelines of the Declaration of Helsinki amended in 2008, and informed consent was obtained from each patient.

HBV testing

HBs Ag, hepatitis B e antigen (HBe Ag) and anti-hepatitis B e antibody (anti-HBe) levels were examined by chemiluminescence immunoassay or enzyme immunoassay. HBV DNA was measured by a PCR-based method (Amplicor HBV monitor; Roche Diagnostics, Tokyo, Japan) or a TMA method (TMA-HPA; Fujirebio, Tokyo, Japan), which have lower detection limits of 2.6 and 3.7 log copies/ml, respectively. The LAM-resistant YMDD mutant virus was examined by a PCR-ELMA method. Serum samples were stored frozen at −80°C.

Diagnosis of HCC and cirrhosis

Ultrasonography was carried out before LAM therapy and every 3–6 months during the follow-up period. New space-occupying lesions detected or suspected at the time of ultrasonography were further examined by computed tomography (CT), magnetic resonance imaging (MRI), or hepatic angiography. HCC was diagnosed by the presence of typical hypervascular characteristics on angiography, in addition to the findings from CT or MRI. If no typical image of HCC was observed, fine-needle aspiration biopsy was carried out with the patient's consent or the patient was carefully followed until a diagnosis was possible with definite observation by CT, MRI, or hepatic angiography. Cirrhosis was diagnosed by liver biopsy or laparoscopy, and for patients without this information, by clinical data, imaging modalities, and portal hypertension.

Statistical analysis

Quantitative variables were expressed as means ± SD. Quantitative variables at the baseline were compared among two groups, the chronic hepatitis and cirrhosis groups, using the Mann–Whitney *U*-test. Categorical data, such as gender and status of HBe Ag, were compared using Fisher's exact test. The cumulative incidence of HCC was evaluated with a Kaplan–Meiercurve and the differences between groups were analyzed by the log-rank test. For multivariate analysis to investigate factors affecting the cumulative incidence of HCC, Cox's regression analysis was carried out. A value of $p < 0.05$ (two-tailed) was considered to be statistically significant. All calculations were performed with SPSS version 15.0J (SPSS, Chicago, IL, USA).

Results

Baseline characteristics of patients

The baseline clinical features of the enrolled patients before LAM administration are shown in Table 1. The mean age of the patients was 48.0 ± 10.7 years, 214 (73%) of the entire group were male, and 163 (56%) tested positive for HBe Ag. Of the 293 patients, 205 (70%) were diagnosed as having chronic hepatitis and 88 (30%) as having cirrhosis. The median HBV-DNA level was 7.0 (range 3.0 to 8.5<) log copies/ml. At baseline, the aspartate aminotransferase (AST) level was 131 ± 151 IU/l, the ALT level was 203 ± 252 IU/l, the total bilirubin level was 1.2 ± 1.6 mg/dl, the albumin (Alb) level was 3.8 ± 0.5 g/dl, and the platelet count was $13.7 \pm 5.4 \times 10^4$/mm^3. The mean follow-up period for all patients was 67.6 ± 27.4 months, with a range of 12–110 months from the start of LAM treatment. There were significant differences between patients with chronic hepatitis and those with liver cirrhosis in age, AST, ALT, total bilirubin, Alb, and platelet counts.

Cumulative incidence of development of HCC

Figure 1a shows the Kaplan–Meier curve of the cumulative HCC incidence for all HBV patients treated with LAM or LAM plus ADV. Of the 293 patients with HBV infection, 32 (10.9%) developed HCC and the cumulative carcinogenesis rate was 6% at 3 years, 12% at 5 years, and 15% at 7 years.

Figure 1b shows the Kaplan–Meier curve of the cumulative HCC incidence according to initial diagnosis (chronic hepatitis vs. cirrhosis). Eight (4%) of the 205 enrolled chronic hepatitis patients developed HCC and the cumulative carcinogenesis rate was 2% at 3 years, 3% at 5 years, and 5% at 7 years. On the other hand, 24 (27%) of the 88 enrolled cirrhosis patients developed HCC and the cumulative carcinogenesis rate was 15% at 3 years, 30% at 5 years, and 35% at 7 years.

Table 1 Patient characteristics

Factor	All	Chronic hepatitis	Cirrhosis	*p* value
Number of patients	293	205	88	
Age (years)	48.0 ± 10.7	46.3 ± 10.7	51.9 ± 9.8	<0.001**
Sex (male/female)	214/79	147/58	67/21	0.475
HBe Ag (positive	163 (56%)	121 (59%)	42 (48%)	0.068
HBV DNA (log copies/ml)[a]	7.0 (3.0 to 8.5<)	6.8±1.1	6.6 ± 1.1	0.162
AST (IU/l)	131 ± 151	143 ± 162	104 ± 120	0.045*
ALT (IU/l)	203 ± 252	235 ± 269	129 ± 189	<0.001**
Total bilirubin (mg/dl)	1.2 ± 1.6	0.9 ± 0.6	1.8 ± 2.7	<0.001**
Alb (g/dl)	3.8 ± 0.5	3.9 ± 0.4	3.5 ± 0.6	<0.001**
Platelets ($\times 10^4$/mm^3)	13.7 ± 5.4	15.6 ± 9.3	9.3 ± 3.8	<0.001**
Follow-up period (months)	67.6 ± 27.4	68.5 ± 26.5	65.5 ± 29.5	0.393

HBe Ag Hepatitis B e antigen, *HBV* hepatitis B virus, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *Alb* albumin

[a] Values are expressed as medians

* $p < 0.05$, ** $p < 0.001$, comparing patients with chronic hepatitis and those with liver cirrhosis using the Mann–Whitney *U*-test for quantitative variables and Fisher's exact test for categorical variables

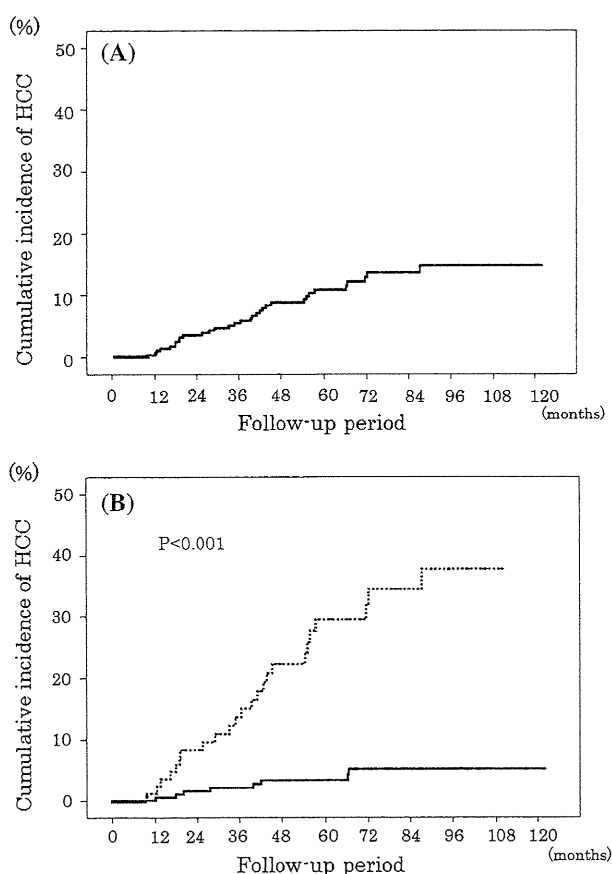

Fig. 1 Cumulative incidence of development of hepatocellular carcinoma (*HCC*) in patients with hepatitis B virus infection treated with lamivudine (LAM). **a** All cases; **b** chronic hepatitis or cirrhosis. *Solid line* Chronic hepatitis, *dotted line* cirrhosis

Risk factors for cumulative incidence of HCC development in all HBV-infected patients

Univariate analysis with the log-rank test was performed for all HBV-infected patients treated with LAM, with the results shown in Table 2. Univariate analysis with the log-rank test showed that the following were significant risk factors for the development of HCC: older age (≥50 years) ($p < 0.001$), cirrhosis ($p < 0.001$), high total bilirubin level (>1.2 g/dl) ($p = 0.004$), low Alb level (<3.8 g/dl) ($p = 0.019$), low platelet count (<14 × 10^4/mm^3) ($p < 0.001$), and non-MVR ($p = 0.035$).

Stepwise multivariate analyses of four of these variables were performed by Cox's regression analysis for all patients treated with LAM with the results shown in Table 3. The analysis indicated the following factors as independent significant risk factors related to the development of HCC: age ≥50 years [hazard ratio (HR) 3.20, 95% confidence interval [CI] 1.08–9.53, $p = 0.036$], platelet count <14.0 × 10^4/mm^3 (HR 4.76, 95% CI 0.05–0.96, $p = 0.045$), cirrhosis (HR 4.64, 95% CI 1.75–12.4, $p = 0.002$), and non-MVR (HR 2.70, 95% CI 1.09–6.56, $p = 0.032$).

Cumulative incidence of and risk factors for HCC development in patients with chronic hepatitis and cirrhosis

The results of univariate analysis with the log-rank test for the development of HCC in chronic hepatitis patients treated with LAM are shown in Table 4, and the following were significant risk factors: older age (≥50 years) ($p = 0.002$), HBe Ag negativity ($p = 0.005$), and low platelet count (<14 × 10^4/mm^3) ($p = 0.004$). Suppression of median HBV-DNA levels to <4.0 log copies/ml by LAM treatment was not associated with the development of HCC in the chronic hepatitis patients. Only non-MVR (median HBV-DNA amount ≥4.0 log copies/ml) was shown to be a significant risk factor for the development of HCC in the cirrhosis patients ($p = 0.029$), while the factors of age, HBe Ag status, and platelet count were not significant in these patients (Table 4).

Table 2 Risk factors for HCC development in all HBV-infected patients by univariate analysis

Factor	95% CI	*p* value
Age (years) (<50/≥50)	2.15–14.5	<0.001
Sex (male/female)	0.33–1.76	0.520
Initial diagnosis (chronic hepatitis/cirrhosis)	3.75–1.176	<0.001
HBe Ag (positive/negative)	0.31–1.29	0.209
HBV DNA (log copies/ml) (<7.0/>7.0)	0.33–1.35	0.262
AST (IU/l) (<40/≥40)	0.33–2.22	0.742
ALT (IU/l) (<40/≥40)	0.17–1.16	0.188
Total bilirubin (mg/dl) (<1.2/>1.2)	1.43–6.72	0.004
Alb (g/dl) (<3.8/≥3.8)	0.19–0.86	0.019
Platelets ($\times 10^4$/mm^3) (<14/≥14)	0.02–0.31	<0.001
Emergence of LAM-resistant viruses (positive/negative)	0.51–2.03	0.968
IVR (positive/negative)	0.52–3.25	0.575
MVR (positive/negative)	1.04–5.95	0.035

HCC Hepatocellular carcinoma, *HBV* hepatitis B virus, *CI* confidence interval, *HBe Ag* hepatitis B e antigen, *HBV* hepatitis B virus, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *Alb* albumin, *IVR* initial viral response, *MVR* maintained viral response, *LAM* lamivudine

Table 3 Risk factors for HCC development in all HBV-infected patients by multivariate analysis

Factor	Category	Risk ratio	95% CI	*p* value
Age (years)	<50	1	1.08–9.53	0.036
	≥50	3.20		
Initial diagnosis	Chronic hepatitis	1	1.75–12.4	0.002
	Cirrhosis	4.64		
Platelets ($\times 10^4$/mm^8) (<14/≥14)	≥14	1	0.05–0.96	0.045
	<14	4.76		
MVR	Negative	1	1.09–6.56	0.032
	Positive	0.37		

HCC Hepatocellular carcinoma, *HBV* hepatitis B virus, *CI* confidence interval, *MVR* maintained viral response

Cumulative incidence of HCC development according to effectiveness of treatment (MVR vs. non-MVR)

Figure 2a shows the Kaplan–Meier curve of cumulative HCC incidence in all HBV-infected patients treated with LAM according to the effectiveness of treatment (MVR vs. non-MVR). The cumulative carcinogenesis rate for MVR-positive patients was 2% at 3 years, 4% at 5 years, and 6% at 7 years. On the other hand, the cumulative carcinogenesis rate for MVR-negative patients was 5% at 3 years, 13% at 5 years, and 16% at 7 years. MVR during LAM significantly suppressed the cumulative HCC incidence compared with non-MVR in all HBV-infected patients ($p = 0.035$).

Table 4 Risk factors for HCC development by univariate analysis (chronic hepatitis/cirrhosis)

	95% CI	*p* value
Chronic hepatitis		
Age (years) (<50/≥50)	0.26–8.38	0.002
Sex (male/female)	0.37–6.42	0.556
HBe Ag (positive/negative)	0.01–0.74	0.005
HBV DNA (log copies/ml) (<7.0/≥7.0)	0.11–1.99	0.296
AST (IU/l) (<40/≥40)	0.11–2.64	0.482
ALT (IU/l) (<40/≥40)	0.06–1.41	0.101
Total bilirubin (mg/dl) (<1.2/≥1.2)	0.67–6.67	0.574
Alb (g/dl) (<3.8/≥3.8)	0.13–8.58	0.960
Platelets ($\times 10^4$/mm^3) (<14/≥14)	0.01–0.72	0.004
Emergence of LAM-resistant viruses (positive/negative)	0.27–4.28	0.927
IVR (positive/negative)	0.29–8.67	0.590
MVR (positive/negative)	0.51–37.10	0.144
Cirrhosis		
Age (years) (<50/≥50)	0.86–6.17	0.089
Sex (male/female)	0.21–1.82	0.380
HBe Ag (positive/negative)	0.80–4.17	0.149
HBV DNA (log copies/ml) (<7.0/≥7.0)	0.40–2.01	0.795
AST (IU/l) (<40/≥40)	0.27–3.07	0.873
ALT (IU/l) (<40/≥40)	0.13–1.47	0.167
Total bilirubin (mg/dl) (<1.2/≥1.2)	0.82–4.80	0.126
Alb (g/dl) (<3.8/≥3.8)	0.28–1.58	0.354
Platelets ($\times 10^4$/mm^3) (<14/≥14)	0.03–1.51	0.084
Emergence of LAM-resistant viruses (positive/negative)	0.44–2.18	0.948
IVR (positive/negative)	0.90–8.32	0.063
MVR (positive/negative)	1.07–0.029	

HCC Hepatocellular carcinoma, *HBV* hepatitis B virus, *CI* confidence interval, *HBe Ag* hepatitis B e antigen, *HBV* hepatitis B virus, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *Alb* albumin, *IVR* initial viral response, *MVR* maintained viral response

Figure 2b shows the Kaplan–Meier curve of the cumulative HCC incidence in chronic hepatitis patients according to the effectiveness of treatment (MVR vs. non-MVR). The cumulative carcinogenesis rate for MVR-positive patients was 0% at 3 years, 0% at 5 years, and 2% at 7 years. On the other hand, the cumulative carcinogenesis rate for MVR-negative patients was 2% at 3 years, 4% at 5 years, and 6% at 7 years. MVR during LAM did not significantly suppress the cumulative HCC incidence compared with non-MVR in the chronic hepatitis patients ($p = 0.144$).

Figure 2c shows the Kaplan–Meier curve of the cumulative HCC incidence in cirrhosis patients according to the effectiveness of treatment (MVR vs. non-MVR).

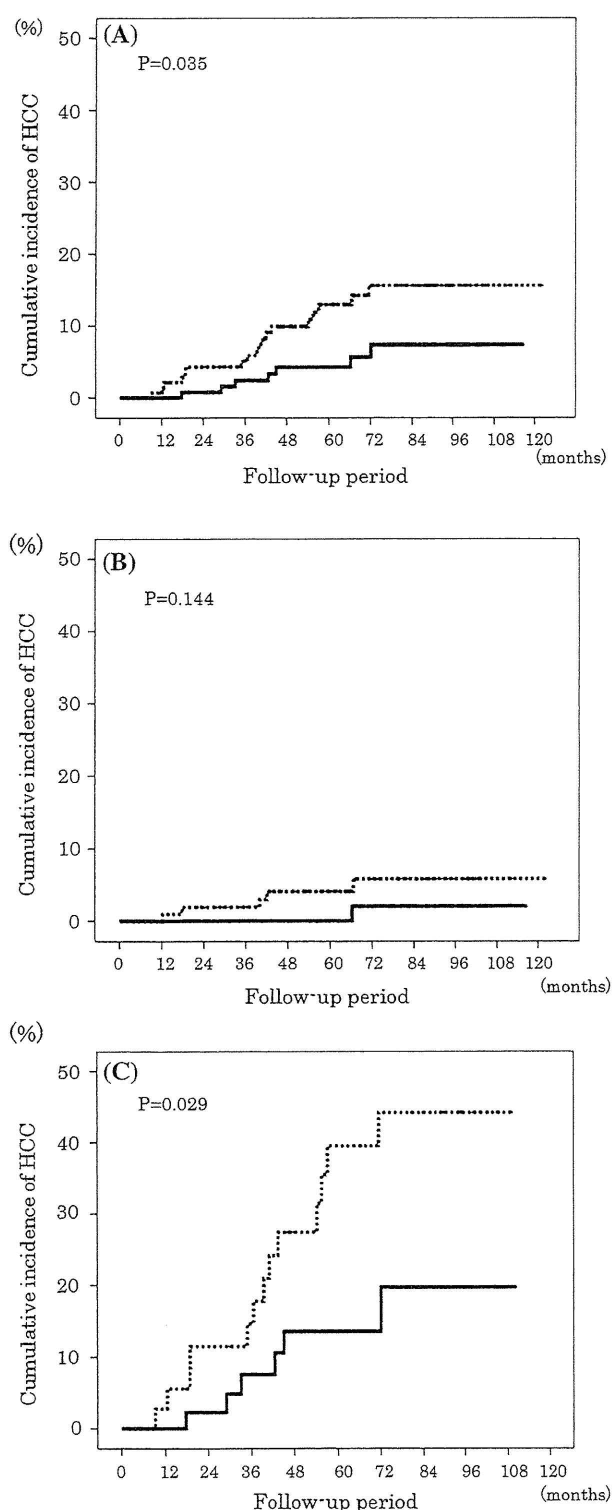

Fig. 2 Cumulative incidence of development of HCC according to the effectiveness of treatment (MVR vs. non-MVR). **a** All cases; **b** chronic hepatitis; **c** cirrhosis. *Solid lines* MVR, *dotted lines* non-MVR. *MVR* Maintained viral response

The cumulative carcinogenesis rate for MVR-positive patients was 8% at 3 years, 14% at 5 years, and 14% at 7 years. On the other hand, the cumulative carcinogenesis rate for MVR-negative patients was 18% at 3 years, 40% at 5 years, and 44% at 7 years. MVR during LAM significantly suppressed the cumulative HCC incidence compared with non-MVR in the cirrhosis patients ($p = 0.029$).

Relationship between IVR and MVR

Maintained viral response (MVR) was achieved by 142 (48%) of the 293 patients enrolled in this study. IVR was achieved by 204 (79%) of the 259 patients who were examined for IVR. The relationship between IVR and MVR is shown in Fig. 3; 60% (122/204) of the IVR-positive patients achieved an MVR, while only 16% (9/55) of the IVR-negative patients achieved an MVR ($p < 0.001$). The LAM-resistant YMDD mutant virus was found in 149 (51%) of all patients during follow-up, and in 52% (107/204) of the IVR-positive patients, a finding which was nearly equal to that for the IVR-negative patients (51%, 28/55). Among the IVR-positive patients, the MVR rate was lower in patients with the YMDD mutation, compared with that in those without the YMDD mutation (44%, 47/107 vs. 77%, 75/97, $p = 0.016$), while the MVR rates were low in the IVR-negative patients, irrespective of their YMDD mutation status (with and without the mutation, 15 vs. 18%, respectively). ADV was added to LAM treatment for 73 (68%) of the 107 IVR-positive patients with the YMDD mutation and 20 (36%) of the 55 IVR-negative patients with the YMDD mutation. However, MVR was only achieved at the low rates of 33% (24/73) for the former patients and 20% (4/20) for the latter.

Discussion

Lamivudine treatment has been shown to improve the liver histological findings in patients with HBV-infected liver disease by reducing the HBV load and stabilizing inflammatory activity [16–18]. One report has shown that LAM effectively reduced the incidence of HCC in patients with chronic hepatitis B, but the study only compared LAM-treated patients with non-treated patients in a matched case-controlled study [19]. However, there have been few detailed reports about the relationship between virological response and HCC development in HBV-infected patients during LAM treatment. In the present study, we retrospectively examined the incidence of HCC to clarify the indicators of LAM therapy, including median HBV-DNA levels, for reducing the risk of HCC in HBV-infected patients.

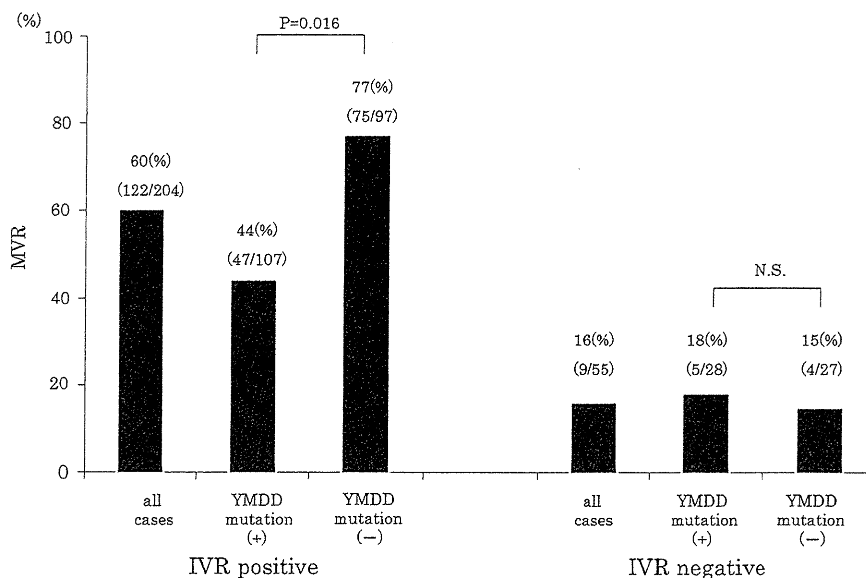

Fig. 3 Relationship between IVR and MVR. *IVR* Initial viral response, *MVR* maintained viral response, *N.S.* not significant

Many investigators have reported that serum HBV DNA levels higher than 4.0–4.5 log copies/ml before HBV treatment serve as a strong risk predictor of HCC [23–25]. Di Marco et al. [26] have reported that the incidence of HCC was higher in patients with serum HBV levels of more than 5.0 log copies/ml, at least once, during LAM therapy than in those in whom serum HBV levels were maintained at 5.0 log copies/ml or less. However, the add-on ADV therapy had not been adopted when the study of Di Marco et al. was reported. When the use of ADV is possible, an evaluation method is needed to measure the antiviral effects of nucleoside/nucleotide analogues against HBV-related liver disease. In the present study, we set the cut-off value for HBV-DNA at 4.0 log copies/ml. The basis of this cut-off value is that a serum HBV DNA level higher than 4.0 log copies/ml before HBV treatment was reported to serve as a strong risk predictor of HCC [23]. MVR, defined as a median HBV-DNA level of less than 4.0 log copies/ml measured every 6 months during therapy, was adopted as an indicator of viral replication, and non-MVR (median HBV-DNA >4.0 log copies/ml) during LAM therapy was shown to be significantly associated with the development of HCC in HBV-infected patients. We also found that a median HBV-DNA level of >4.0 log copies/ml during LAM therapy was a risk factor for HCC development. On the other hand, IVR, defined as HBV-DNA of <4.0 log copies/ml in the first 6 months of the follow-up period after the initiation of therapy, was not associated with the development of HCC in HBV patients in this study. As shown in Fig. 3, 84% of the IVR-negative patients could not achieve an MVR, suggesting that it is crucial to achieve an IVR in order to achieve an MVR. The reason why IVR was not a significant factor for MVR seemed to be the appearance of the YMDD mutation, which reduced the antiviral effect of LAM for HBV in IVR-positive patients. The LAM-resistant YMDD mutant virus was found in 52% of the IVR-positive patients. Although ADV was added to LAM treatment for 73 patients, only 33% of these patients could achieve an MVR. We speculate that the antiviral effect of ADV is not very strong [27] and it takes time to reduce the YMDD mutant virus, which may explain the low MVR rate (33%) in patients with the add-on ADV therapy. The immediate administration of ADV when the LAM-resistant YMDD mutant virus appears can be important [28]. A switch to ETV, which induces resistant virus less frequently, could also raise MVR rates among IVR-positive patients without the YMDD mutant virus.

As the duration of the add-on ADV therapy was included in this study, we compared the cumulative incidence of HCC in patients receiving LAM monotherapy with that in patients who also received the add-on ADV therapy. Sixteen (10%) of the 164 patients who received the LAM monotherapy developed HCC and the cumulative carcinogenesis rate was 6% at 3 years, 10% at 5 years, and 15% at 7 years. On the other hand, 16 (12%) of the 129 patients who received LAM plus ADV developed HCC and the cumulative carcinogenesis rate was 6% at 3 years, 12% at 5 years, and 14% at 7 years. No significant difference was found between these two groups ($p = 0.986$). In addition, we examined the cumulative incidence of HCC development according to the effectiveness of treatment (MVR vs. non-MVR) in patients for whom the observation period was terminated when ADV was added, and the same results were obtained (data not shown).

Older age (≥50 years), cirrhosis, and low platelet count ($<14 \times 10^4/mm^3$) were shown to be significantly associated with the development of HCC in patients with HBV infection. These results were consistent with those of

previous reports [29–31], suggesting that patients of older age with advanced fibrosis should be followed up carefully for longer periods in order to detect early stages of HCC even if LAM therapy does effectively suppress HBV. Of note, in the present study we estimated the cumulative HCC incidence according to the initial diagnosis of chronic hepatitis or cirrhosis. In the chronic hepatitis patients, older age ($\geq$50 years), HBe Ag negativity, and low platelet count ($<14 \times 10^4$/mm^3) were significant risk factors for the development of HCC, but this was not the case in the cirrhosis patients. Because liver biopsies had not been performed, the liver fibrosis stage could not be evaluated with respect to the risk factors for HCC in this study. Instead, the factors of age, HBe Ag status, and platelet count may reflect the degree of liver fibrosis in chronic hepatitis patients. In fact, cirrhotic patients, in comparison with chronic hepatitis patients, were of older age (chronic hepatitis vs. cirrhosis: 46.3 ± 10.7 vs. 51.9 ± 9.8 years, $p < 0.001$), had higher rates of HBe Ag negativity (chronic hepatitis vs. cirrhosis: 39 vs. 51%, $p = 0.065$), and had lower platelet counts (chronic hepatitis vs. cirrhosis: 15.6 ± 4.9 vs. $9.3 \pm 3.8 \times 10^4$/mm^3, $p < 0.001$). This seems to explain why none of these factors were significant risk factors for HCC in cirrhotic patients. On the other hand, in the chronic hepatitis patients, MVR was not a significant factor for HCC development, while MVR was a significant factor for HCC development in the cirrhotic patients. We speculate that HBV suppression induced by LAM therapy could reduce the incidence of HCC in patients infected with HBV, especially those with cirrhosis, who displayed higher malignant potential. Investigation over a longer period is needed to clarify the effect of HBV suppression on the development of HCC in chronic hepatitis patients.

In conclusion, the present study shows that the attainment of an MVR induced by LAM therapy has a significant beneficial effect on the clinical course of HBV-infected patients by decreasing the incidence of HCC. The newer nucleotide analogues, such as ETV and tenofovir, should be able to further reduce the incidence of HCC, given their greater potency.

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Conflict of interest The authors declare that they have no conflict of interest.

References

1. Lok AS, McMahon BJ. Chronic hepatitis B. Hepatology. 2007;45:507–39.
2. Lavanchy D. Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. J Viral Hepat. 2004;11:97–107.
3. Lee WM. Hepatitis B virus infection. N Engl J Med. 1997;337:1733–45.
4. Beasley RP. Hepatitis B virus: the major etiology of hepatocellular carcinoma. Cancer. 1988;61:1942–56.
5. Lok SF. Chronic hepatitis B. N Engl J Med. 2002;346:1682–3.
6. Tang B, Kruger WD, Chen G, Shen F, Lin WY, Mboup S, et al. Hepatitis B viremia is associated with increased risk of hepatocellular carcinoma in chronic careers. J Med Virol. 2004;72:35–40.
7. Kim SR, Kudo M, Hino O, Han KH, Chung YH, Lee HS. Epidemiology of hepatocellular carcinoma in Japan and Korea. A review. Oncology. 2008;75:13–6.
8. Marcellin P, Chang TT, Lim SG, Tong MJ, Sievert W, Shiffman ML, et al. Adefovir dipivoxil for the treatment of hepatitis B e antigen-positive chronic hepatitis B. N Engl J Med. 2003;348:808–16.
9. Taura K, Ikai I, Hatano E, Fujii H, Uyama N, Shimahara Y. Implication of frequent local ablation therapy for intrahepatic recurrence in prolonged survival of patients with hepatocellular carcinoma undergoing hepatic resection: an analysis of 610 patients over 16 years old. Ann Surg. 2006;244:265–73.
10. Shimada K, Sano T, Sakamoto Y, Kosuge T. A long-term follow-up and management study of hepatocellular carcinoma patients surviving for 10 years or longer after curative hepatectomy. Cancer. 2005;104:1939–47.
11. Takayasu K, Arii S, Ikai I, Omata M, Okita K, Ichida T, et al. Prospective cohort study of transarterial chemoembolization for unresectable hepatocellular carcinoma in 8510 patients. Gastroenterology. 2006;131:461–9.
12. Dienstag JL, Perrillo RP, Schiff ER, Bartholomew M, Vicary C, Rubin M. A preliminary trial of lamivudine for chronic hepatitis B infection. N Engl J Med. 1995;333:1657–61.
13. Nevens F, Main J, Honkoop P, Tyrrell DL, Barber J, Sullivan MT, et al. Lamivudine therapy for chronic hepatitis B: a six-month randomized dose-ranging study. Gastroenterology. 1997;113:1258–63.
14. Rapti I, Dimou E, Mitsouka P, Hadziyannis SJ. Adding-on versus switching-to adefovir therapy in lamivudine-resistant HBVAg-negative chronic hepatitis B. Hepatology. 2007;45:307–13.
15. Chang TT, Lai CL, Kew Yoon S, Lee SS, Coelho HS, Carriho FJ, et al. Entecavir treatment for 5 years in patients with hepatitis Be antigen-positive chronic hepatitis B. Hepatology. 2010;51:422–30.
16. Kiyosawa K, Tanaka E, Sodeyama T. Hepatitis C virus and hepatocellular carcinoma. Curr Stud Hematol Blood Transfus. 1998;62:161–80.
17. Ohkoshi S, Norio O, Ichida T. The long-term clinical outcome of 1-year treatment of chronic hepatitis B with lamivudine-5 years observation. Hepatol Res. 2003;27:13–7.
18. Chu CM, Liaw YF. Hepatitis B virus-related cirrhosis: natural history and treatment. Semin Liver Dis. 2006;26:142–52.
19. Matsumoto A, Tanaka E, Rokuhara A, Kiyosawa K, Kumada H, Omata M, et al. Efficacy of lamivudine for preventing hepatocellular carcinoma in chronic hepatitis B: a multicenter retrospective study of 2795 patients. Hepatol Res. 2005;32:173–84.
20. Shamliyan TA, MacDonald R, Shaukat A, Taylor BC, Yuan JM, Johnson JR, et al. Antiviral therapy for adults with chronic hepatitis B: a systematic review for a National Institutes of Health Consensus Development Conference. Ann Intern Med. 2009;150(2):111–24.
21. Liaw YF, Sung JJ, Chow WC, Farrell G, Lee CZ, Yuen H, et al. Lamivudine for patients with chronic hepatitis B and advanced liver disease. N Engl J Med. 2004;351(15):1521–31.

22. Perrillo R, Hann HW, Mutimer D, Willems B, Leung N, Lee WM, et al. Adefovir dipivoxil added to ongoing lamivudine in chronic hepatitis B with YMDD mutant hepatitis B virus. Gastroenterology. 2004;126:81–90.
23. Chen CJ, Yang HI, Su J, Jen CL, You SL, Lu SN, et al. Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. JAMA. 2006;295:65–73.
24. Chan HL, Hui AY, Wong ML, Tse AM, Hung LC, Wong VW, et al. Genotype C hepatitis B virus infection is associated with increased risk of hepatocellular carcinoma. Gut. 2004;53:1494–8.
25. Chan HL, Tse CH, Koh FM, Wong VW, Wong GL, Chan SL, et al. High viral load and hepatitis B virus subgenotype Ce are associated with increased risk of hepatocellular carcinoma. J Clin Oncol. 2008;26:177–82.
26. Di Marco V, Marzano A, Lampertico P, Andreone P, Santantonio T, Almasio PL, et al. Clinical outcome of HBeAg-negative chronic hepatitis B in relation to virological response to lamivudine. Hepatology. 2004;40:883–91.
27. Marcellin P, Chang TT, Lim SG, Tong MJ, Sievert W, Shiffman ML, et al. Adefovir dipivoxil for the treatment of hepatitis B e antigen-positive chronic hepatitis B. N Engl J Med. 2003;348:808–16.
28. Kurashige N, Hiramatsu N, Ohkawa K, Yakushijin T, Kiso S, Kanto T, et al. Factors contributing to antiviral effect of adefovir dipivoxil therapy added to ongoing lamivudine treatment in patients with lamivudine-resistant chronic hepatitis B. J Gastroenterol. 2009;44(6):601–7.
29. Chen CJ, Yu MW, Liaw YF. Epidemiological characteristics and risk factors of hepatocellular carcinoma. J Gastroenterol Hepatol. 1997;12:5294–308.
30. Yun LF. Natural history of chronic hepatitis B virus infection and long-term outcome under treatment. Liver Int. 2009;29:100–7.
31. Sung JJ, Tsoi KK, Wong VW, Li KC, Chan LL. Meta-analysis: treatment of hepatitis B infection reduces risk of hepatocellular carcinoma. Aliment Pharmacol Ther. 2008;28:1067–77.

Delayed-Onset Caspase-Dependent Massive Hepatocyte Apoptosis upon Fas Activation in Bak/Bax-Deficient Mice

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The proapoptotic Bcl-2 family proteins Bak and Bax serve as an essential gateway to the mitochondrial pathway of apoptosis. When activated by BH3-only proteins, Bak/Bax triggers mitochondrial outer membrane permeabilization leading to release of cytochrome c followed by activation of initiator and then effector caspases to dismantle the cells. Hepatocytes are generally considered to be type II cells because, upon Fas stimulation, they are reported to require the BH3-only protein Bid to undergo apoptosis. However, the significance of Bak and Bax in the liver is unclear. To address this issue, we generated hepatocyte-specific Bak/Bax double knockout mice and administered Jo2 agonistic anti-Fas antibody or recombinant Fas ligand to them. Fas-induced rapid fulminant hepatocyte apoptosis was partially ameliorated in Bak knockout mice but not in Bax knockout mice, and was completely abolished in double knockout mice 3 hours after Jo2 injection. Importantly, at 6 hours, double knockout mice displayed severe liver injury associated with repression of XIAP, activation of caspase-3/7 and oligonucleosomal DNA breaks in the liver, without evidence of mitochondrial disruption or cytochrome c–dependent caspase-9 activation. This liver injury was not ameliorated in a cyclophilin D knockout background nor by administration of necrostatin-1, but was completely inhibited by administration of a caspase inhibitor after Bid cleavage. Conclusion: Whereas either Bak or Bax is critically required for rapid execution of Fas-mediated massive apoptosis in the liver, delayed onset of mitochondria-independent, caspase-dependent apoptosis develops even in the absence of both. The present study unveils an extrinsic pathway of apoptosis, like that in type I cells, which serves as a backup system even in type II cells. (HEPATOLOGY 2011;54:240-251)

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Fas, also called APO-1 and CD95, is one of the death receptors that are potent inducers of apoptosis and constitutively expressed by every cell type in the liver.[1] Dysregulation of Fas-mediated apoptosis is involved in several liver diseases.[2] In the liver of patients with chronic hepatitis C, Fas is overexpressed in correlation with the degree of hepatitis, and Fas ligand can be detected in liver-infiltrating mononuclear cells.[3,4] Fas is also strongly expressed in the livers of patients with chronic hepatitis B, autoimmune hepatitis, and nonalcoholic steatohepatitis.[4,5] Moreover, in the liver of patients with fulminant hepatitis, Fas is up-regulated with strong detection of Fas ligand.[6] In mice, injection of Jo2 agonistic anti-Fas antibody leads

Abbreviations: ALT, alanine aminotransferase; CypD, cyclophilin D; DISC, death-inducing signaling complex; DKO, double knockout; DMSO, dimethylsulfoxide; IAP, inhibition of apoptosis protein; KO, knockout; PARP, poly(adenosine diphosphate ribose) polymerase; RIP, receptor-interacting protein; TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling; WT, wild-type.

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to massive hepatocyte apoptosis and lethality, suggesting that the hepatocyte is one of the most sensitive cell types to Fas stimulation.[7] This model is considered to at least partly mimic human fulminant liver failure.

Fas, upon ligation by Fas ligand, activates caspase-8 through the recruitment of Fas-associated protein with a death domain and formation of the death-inducing signaling complex (DISC).[1,2] Whereas activated caspase-8 directly activates effector caspases such as caspase-3 and caspase-7 through the so-called extrinsic pathway, leading to apoptosis in type I cells, it activates caspase-3/7 through the mitochondrial pathway in type II cells. In type II cells, activated caspase-8 cleaves the BH3-only protein Bid into its truncated form, which in turn directly or indirectly activates and homo-oligomerizes Bak and/or Bax to form pores at the mitochondrial outer membrane, leading to the release of cytochrome c. After being released, cytochrome c assembles with Apaf-1 to form apoptosomes which promote self-cleavage of procaspase-9 followed by activation of caspase-3/7 to cleave a variety of cellular substrates such as poly(adenosine diphosphate ribose) polymerase (PARP) and finally to execute apoptosis.[8,9] Hepatocytes are considered to be typical type II cells, because Bid knockout (KO) mice were reported to be resistant to hepatocyte apoptosis upon Fas activation.[10,11] Although Bak and Bax are crucial gateways to apoptosis of the mitochondrial pathway, little information is available about their significance in hepatocyte apoptosis because most traditional Bak/Bax double knockout (DKO) mice ($bak^{-/-}$ $bax^{-/-}$) die perinatally.[12]

In the present study, we tried to address this issue by generating hepatocyte-specific Bak/Bax DKO mice. We demonstrate that either Bak or Bax is required and sufficient to induce Fas-mediated early-onset hepatocyte apoptosis and lethal liver injury. Importantly, even if deficient in both Bak and Bax, Bak/Bax DKO mice still develop delayed-onset caspase-dependent massive hepatocyte apoptosis, suggesting that the mitochondria-independent pathway of apoptosis, as observed in type I cells, works as a backup system when the mitochondrial pathway of apoptosis in the liver is absent. This study is the first to demonstrate the significant but limited role of Bak and Bax in executing Fas-induced apoptosis in the liver.

Materials and Methods

Mice. Heterozygous Alb-Cre transgenic mice expressing Cre recombinase gene under the promoter of the albumin gene were described.[13] We purchased Bak KO mice ($bak^{-/-}$), Bax KO mice ($bax^{-/-}$), and Bak KO mice carrying the *bax* gene flanked by 2 *lox*P sites ($bak^{-/-}$ $bax^{flox/flox}$) from the Jackson Laboratory (Bar Harbor, ME). Traditional cyclophilin D (CypD) KO mice have been described.[14] All mice strains that we used were created from a mixed background (C57BL/6 and 129). We generated hepatocyte-specific Bak/Bax DKO mice ($bak^{-/-}$ $bax^{flox/flox}$ *Alb-Cre*) or hepatocyte-specific CypD/Bak/Bax triple KO mice ($cypd^{-/-}$ $bak^{-/-}$ $bax^{flox/flox}$ *Alb-Cre*) by mating the strains. Mice were injected intraperitoneally with 1.5 or 0.5 mg/kg Jo2 anti-Fas antibody (BD Bioscience, Franklin Lakes, NJ) or intravenously with 0.25 mg/kg recombinant Fas ligand (Alexis Biochemicals, San Diego, CA) cross-linked with 0.5 mg/kg anti-Flag M2 antibody (Sigma-Aldrich, St. Louis, MO) to induce apoptosis. In some experiments, mice were intraperitoneally injected with 2 mg/kg necrostatin-1 (Sigma-Aldrich) or 40 mg/kg Q-VD-Oph (R&D Systems, Minneapolis, MN). They were maintained in a specific pathogen-free facility and treated with humane care with approval from the Animal Care and Use Committee of Osaka University Medical School.

Apoptosis Assay. Measurement of serum alanine aminotransferase (ALT) levels, hematoxylin and eosin staining, and terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) of liver sections have been described.[15] Analysis of cytochrome c release from isolated mitochondria have also been described.[16] To detect DNA fragmentation, 1.5 μg DNA extracted from 30 mg liver tissue by Maxwell16 (Promega, Madison, WI) was incubated with 0.5 μg RNase A (Qiagen, Tokyo, Japan) and separated by way of electrophoresis on a 1.5% agarose gel.

Western Blot Analysis. For western immunoblotting, the following antibodies were used: anti–full-length Bid, anti–Cox IV, anti–cleaved caspase-3, anti–caspase-7, anti–caspase-8, anti–caspase-9, anti-PARP, anti-Bax, anti-cIAP1, and anti-XIAP antibodies were

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obtained from Cell Signaling Technology (Beverly, MA); anti-Bax and anti-cIAP2 antibodies were obtained from Millipore (Billerica, MA); anti-Bid antibody, which detects truncated Bid, was generously provided by Xiao-Ming Yin (Indiana University School of Medicine, Indianapolis, IN)[17]; and anti–β-actin antibody was obtained from Sigma-Aldrich. For isolation of the mitochondria-rich fraction, a Mitochondrial Isolation Kit (Thermo Scientific, Rockford, IL) was used. The isolation of hepatocytes from whole liver has been described.[13]

Detection of Bax Oligomerization. Liver tissue was lysed with HCN buffer (25 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid, 300 mM NaCl, 2% CHAPS, protease inhibitor cocktail, phosphatase inhibitor cocktail, 100 μM BOC-Asp(OMe)CH2F [MP Biomedicals, Solon, OH]; pH 7.5). After the liver lysate was sonicated and centrifuged, the supernatant was collected and the concentration was adjusted. For cross-linking, 100 μL of the lysate was incubated with 5 μL 100 mM bis(maleimido)hexane (Thermo Scientific) and 5 μL 100 mM BS^3 (Thermo Scientific) for 30 minutes at room temperature as described.[18] After quenching the cross-linkers by way of incubation with 12 μL 1 M Tris-HCl (pH 7.5) for 15 minutes at room temperature, the lysate was boiled with sample buffer followed by western blot analysis for Bax.

Electron Microscopy. Livers were fixed by perfusion of phosphate-buffered saline with 2.5% glutaraldehyde solution buffered at pH 7.4 with 0.1 M Millonig's phosphate, postfixed in 1% osmium tetroxide solution at 4°C for 1 hour, dehydrated in graded concentrations of ethanol, and embedded in Quetol 812 epoxy resin (Nisshin EM, Tokyo, Japan). Ultrathin sections (80 nm) cut on ultramicrotome were stained with uranyl acetate and lead citrate and examined with an H-7650 electron microscope (Hitachi Ltd., Tokyo, Japan) at 80 kV.

Statistical Analysis. Data are presented as the mean $\pm$ SE. Differences between two groups were determined using the Mann-Whitney U test for unpaired observations. The survival curves were estimated using the Kaplan-Meier method and were tested by way of log-rank test. $P < 0.05$ was considered statistically significant.

Results

Bak Deficiency Partially Ameliorates Fas-Induced Hepatocellular Apoptosis but Fails to Prevent Animal Death. First, to examine the significance of Bak in hepatocellular apoptosis induced by Fas stimulation, Bak KO mice ($bak^{-/-}$) and wild-type (WT) littermates ($bak^{+/+}$) were intraperitoneally injected with 1.5 mg/kg Jo2 anti-Fas antibody and analyzed 3 hours later. Consistent with previous reports,[10,19] WT mice showed severe elevation of serum ALT levels with massive hepatocellular apoptosis (Fig. 1A,B). Bak KO mice also developed liver injury, but the levels of serum ALT and the number of TUNEL-positive hepatocytes were significantly lower in Bak KO mice than in WT mice (Fig. 1A-C). Western blotting for cleaved caspase-3, caspase-7, and PARP revealed that activation of effector caspases were partially inhibited in KO livers compared with WT livers (Fig. 1D). Cleavage of procaspase-9, which is initiated by mitochondrial release of cytochrome c, was also suppressed in Bak KO livers compared with WT liver (Fig. 1D). The cleaved form of caspase-8, a direct downstream target of Fas activation, was detected in both mice, but its levels were reduced in Bak KO mice compared with WT mice (Fig. 1D). This reduction may be explained by the lesser activation of caspase-3/7, because it has been reported that caspase-3/7 could activate caspase-8 through an amplification loop during apoptosis.[20] Collectively, these findings demonstrated that Bak deficiency partially ameliorated Fas-induced hepatocellular apoptosis associated with reduced cleavage of caspase-9, caspase-3/7, and PARP. We then compared survival of mice after Jo2 injection but found that Bak KO mice also rapidly died with kinetics similar to those of WT mice, suggesting that partial amelioration of hepatocellular apoptosis induced by Bak deficiency did not lead to survival benefit under our experimental conditions (Fig. 1E). Because Bax residing in the cytosol moves to the mitochondria upon activation, where it undergoes oligomerization,[21] we analyzed its translocation and oligomerization in the liver at 3 hours after Jo2 injection. Western blot analysis revealed that the levels of Bax expression clearly increased in the mitochondrial fraction in both WT livers and Bak KO livers (Fig. 1F). Signals for the Bax dimer were also detected in both livers (Fig. 1F). These findings indicate that Bax is also activated after Fas stimulation, raising the possibility of its involvement in hepatocellular apoptosis.

Bax Deficiency Fails to Ameliorate Fas-Induced Hepatocellular Apoptosis. Next, to examine the significance of Bax in hepatocellular apoptosis induced by Fas stimulation, Bax KO mice ($bax^{-/-}$) and WT littermates ($bax^{+/+}$) were injected with Jo2 and examined 3 hours later. There was no significant difference in the levels of serum ALT or the number of TUNEL-positive hepatocytes between the two groups (Fig. 2A-C), which is consistent with a previous report.[22] The levels of the cleaved forms of caspase-8, -9, -3, -7, and

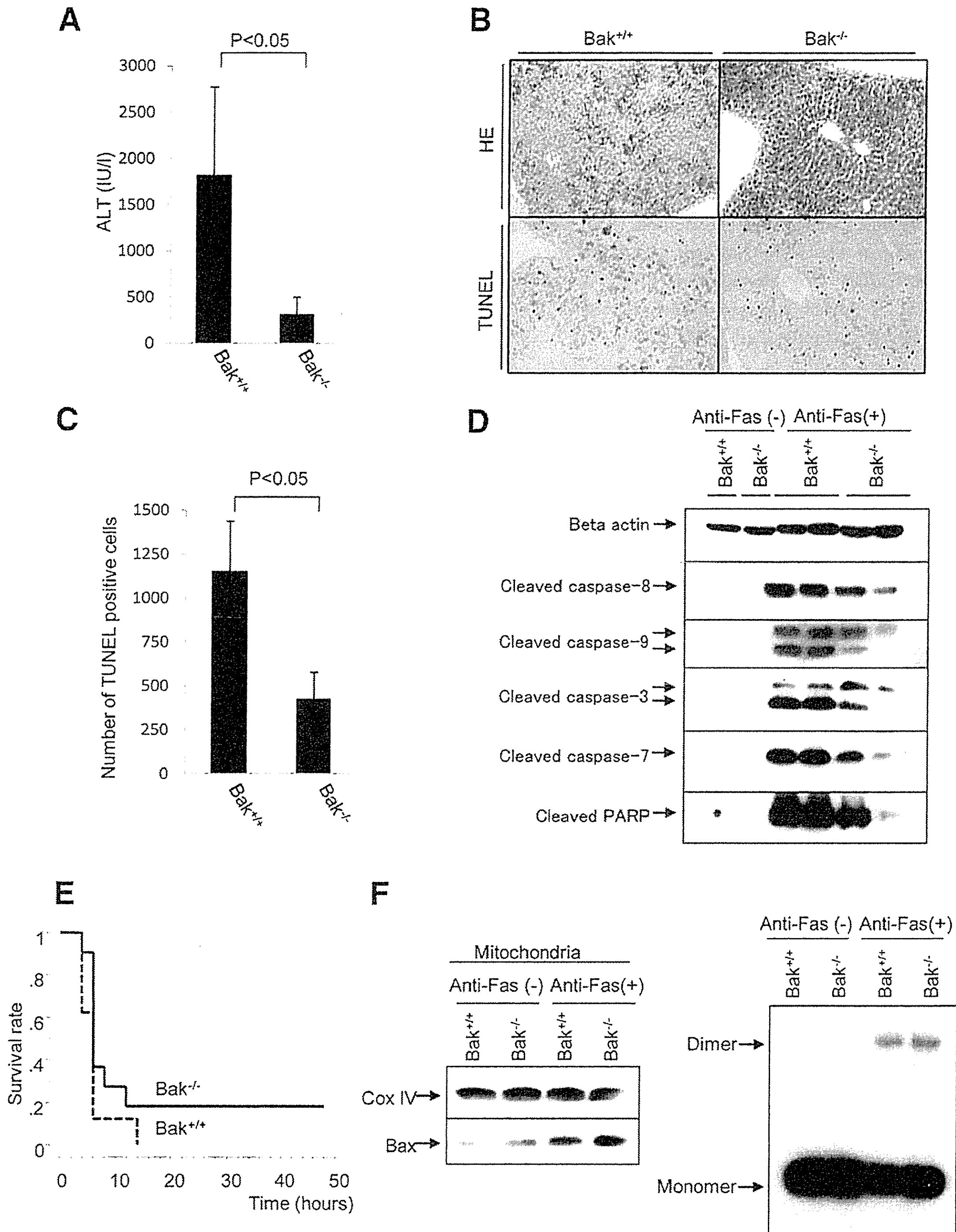

Fig. 1. Bak KO mice are partially resistant to Fas-induced hepatocellular apoptosis. Bak KO mice ($Bak^{-/-}$) or control WT littermates ($Bak^{+/+}$) were analyzed at 3 hours after intraperitoneal injection of 1.5 mg/kg Jo2 anti-Fas antibody. (A) Serum ALT levels (n = 10 or 11, respectively). (B) Hematoxylin and eosin (HE) and TUNEL staining of the liver sections. (C) Number of TUNEL-positive cells (n = 8 or 9, respectively). (D) Western blot analysis for the expressions of cleaved caspase-8, 9, -3, -7 and PARP. (E) Bak KO mice or control WT littermates were intraperitoneally injected with 1.5 mg/kg Jo2 anti-Fas antibody (n = 8 or 11, respectively). Survival rates after Jo2 injection are shown. (F) Bak KO mice or control WT littermates were analyzed 3 hours after intraperitoneal injection of Jo2 anti-Fas antibody (1.5 mg/kg) or vehicle. Left: Western blot analysis of the mitochondrial fraction of the liver for the expression of Bax. Right: Western blot analysis for the expression of Bax monomer and dimer in the liver.