表1　ベースライン時の患者背景（102試験）

CHARACTERISTIC	TDF (N=250)	ADV (N=125)
Mean Age (years)	44	43
Race Caucasian Asian	 64% 25%	 65% 24%
Male	77%	78%
Prior Lamivudine Experience	17%	18%
Mean HBV DNA (log_{10} copies/mL)	6.86	6.98
Mean ALT (U/L)	128	164
Mean Knodell Necroinflammatory Score Mean Knodell Fibrosis Score	7.8 2.3	7.8 2.4
Knodell Fibrosis Score=4 (Cirrhosis)	19%	20%
Viral Genotype A B C D	 12% 9% 12% 64%	 11% 14% 10% 63%

化を伴わず，投与後48週に完全寛解（血清HBV DNA＜400 copies/mL，かつ組織学的改善：Knodell壊死炎症スコア2ポイント以上の低下）に至った症例の比率である。48週以降，TDF群はTDFを継続投与（TDF-TDF群），ADV群はTDFに切り替えて（ADV-TDF群），さらに4年間（計5年間=240週間）の観察を行うが，72週までの結果が得られている。

ベースライン時の患者背景は，両群ともに同様であった（表1）。平均HBV DNA量はTDF群6.86 log_{10} copies/mL，ADV群 6.98 log_{10} copies/mLであった。平均ALT値はTDF群 128 U/L，ADV群 164 U/Lであり，Knodell壊死炎症スコアは両群ともに7.8，Knodell線維化スコアはTDF群 2.3，ADV群 2.4であり，両群ともに約20%が肝硬変を有していた。

投与後48週における主要評価項目および副次的評価項目のまとめを図1に示した。完全寛解に至った症例の比率は，TDF群は71%であり，ADV群の49%に比較して，有意に高率であった（$p<0.001$）。また，HBV DNA＜400 copies/mLに至った症例の比率は，TDF群が93%であり，ADV群の63%に比較して，有意に高率であった（$p<0.001$，図1）。一方，組織学的改善においては，TDF群が72%，ADV群が69%であり，両群間で明らかな差は認められなかった（図1）。

投与後48週以降の結果を図2～4に示す。図2は，HBV DNA＜400 copies/mLに至った症例の比率の推移であるが，48週時ではADV群は63%とTDF群（93%）に比べ，有意に低い値であった。しかし，48週以降にADVからTDFに切り替えたことによって，72週時にはADV-TDF群の88%がHBV DNA＜400 copies/mLに至った（TDF-TDF群：91%，$p=0.315$，図2）。図3は，平均HBV DNA量の推移であるが，投与後48週時ではTDF群の方が有意に減少が大きく（$p<0.001$），ADV群では投与後48週までに検出限界未満に至らなかった。TDFに切り替えた後，ADV-TDF群は56週以降，検出限界未満に至った（図3）。また，ALTの正常化は，ADVからTDFに切り替えた後も77%で維持されていた（TDF-TDF群：79%，図4）。TDF-TDF群において，HBV DNA＜400 copis/mLであった2例が，投与後72週までに，ウイルス量のリバウンド（≧400 copies/mL）を示した。2例ともアドヒアランス不良で

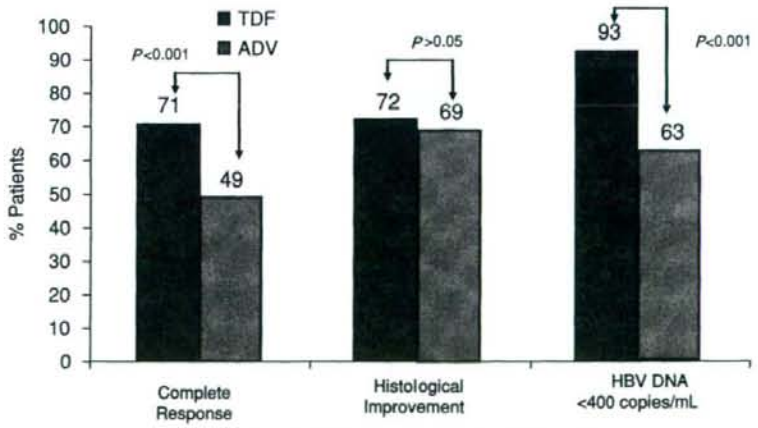

図1　102試験　主要評価項目および副次的評価項目

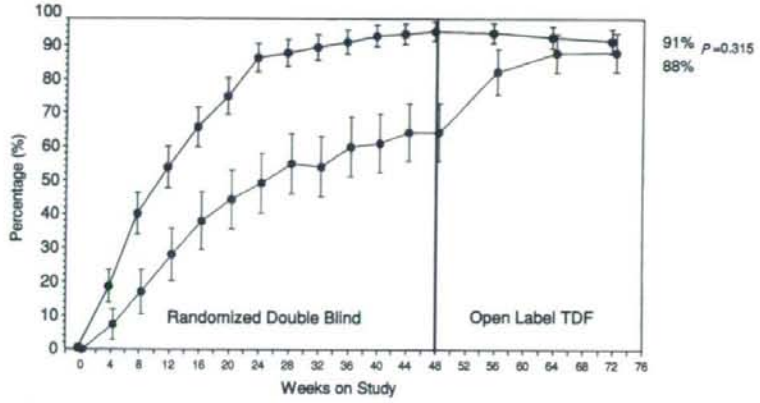

図2　HBV DNA＜400 copies/mLに至った症例比率（Missing=Failure，102試験）

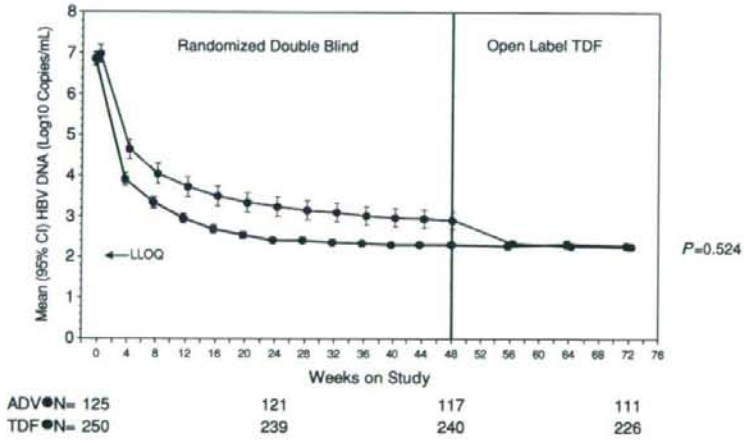

図3　平均HBV DNA量の推移（102試験）

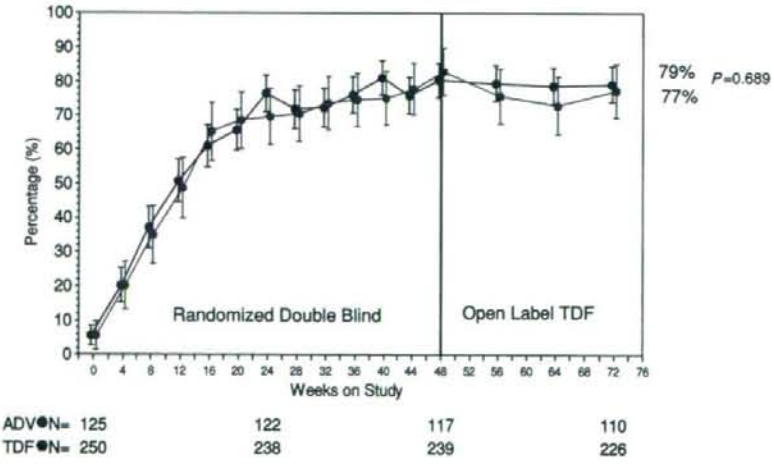

図4　ALTの正常化に至った症例比率（Missing=Failure，102試験）

表2　ADV-TDF群におけるTDF切り替えに対するHBV DNAの反応性（102試験）

Week 48 ADV Viral Response	Week 72 TDF Complete Viral Response
Complete Response (<400 copies/mL)	76 (100%)
Incomplete Response (≥400 copies/mL)	33 (94%)

表3　TDF-TDF群における24週時ウイルス不応例に対する転帰（102試験）

Week 24 TDF Viral Response	W48 Complete Response (<400 copies/mL)	W72 Complete Response (<400 copies/mL)
Complete Response (<400 copies/mL)	205 (99%)	197 (99%)
Incomplete Response (≥400 copies/mL)	24 (86%)	23 (92%)

あり，耐性検査を実施したが，TDF耐性にかかわる変異は認められなかった。ADV-TDF群において，TDFへの切り替えに対する反応性をみたところ，ADV投与後48週時にHBV DNA＜400 copies/mLであった症例は，TDFに切り替えても全例がHBV DNA＜400 copies/mLを維持していた（表2）。また，ADV投与後48週時にHBV DNA≧400 copies/mLであった症例の94%は，TDFに切り替えたことによって，ウイルスが速やかに抑制された（表2）。TDF-TDF群において，TDF投与後24週時にHBV DNA＜400 copies/mLであった症例は，その後も99%の症例がHBV DNA＜400 copies/mLを維持していた（表3）。また，TDF投与後24週時にHBV DNA≧400 copies/mLであった症例は，その後のTDF投与継続により，48週時には86%，72週時には92%がHBV DNA＜400 copies/mLに至った（表3）。

本試験における安全性については，投与後72週までの薬剤に起因する重篤な有害事象およびグレード3/4の臨床検査値異常については，両群間で同様であった（表4）。また，両群とも，クレアチニン0.5 mg/dL以上の増加やクレアチニンクリアランス＜50 mL/minを示す症例は認められず，腎関連の有害事象による投与中止例も認められなかった。

以上より，HBe抗原陰性のHBV単独感染患者において，TDFは効果が強力であり，HBV DNAの抑制に関しては，TDFの方が，ADVより優れていることが示された。また，TDFは，ADVと同様に忍容性や安全性が良好であることが示された。

2. HBe抗原陽性のHBV単独感染患者におけるTDFの抗HBV作用（Study GS-US-174-0103；103試験）[3, 4]

本試験は，未治療のHBV単独感染患者（HBe抗原陽性）266例を対象に，TDF 300mgの1日1回投与（以下，TDF群。N=176）とADV 10mgの1日1回投与（以下，ADV群。N=90）を比較した多施設二重盲検の無作為化コントロール試験である。主要評価項目としては，102試験と同様に，肝線維化の悪化を伴わず，投与後48週に完全寛解（血清HBV DNA＜400 copies/mL，かつ組織学的改善：Knodell壊死炎症スコア2ポイント以上の低下）に至った症例の比率である。48週以降，102試験と同様に，TDF群はTDFを継続投与（TDF-TDF群），ADV群はTDFに切り替えて（ADV-TDF群），さらに4年間（計5年間=240週間）の観察を行うが，72週までの結果が得られている。

表4　HBe抗原陰性のHBV単独感染患者における72週までの安全性データ（102試験）

	TDF-TDF (N=250)	ADV-TDF (N=125)
Study Drug-Related SAE	0.4%	0.8%
G3 Laboratory G4 Laboratory	14% 5%	13% 2%
Confirmed ↓phosphorus < 2mg/dL	2%	0
Confirmed 0.5 mg/dL↑ in creatinine	0	0
Confirmed creatinine clearance <50 mL/min	0	0

表5　ベースライン時の患者背景（103試験）

	TDF (N=176)	ADV (N=90)
Mean Age	34	34
Race Caucasian Asian	 52% 36%	 51% 36%
Male	68%	71%
Mean HBV (log10 copies/mL)	8.64	8.88
Mean ALT (U/mL)	142	155
Mean Knodell Necroinflammatory Score	8.3	8.5
Knodell Fibrosis (Score = 4)	20%	21%
Viral Genotype A B C D	 24% 15% 25% 32%	 21% 11% 30% 35%

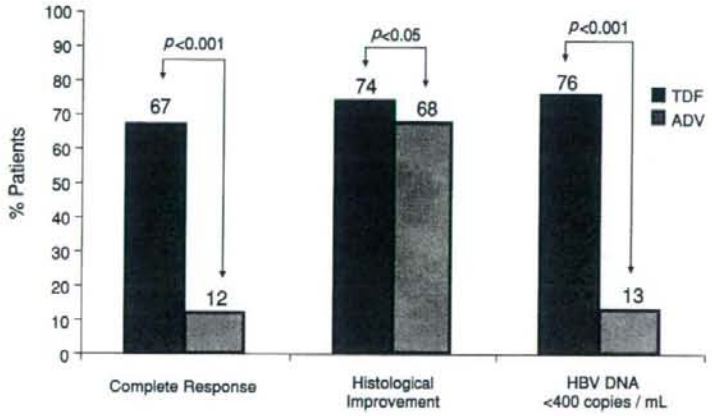

図5　103試験　主要評価項目および副次的評価項目

ベースライン時の患者背景は，両群ともに同様であった（表5）。平均HBV DNA量はTDF群8.64 $\log_{10}$ copies/mL，ADV群 8.88 $\log_{10}$ copies/mLであり，平均ALT値はTDF群 142 U/L，ADV群 155 U/Lであった。Knodell壊死炎症スコアはTDF群 8.3，ADV群 8.5であり，両群ともに約20%が肝硬変を有していた。

投与後48週において完全寛解に至った症例の比率は，TDF群は67%であり，ADV群の12%に比較して，有意に高率であった（$p<0.001$，図5）。また，HBV DNA＜400 copies/mLに至った症例比率は，TDF群が76%であり，ADV群の13%に比較して，有意に高率であった（$p<0.001$，図5）。一方，組織学的改善においては，両群で明らかな差は認められなかった（74% vs 68%, $p>0.05$，図5）。

投与後48週以降の結果を図6～10に示す。図6は，HBV DNA＜400 copies/mLに至った症例の比率の推移であるが，48週時ではADV群は13%とTDF群（76%）に比べ，有意に低い値であった。48週以降のADVからTDFへの切り替えによって，72週時には76%となった（TDF-TDF群：79%，$p=0.617$，図6）。図7は，平均HBV DNA量の減少の推移であるが，投与後48週時では，TDF群の方がADV群に比べ，有意に減少の程度は大きかった（$p<0.001$）。TDFに切り替えた後，ADV群のプラトー状態であった平均HBV DNA

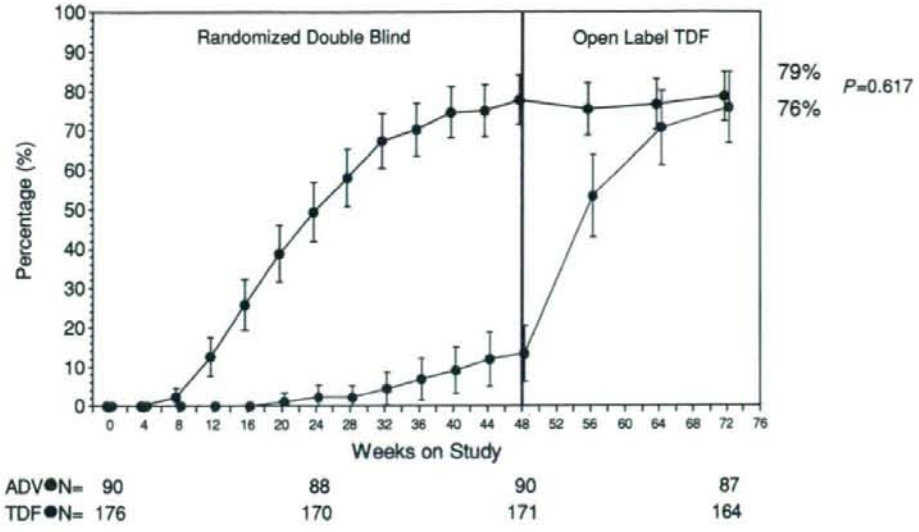

図6　HBV DNA＜400 copies/mLに至った症例比率（Missing=Failure，103試験）

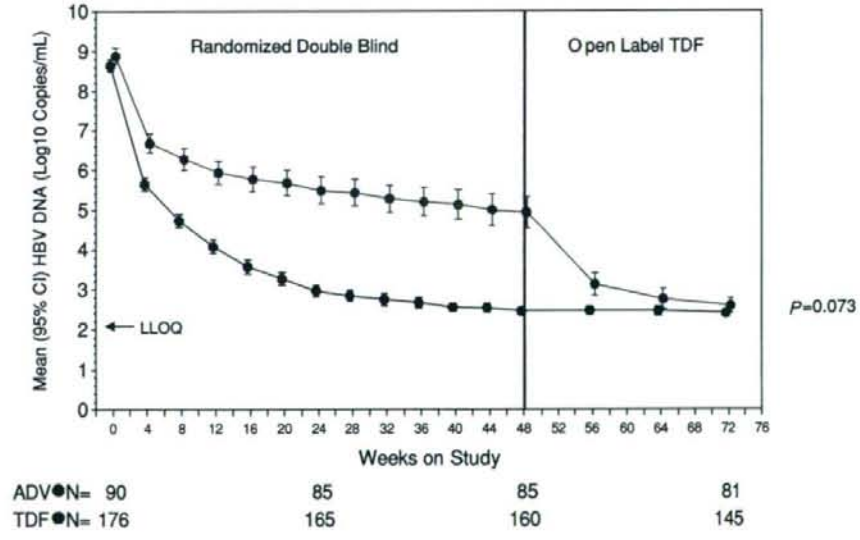

図7　平均HBV DNA量の推移（103試験）

量は，さらに減少を認め，72週時にはTDF-TDF群と同程度までに至った（$p = 0.073$）。ALTの正常化を示した症例の比率は，TDF-TDF群では72週時，77%であったが，ADV-TDF群では依然，72週時，TDFに切り替え以降も61%と有意に低率であった（$p = 0.014$，図8）。ADV-TDF群において，TDFへの切り替えに対する反応性をみたところ，ADV投与後48週時にHBV DNA＜400 copies/mLであった症例は，TDFに切り替えても全例がHBV DNA＜400 copies/mLを維持しており，また，ADV投与後48週時にHBV DNA≧400

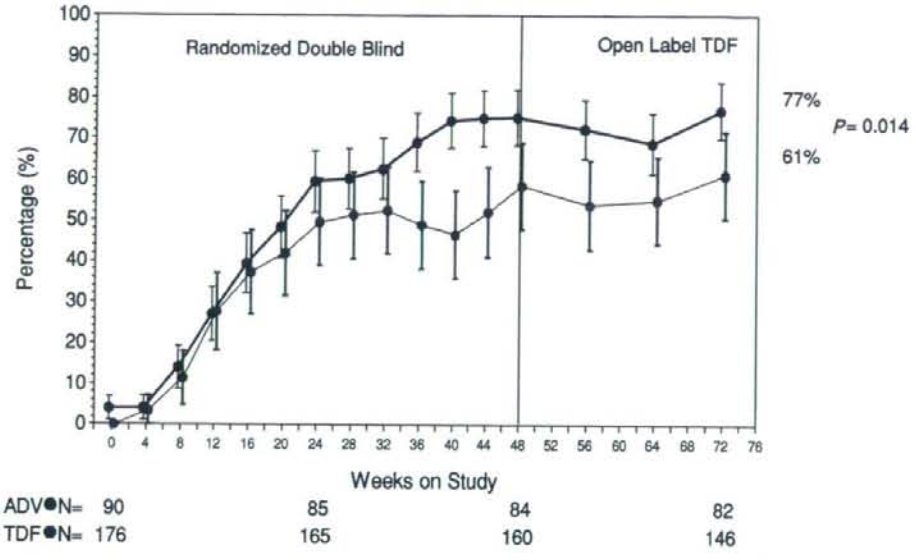

図8　ALTの正常化に至った症例比率（Missing=Failure，103試験）

表6　ADV-TDF群におけるTDF切り替えに対するHBV DNAの反応性（103試験）

Week 48 ADV Viral Response	Week 72 TDF complete Viral Response
Complete Response (＜400 copies/mL)	12 (100%)
Incomplete Response (≥400 copies/mL)	54 (78%)

表7　TDF-TDF群における24週時ウイルス不応例に対する転帰（103試験）

Week 24 Viral Response	W48 Complete Viral Response (＜400 copies/mL)	W72 Complete Viral Response (＜400 copies/mL)
Complete Response (＜400 copies/mL)	80 (100%)	71 (100%)
Incomplete Response (≥400 copies/mL)	50 (66%)	56 (79%)

copies/mLであった症例の78%が，TDFに切り替えたことによって，HBV DNA＜400 copies/mLに至った（表6）。TDF-TDF群において，TDF投与後24週時にHBV DNA＜400 copies/mLであった症例は，その後も100%の症例がHBV DNA＜400 copies/mLを維持していた（表7）。また，TDF投与後24週時にウイルスがコントロールされていなかった症例は，その後のTDF投与継続により，48週時には66%，72週時には79%がHBV DNA＜400 copies/mLに至った（表7）。図9に投与後48週および64週（ADV群ではTDFに切り替え後24週）のHBeセロコンバージョンの比率を示した。64週時のTDF-TDF群におけるHBeセロコンバージョン率は26%であり，ADV-TDF群の21%と比較して，明らかな差はなかった（p = NS）。一方，HBsセロコンバージョン（図10）については，「HBs抗原の消失」に至った症例は，64週時，ADV-TDF群では0%であったが，TDF-TDF群では48週以降，3%から5%に増加し，有意差が認められた（p = 0.004）。一方，「HBs抗体の出現」まで至った症例は，ADV-TDF群では64週時に0%であったが，TDF-TDF群では48週以降，1%から2%に増加していた（p=NS）。

本試験における安全性については，投与後72週までの薬剤に起因する重篤な有害事象およびグレード3/4の臨床検査値異常については，両群間で顕著な差は認められなかった（表8）。また，ADV-TDF群で1%にクレアチニン0.5 mg/dL以上の増加が認められたが，これ以外には，両群においてクレアチニンクリアランス＜50 mL/minを示す症例は認められず，腎関連の有害事象による投与中止例も認められなかった。

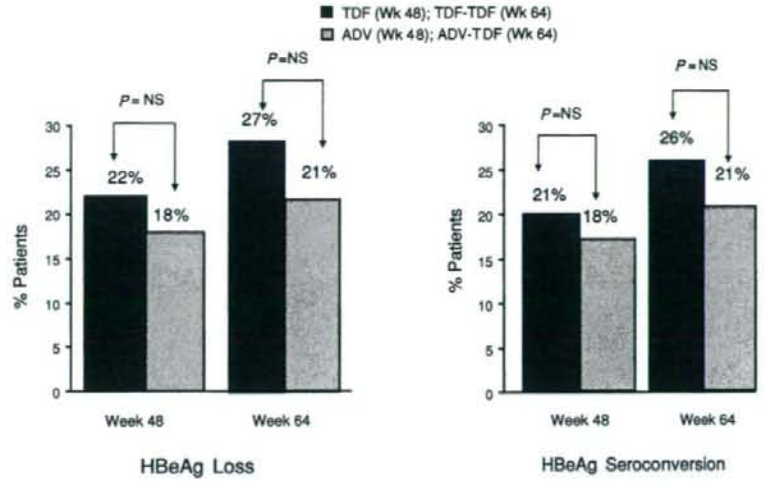

図9　投与後48週および64週におけるHBeセロコンバージョン（103試験）

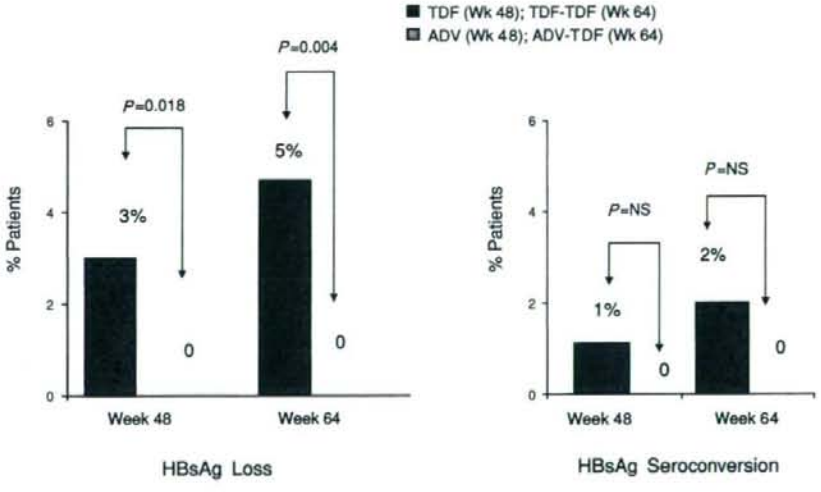

図10　投与後48週および64週におけるHBsセロコンバージョン（103試験）

以上より，HBe抗原陽性のHBV単独感染患者において，TDFは効果が強力であり，HBV DNAの抑制に関しては，TDFの方が，ADVより優れていることが示された。また，TDFは，ADVと同様に忍容性や安全性が良好であることが示された。さらに，102試験および103試験の結果より，TDF投与後24週時にウイルスが検出限界未満に至っていないということは，TDFの治療失敗を予知させるものではなく，TDFを継続投与することにより，ウイルスが検出限界未満にコントロールされるというベネフィットがある。

3．TDFの安全性

概説した2試験の安全性については試験毎に記載したが，これらの試験において，TDFの安全性はADVと同様であり，忍容性が高いことが示された。これまでのHIV/HBV合併例におけるTDFの使用経験においても，特記すべき重篤な副作用は報告されていない。また，TDF投与中，ALTの上昇を示した症例が認められているが，肝不全に至った症例はない。

TDFの最も注意すべき副作用として，腎障害がHIV感染症患者で報告されているが，今回の2試験を

表8　HBe抗原陽性のHBV単独感染患者における72週までの安全性データ（103試験）

	TDF-TDF (N=176)	ADV-TDF (N=90)
Study Drug-Related SAE (ALT Flare)	4% (3%)	7% (7%)
G3 Laboratory excluding ALT G3 ALT	18% 15%	10% 10%
G4 Laboratory	12%	11%
Confirmed ↓ phosphorus <2 mg/dL	1%	1%
Confirmed 0.5 mg/dL ↑ in creatinine	0	1%
Confirmed creatinine clearance <50 mL/min	0	0

含め，これまでHBV感染症に対するTDFの使用において腎機能の明らかな変動の報告はない。

TDF投与中に発現した腎障害の多くは可逆的であることが報告されているが，TDFを投与する場合には，血中クレアチニンや血中リン等の変動を注意深く観察し，異常が認められた場合には，投与を中止する等，適切な処置を行うことが必要である。最近，リスク因子の検討が進んでいるが，腎機能障害の既往がある患者や腎毒性のある薬剤が投与されている患者，あるいは糖尿病を合併している患者では注意すべきである。また，腎機能が低下している患者においては，テノホビルの血中濃度が上昇することによる副作用を発現する可能性があることから，添付文書通りの投与間隔の調節が必要である。なお，この他，TDFの主な副作用としては，鼓腸，下痢などの消化器系の症状が報告されている。

また，TDFの投与を中断する場合には，HBVが再増殖するおそれがあるので，十分注意が必要である。特に，非代償性の場合，重症化するおそれがあるので注意が望まれる。

おわりに

現在，本邦におけるB型慢性肝炎に対する抗ウイルス薬としてインターフェロンとLAM，ADVおよびETVの4剤が使用可能である。TDFは，既に，HIV/HBV合併例に対する多剤併用療法（HAART）として，海外の主要なガイドラインで選択されるべき薬剤として位置付けられており，本邦においても第一選択薬の一つと位置付けられている。しかしながら，TDFのHBV単独感染症に対する適応は，本邦では，まだ得られていない。

今回，5年間の観察が計画されているTDFの第Ⅲ相試験の中間成績を報告したが，これらの試験はさらに8年間の観察がされるようである。核酸アナログの耐性プロファイルや完全寛解に至る効果の強さは，薬剤によって異なっていることから，どのような使い方が，長期的に耐性の発現を最小にし，最大のウイルス抑制作用を持続させるのにベストであるのかを明らかにすることが課題である。今後，核酸アナログの長期使用成績が集積することで，「ベストな長期核酸アナログ療法」の評価が可能になると考えられる。

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Transient elastography for measurement of liver stiffness measurement can detect early significant hepatic fibrosis in Japanese patients with viral and nonviral liver diseases

Noriyuki Obara[1], Yoshiyuki Ueno[1], Koji Fukushima[1], Yu Nakagome[1], Eiji Kakazu[1], Osamu Kimura[1], Yuta Wakui[1], Osamu Kido[1], Masashi Ninomiya[1], Takayuki Kogure[1], Jun Inoue[1], Yasuteru Kondo[1], Masaaki Shiina[1], Takao Iwasaki[1], Takeshi Yamamoto[2], and Tooru Shimosegawa[1]

[1] Division of Gastroenterology, Tohoku University Graduate School of Medicine, 1-1 Seiryo, Aoba, Sendai 980-8574, Japan
[2] Department of Gastroenterology, Tohoku Koseinenkin Hospital, Miyagino, Sendai, Japan

Background. Many studies have reported the efficiency of transient elastography, a noninvasive, reproducible, and reliable method for predicting liver fibrosis, in patients with chronic hepatitis C (CHC) and B (CHB), but there are few reports about nonviral chronic liver disease (CLD) such as primary biliary cirrhosis (PBC), nonalcoholic steatohepatitis (NAFLD), and autoimmune hepatitis (AIH). We therefore compared the efficiency of transient elastography between CHC and nonviral CLD. ***Methods.*** We assessed the accuracy of liver stiffness measurement (LSM) using Fibroscan, and compared these values with those of hyaluronic acid, type 4 collagen, platelet count, prothrombin index, and AST/platelet ratio index (APRI) as indices for the diagnosis of liver fibrosis in 114 patients with a variety of chronic liver diseases: CHC (n = 51), CHB (n = 11), NAFLD (n = 17), PBC (n = 20), and AIH (n = 15). The histology was assessed according to the METAVIR score by two pathologists. ***Results.*** The number of fibrosis stage (F0/1/2/3/4) with CHC was 9/15/12/6/10, and that with nonviral CLD was 10/21/11/4/6, respectively. The ability, assessed by area under receiver operating characteristic (AUROC) curve, to predict liver fibrosis F ≥ 2 for LSM, HA, type 4 collagen, platelet count, prothrombin index, and APRI, was 0.92, 0.81, 0.87, 0.85, 0.85, and 0.92 in CHC patients, respectively; and 0.88, 0.72, 0.81, 0.67, 0.81, and 0.77 in nonviral CLD patients, respectively. ***Conclusions.*** In patients with nonviral CLD, LSM was most helpful in predicting significant fibrosis (F ≥ 2). Transient elastography is a reliable method for predicting significant liver fibrosis, not only in CHC patients but also in nonviral CLD patients.

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Reprint requests to: Y. Ueno

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Introduction

The prognosis and clinical management of chronic liver disease (CLD) are strongly influenced by the extent of liver fibrosis, because life-threatening complications mainly occur in patients with cirrhosis. The annual incidences of hepatocellular carcinoma, decompensation, and death are approximately 3%, 4%, and 3%, respectively,[1–5] underscoring the need for early identification of developing liver fibrosis to prevent complications. Although liver biopsy (LB) remains the gold standard for the assessment of the degree of fibrosis, this procedure is essentially invasive and with potentially fatal complications. Moreover, LB is an expensive procedure difficult for some patient to accept.[6,7] In addition, sampling error is common because only 1/50 000 of the organ is analyzed and thus up to 30% of results are false negatives.[8]

Liver stiffness measurement (LSM) by transient elastography is a rapid, noninvasive, and reproducible method[9–16] and has been compared with classical hematological markers, such as serum hyaluronic acid level,[17] type 4 collagen,[18] and the aspartate transaminase to platelet ratio index (APRI),[9,19] which have been reported to accurately represent the state of liver fibrosis.

However, a thorough comparison of the diagnostic ability of transient elastography to detect significant fibrosis between patients with chronic hepatitis C (CHC) and those with nonviral CLD, such as primary biliary cirrhosis (PBC), nonalcoholic steatohepatitis (NAFLD), and autoimmune hepatitis (AIH), has not been performed, especially in the Japanese population. The aim of the current study was to compare the diagnostic performance of transient elastography between CHC and nonviral CLD for the evaluation of liver fibrosis.

Material and methods

Patients

One hundred thirty-three consecutive patients of chronic liver disease (CLD) who underwent a liver biopsy (LB) and liver stiffness measurement (LSM) within a period of less than 6 months at the Division of Gastroenterology of Tohoku University Hospital from January 2006 to November 2007 were included in this study. Written informed consent was obtained from all patients before the procedures. Twelve patients had unsuitable biopsy specimens, and 7 patients had an unreliable LSM; thus, the analysis was performed in 114 patients with CHC (n = 51), chronic hepatitis B (CHB, n = 11), NAFLD (n = 17), PBC (n = 20), and AIH (n = 15).

CHC and CHB were diagnosed by the presence of serological hepatitis C virus (HCV)-RNA and hepatitis B virus (HBV)-DNA measured by polymerase chain reaction, respectively. The diagnosis of NAFLD was based on the following criteria: (1) elevated aminotransferases [aspartate aminotransferase (AST) or alanine aminotransferase (ALT)]; (2) liver biopsy showing steatosis in at least 10% of hepatocytes; and (3) exclusion of other etiological liver disease, including alcohol-induced or drug-induced liver disease, autoimmune or viral hepatitis, and cholestatic or metabolic/genetic liver disease. PBC was diagnosed by at least two of the following criteria: serum alkaline phosphatase (ALP) more than 1.5 times the upper limit of normal, a positive antimitochondrial antibody (>1:40), and compatible liver histology. The diagnosis of AIH was based on the revised descriptive criteria for the diagnosis of AIH reported by the International Autoimmune Hepatitis Group (IAHG) in 1999.[20] Criteria for exclusion from the study were the coexistence of hepatocellular carcinoma, a history of alcoholic abuse, receiving therapy of anticoagulants, obvious existence of ascites observed with sonography, and unsuitable LB and LSM.

Blood liver tests [i.e., serum bilirubin and albumin levels, gamma-glutamyl transpeptidase (GGT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), platelet count, prothrombin index, hyaluronic acid level, and type 4 collagen level] were performed before LB within a period of 1 week. Serum hyaluronic acid (HA) and type 4 collagen were evaluated using a Latex Agglutination-Turbidimetric Immunoassay.

Liver stiffness measurement (LSM)

Measurement of the liver stiffness by transient elastography was performed using a FibroScan (EchoSens, Paris, France), as reported previously.[14] In brief, vibrations of mild amplitude and low frequency are transmitted from the vibrator toward the tissues by the transducer itself, inducing an elastic shear wave that propagates through the tissue. All measures were performed on the right lobes of the liver through intercostal spaces while the patients were lying on their backs with the right arm in maximal abduction. An ultrasound guide was used to identify a target liver area, at least 6 cm thick without major vascular structures. The measurement depth was between 25 and 65 mm below the skin surface. The procedure was based on at least ten successful measurements, and a greater than 60% success rate (ratio between numbers of successful and total measurements) was obtained. Liver stiffness was recorded in kilopascals (kPa) as the median value of all measurements. The measurement of this liver stiffness was approved by the Institutional Review Board (IRB) (#2006-69).

Serum surrogate markers

The following parameters were determined using blood samples within 1 week before the LB: hyaluronic acid level,[17] type 4 collagen level,[18] platelet count, prothrombin index, and AST/platelet ratio index (APRI).[9,19] The APRI was calculated because it was previously reported to be associated with the severity of fibrosis in patients with CHC infection. The formula used was AST (per upper limit of normal; 35 IU/L) × 100/platelet count (10^9/l).[9,19]

Liver histology and staging

Liver biopsy was performed under sonography or laparoscopy. A Silverman needle (14-gauge) under laparoscopy or a Tru-cut needle (16-gauge) under sonography was used to obtain an adequate specimen of hepatic tissue. LB specimens were fixed in paraformaldehyde and embedded in paraffin. Sequentially, they were stained with hematoxylin and eosin or elastic Masson's trichrome staining. All specimens were analyzed by two independent, experienced liver pathologists blinded to the results of LSM and clinical data. LB specimens with a length of less than 10 mm, and those considered as unsuitable for fibrosis assessment by the pathologists, were excluded. Liver fibrosis was evaluated according to the METAVIR scoring system with some modifications.[21] Fibrosis was staged into a 0–4 scale as follows: F0 = no fibrosis; F1 = portal fibrosis, periportal fibrosis, or perisinusoidal fibrosis without septa; F2 = few septa; F3 = numerous septa without cirrhosis; and F4 = cirrhosis.

Statistical analysis

Patients were divided according to their consensus fibrosis stage. Differences between quantitative

Table 1. Characteristics of patients

	CHB (n = 11)	CHC (n = 51)	NAFLD (n = 17)	PBC (n = 20)	AIH (n = 15)	Total (n = 114)
Sex (male/female)	11/0	28/23	11/6	3/17	2/13	55/59
Age (years)	46 (24–64)	57.5 (27–74)	50 (25–71)	55.5 (38–75)	55 (18–63)	56 (18–75)
Height (cm)	171 (159–178)	161 (143–180)	160 (147–178)	155 (144–181)	155 (146–171)	160 (143–181)
Body weight (kg)	68 (58–82)	57 (40–128)	68 (44–107)	53 (41–71)	61 (38–67)	61 (38–128)
Stage of liver fibrosis F: 0/1/2/3/4	0/2/4/2/3	9/15/12/6/10	6/3/3/1/4	3/11/4/0/2	1/7/4/3/0	19/37/27/12/19
Total bilirubin (mg/dl)	1.1 (0.7–1.8)	0.9 (0.4–1.8)	1.2 (0.6–2.9)	0.9 (0.5–12.3)	0.9 (0.4–2.9)	1.0 (0.4–12.3)
AST (IU/l)	56 (20–420)	48 (13–291)	63 (27–160)	59 (16–241)	75 (31–351)	59 (13–420)
ALT (IU/l)	74 (26–800)	58 (9–433)	88 (18–245)	60 (14–175)	75 (33–972)	64 (9–972)
ALP (IU/l)	307 (214–726)	252 (85–782)	312 (158–727)	582 (151–3806)	386 (171–569)	4.0 (2.6–5.1)
GGT (IU/l)	63 (24–159)	48 (10–291)	77 (40–911)	189 (30–1861)	80 (13–406)	70 (10–1861)
Albumin (g/dl)	3.7 (3.1–4.9)	4.1 (2.7–5.0)	4.2 (3.0–5.1)	3.9 (2.6–4.3)	3.8 (2.8–4.7)	4.0 (2.6–5.1)
Platelet count (10^4/l)	166 (91–293)	159 (78–378)	224 (75–343)	221 (101–411)	205 (70–419)	184 (70–419)
Prothrombin index (%)	83 (63–120)	87 (63–120)	88 (40–118)	103 (63–120)	90 (57–109)	88 (40–120)
Hyaluronic acid (ng/ml)	90 (12–374)	110 (10–1307)	73 (10–8000)	75 (12–1053)	107 (10–885)	95 (10–8000)
Type 4 collagen (ng/ml)	216 (59–447)	152 (59–437)	136 (77–633)	150 (61–688)	151 (102–447)	150 (59–688)
Liver stiffness (kPa)	18.0 (5.0–27.4)	10.5 (3.3–36.3)	7.4 (4.8–69.1)	8.1 (4.2–75.0)	8.2 (4.8–35.3)	9.6 (3.3–75.0)

Median values are shown (ranges shown in parentheses)
CHC, chronic hepatitis C; CHB, chronic hepatitis B; NAFLD, nonalcoholic steatohepatitis; PBC, primary biliary cirrhosis; AIH, autoimmune hepatitis; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transpeptidase

variables were analyzed by a nonparametric test (Mann–Whitney U test). The trend between LSM and serum surrogate markers and ordinate fibrosis stages was estimated by the Spearman's ρ coefficient. The diagnostic performance of LSM and serum surrogate markers was determined in terms of sensitivity, specificity, positive predictive value (PPV), negative predictive values (NPV), and area under receiver operating characteristics (AUROC) curves. Optimal cutoff values between fibrosis categories were determined at the maximum sum of sensitivity plus specificity. The significance level was set at 0.05, and all P values were two-tailed. Statistical analyses were performed with Dr. SPSS II software (SPSS Japan, Tokyo, Japan).

Results

Patients

Among the 133 patients, 1 patient with CHB, 5 patients with CHC, 2 patients with NAFLD, 3 patients with PBC, and 1 patient with AIH were excluded because of unsuitable LB (n = 12). Similarly, 1 patient with CHB, 3 patients with CHC, and 3 patients with NAFLD were excluded because of defective LSM (n = 7). Data analysis was performed on the basis of the 114 remaining patients (CHB, n = 11; CHC, n = 51; NAFLD, n = 17; PBC, n = 20; AIH, n = 15). Their characteristics are provided in Table 1.

Liver biopsies

The median length of the liver biopsy samples was 17 mm, and the median number of fragments was one. The fibrosis grades of the 114 biopsy specimens of all patients were as follows: F0, n = 19; F1, n = 37; F2, n = 27; F3, n = 12; F4, n = 19. Those of the CHC patients were F0, n = 9; F1, n = 15; F2, n = 12; F3, n = 6; F4, n = 10. Those of the nonviral CLD patients were F0, n = 10; F1, n = 21; F2, n = 11; F3, n = 4; F4, n = 6 (see Table 1).

Relationship between the fibrosis stage and liver stiffness measurement

The liver stiffness values ranged from 3.3 to 75.0 kPa (median, 9.6 kPa). The median liver stiffness values in all patients with F0 (n = 19), F1 (n = 37), F2 (n = 27), F3 (n = 12), and F4 (n = 19) were 5.4, 6.8, 10.9, 19.7, and 27.4 kPa, respectively (Fig. 1A). Subgroup analysis demonstrated that the values in CHC patients with F0 (n = 9), F1 (n = 15), F2 (n = 12), F3 (n = 6), and F4 (n = 10) were 4.3, 6.7, 14.7 13.7, and 21.9 kPa, respectively; and in patients with nonviral CLD with F0

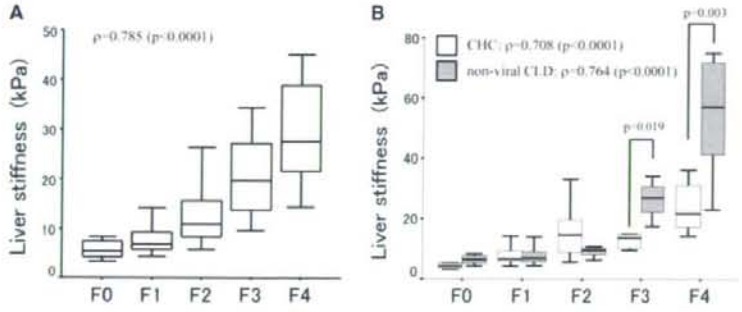

Fig. 1. Box plots of liver stiffness according to METAVIR fibrosis stage in all patients (**A**) and according to liver disease etiology (**B**). The lengths of the boxes represent the interquartile range within which 50% of the values were located. The *lines* in the boxes represent the median values. *Upper and lower error bars* are computed as upper quartile + 1.5* (interquartile range) and lower quartile + 1.5* (interquartile range), respectively. The ρ values are those obtained by the Spearman's ρ coefficient. There were statistically significant differences between chronic hepatitis C (*CHC*) and nonviral chronic liver disease (*CLD*) by the Mann–Whitney *U* test with F = 3 ($P = 0.019$) and F = 4 ($P = 0.003$)

Table 2. Correlation between liver fibrosis stage and surrogate markers

	LSM	Hyaluronic acid	Tyep 4 collagen	Platelet count	Prothrombin index	APRI
All patients	0.785	0.589	0.605	–0.579	–0.651	0.631
CHC patients	0.764	0.583	0.676	–0.662	–0.559	0.750
Nonviral CLD patients	0.708	0.528	0.550	–0.443	–0.619	0.492

Spearman's ρ coefficient
LSM, liver stiffness measurement; APRI, AST/platelet ratio index; CLD, chronic liver disease

Table 3. Performance of transient elastography for the determination of fibrosis stage in all patients, patients with chronic hepatitis C, and nonviral chronic liver disease

	All ($n = 114$)				CHC ($n = 51$)				Nonviral ($n = 52$)			
	F ≥ 1	F ≥ 2	F ≥ 3	F = 4	F ≥ 1	F ≥ 2	F ≥ 3	F = 4	F ≥ 1	F ≥ 2	F ≥ 3	F = 4
AUROC	0.84	0.94	0.94	0.94	0.87	0.92	0.85	0.90	0.76	0.88	0.99	0.99
Optimal cutoff (kPa)	8.4	9.5	14.2	17.2	5.6	9.5	10.3	17.2	8.4	8.1	15.8	21.8
Sensitivity	0.64	0.84	0.90	0.94	0.93	0.89	0.94	0.80	0.55	0.91	1.00	1.00
Specificity	0.95	0.83	0.84	0.85	0.78	0.83	0.69	0.88	1.00	0.76	0.95	0.91
Positive predictive value	0.64	0.84	0.71	0.55	0.95	0.86	0.96	0.95	1.00	0.73	0.83	0.60
Negative predictive value	0.95	0.91	0.96	0.98	0.70	0.86	0.58	0.62	0.31	0.92	1.00	1.00

AUROC, area under receiver operating characteristic

($n = 10$), F1 ($n = 21$), F2 ($n = 11$), F3 ($n = 4$), and F4 ($n = 6$), they were 6.6, 6.9, 9.6, 27.2, and 57.1 kPa, respectively (Fig. 1B). There were significant differences between CHC and non-viral CLD with F = 3 (Mann–Whitney *U* test, $P = 0.019$) and F = 4 ($P = 0.003$), respectively.

There were significant correlations between the liver fibrosis and LSM by the Spearman's ρ coefficient in all patients ($\rho = 0.785$; $P < 0.0001$), in patients with CHC ($\rho = 0.764$; $P < 0.0001$), and in patients with nonviral CLD ($\rho = 0.708$; $P < 0.0001$) (Table 2). The optimal cutoff values are those giving the highest sum of sensitivity plus specificity. The AUROC curve in all patients for the diagnosis of fibrosis F ≥ 1, F ≥ 2, F ≥ 3, and F = 4 was 0.84, 0.94, 0.94, and 0.94, respectively; and the optimal cutoff values were 8.4, 9.5, 14.2, and 17.2 kPa for F ≥ 1, F ≥ 2, F ≥ 3, and F = 4, respectively (Fig. 2A, Table 3). The AUROC curve in patients with CHC for diagnosis of fibrosis F ≥ 1, F ≥ 2, F ≥ 3, and F = 4 was 0.87, 0.92, 0.85, and 0.90, respectively, and the optimal cutoff values were 5.6, 9.5, 10.3, and 17.2, respectively (Fig. 2B, Table 3). The AUROC curve in patients with nonviral CLD for the diagnosis of fibrosis F ≥ 1, F ≥ 2, F ≥ 3, and F = 4 was 0.76, 0.88, 0.99, and 0.99, respectively, and the optimal cutoff values were 8.4, 8.1, 15.8, and 21.8, respectively (Fig. 2C, Table 3).

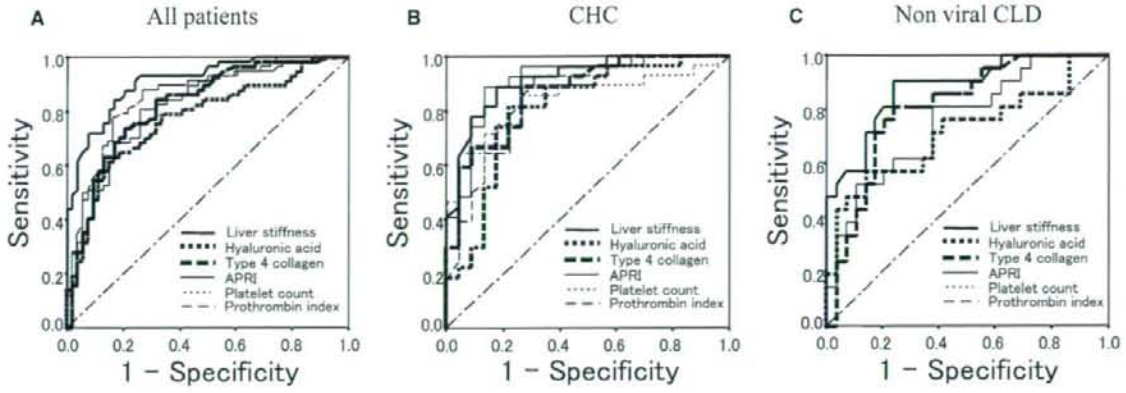

Fig. 2. Receiver operating characteristic curves for diagnosis of METAVIR fibrosis F ≥ 2 by transient elastography (*bold black line*), hyaluronic acid (*bold dotted line*), type 4 collagen (*bold dashed line*), AST/platelet ratio index (APRI) (*thin black line*), platelet count (*thin dotted line*), and prothrombin index (*thin dashed line*) in all patients (**A**), in patients with chronic hepatitis C (CHC) (**B**), and in nonviral chronic liver disease (CLD) (**C**)

Table 4. Comparisons of parameters associated with significant fibrosis (F ≥ 2) on liver biopsy

	F0–F1 (n = 56) median (range)	F2–F4 (n = 58) median (range)	P
Sex (male/female)	22/34	33/25	0.061
Age (years)	55.0 (25–75)	57 (18–74)	0.277
Height (cm)	158.5 (144–181)	164.0 (143–180)	0.340
Body weight (kg)	57.0 (38–107)	62.5 (41–128)	0.104
Total bilirubin (mg/dl)	0.85 (0.4–4.2)	1.1 (0.6–12.3)	<0.001
AST (IU/l)	40.0 (13–160)	71.5 (26–420)	<0.001
ALT (IU/l)	54.5 (9–245)	74.0 (18–972)	0.004
ALP (IU/l)	276.0 (85–1102)	367.0 (175–3806)	0.009
GGT (IU/l)	57.5 (10–932)	76.0 (19–1861)	0.046
Albumin (g/dl)	4.1 (3.0–5.1)	3.7 (2.6–4.9)	0.001
Platelet count (10^3/mm^3)	224.5 (101–378)	127.0 (70–419)	<0.001
Prothrombin index (%)	98.5 (63.7–120)	79.5 (40.4–120)	<0.001
Hyaluronic acid (ng/ml)	41.4 (10–427.5)	144.5 (11.5–8000)	<0.001
Type 4 collagen (ng/ml)	117.0 (59–688)	209.0 (84–652)	<0.001
APRI	0.52 (0.12–3.51)	1.59 (0.38–14.33)	<0.001
Liver stiffness (kPa)	6.45 (3.3–20.4)	17.2 (5.8–75.0)	<0.001

Mann-Whitney U-test

Relationship between the fibrosis stage and serum surrogate markers

To assess the predictive performance of the serum surrogate markers for significant fibrosis (F ≥ 2), we compared the minimal fibrosis group (F0–F1) and the significant fibrosis group (F2–F4). Significant differences were found in total bilirubin, AST, platelet count, prothrombin index, hyaluronic acid, type 4 collagen, and APRI between the minimal fibrosis group (F0–F1) and significant fibrosis group (Mann–Whitney U test, $P < 0.001$) (Table 4). We evaluated sequentially the correlations between the liver fibrosis by Spearman's ρ coefficient. Hyaluronic acid (ρ = 0.589; $P < 0.0001$), type 4 collagen (ρ = 0.605; $P < 0.0001$), and APRI (ρ = 0.631; $P < 0.0001$) were significantly positively correlated with the fibrotic stage, whereas the platelet count (ρ = –0.579; $P < 0.0001$) and prothrombin index (ρ = –0.651; $P < 0.0001$) had a significant negative correlation (see Table 2). Accordingly, we use those values to assess the predicting performance for significant fibrosis (F ≥ 2). The median hyaluronic acid level in all patients with F0, F1, F2, F3, and F4 was 27.4, 43.8, 93.6, 180.8, and 329.0 ng/ml, respectively. The median type 4 collagen level in all patients with F0, F1, F2, F3, and F4 was 104, 125, 165, 216, and 303 ng/ml, respectively. The median platelet

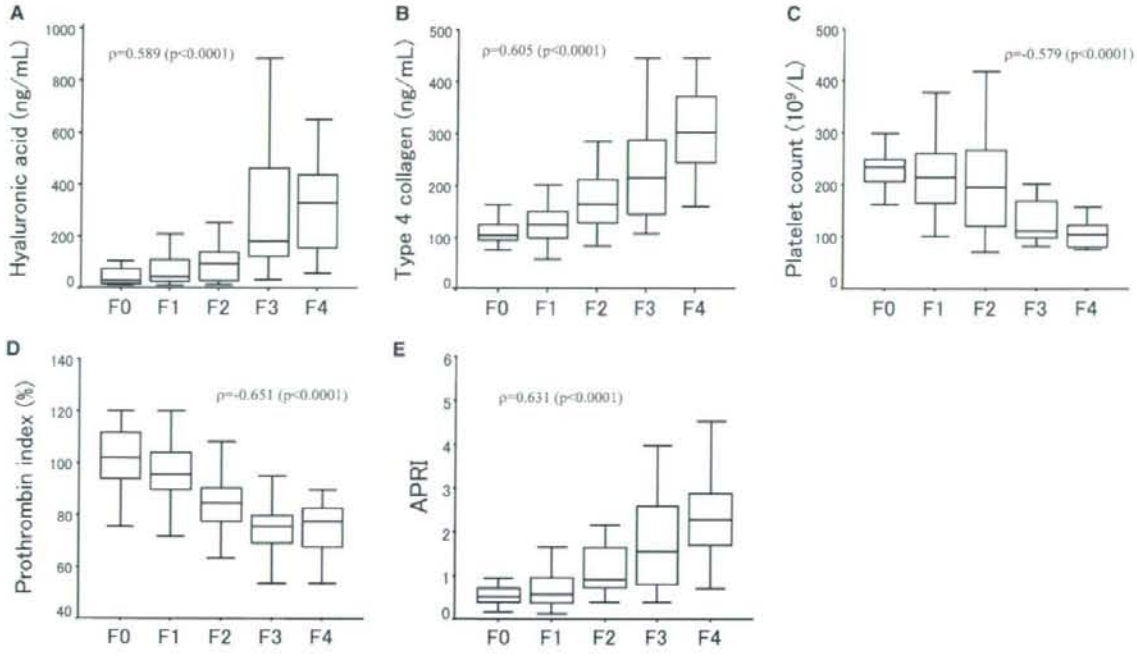

Fig. 3. Box plots of hyaluronic acid (**A**), type 4 collagen (**B**), platelet count (**C**), prothrombin index (**D**), and APRI (**E**) according to METAVIR fibrosis stage in all patients. The lengths of the boxes represent the interquartile range within which 50% of the values were located. The lines in the boxes represent the median values. *Upper and lower error bars* are computed as upper quartile + 1.5* (interquartile range) and lower quartile − 1.5* (interquartile range), respectively

count in all patients with F0, F1, F2, F3, and F4 was 233, 214, 195, 112, and 105 × 10^9/l, respectively. The median prothrombin index in all patients with F0, F1, F2, F3, and F4 was 101.9%, 95.5%, 84.7%, 75.4%, and 77.6%, respectively (Fig. 3A–E). The medial APRI in all patients with F0, F1, F2, F3, and F4 was 0.51, 0.56, 0.91, 1.55, and 2.29, respectively.

Comparison of transient elastography with the hyaluronic acid level, type 4 collagen level, platelet count, prothrombin index, and APRI for the diagnosis of significant fibrosis (F ≥ 2)

ROC curve analysis for predicting significant fibrosis (F ≥ 2) was performed on overall patients, CHC patients, and nonviral CLD patients, respectively (see Fig. 2). The AUROC curve of LSM, hyaluronic acid, type 4 collagen, platelet count, prothrombin index, and APRI was 0.94, 0.77, 0.82, 0.77, 0.85, and 0.83 for overall patients (n = 114), respectively. The highest AUROC curve was obtained by LSM. For patients with CHC (n = 51), the AUROC curve of LSM, hyaluronic acid, type 4 collagen, platelet count, prothrombin index, and APRI was 0.92, 0.81, 0.87, 0.85, 0.85, and 0.92, respectively; thus, LSM and APRI should be nearly the highest AUROC curve. In contrast, in patients with nonviral CLD (n = 52), the AUROC of LSM, hyaluronic acid, type 4 collagen, platelet count, prothrombin index, and APRI was 0.88, 0.72, 0.81, 0.67, 0.81, and 0.77, respectively. Finally, in comparison with the surrogate serum markers, LSM had the highest AUROC and correlated not only with the patients with CHC but also those with nonviral CLD (Fig. 2, Table 2, Table 5).

Discussion

In the present study we examined the ability of transient elastography to predict liver fibrosis in each stage. In nonviral CLD patients, the AUROC curves for the diagnosis of significant fibrosis (F ≥ 2), severe fibrosis (F ≥ 3), and cirrhosis were 0.88, 0.99, and 0.99, respectively (see Table 3). Although it seems to be more efficient to predict severe fibrosis and cirrhosis, it is more important to detect early-stage liver fibrosis from a clinical point of view for preventing developing cirrhosis and its complications. Thus, we compared the ability

Table 5. Diagnostic performance in predicting liver fibrosis (F ≥ 2)

	LSM	Hyaluronic acid	Type 4 collagen	Platelet count	Prothrombin index	APRI
All patients (n = 114)						
AUROC	0.94	0.77	0.82	0.77	0.85	0.83
Optimal cutoff	9.5	125.2	152.5	152.5	87.6	0.77
Sensitivity	0.84	0.61	0.74	0.59	0.78	0.81
Specificity	0.83	0.85	0.80	0.88	0.84	0.75
PPV	0.84	0.82	0.78	0.83	0.85	0.74
NPV	0.91	0.68	0.76	0.67	0.94	0.80
CHC patients (n = 51)						
AUROC	0.92	0.81	0.87	0.85	0.85	0.92
Optimal cutoff	9.5	96.6	128.0	122.0	90.7	0.70
Sensitivity	0.89	0.82	0.89	0.64	0.93	0.89
Specificity	0.83	0.78	0.74	0.96	0.70	0.87
PPV	0.86	0.82	0.80	0.95	0.79	0.89
NPV	0.86	0.78	0.81	0.69	0.89	0.87
Nonviral patients (n = 52)						
AUROC	0.88	0.72	0.81	0.67	0.81	0.77
Optimal cutoff	8.1	146.4	149.5	213.0	79.4	0.77
Sensitivity	0.91	0.48	0.81	0.71	0.62	0.81
Specificity	0.76	0.93	0.76	0.65	0.94	0.65
PPV	0.73	0.83	0.68	0.58	0.76	0.61
NPV	0.92	0.71	0.85	0.77	0.78	0.83

PPV, positive predictive value; NPV, negative predictive value

of transient elastography and surrogate serum markers to predict significant fibrosis (F ≥ 2) in patients with CHC and those with nonviral CLD. The AUROC curve predicting significant fibrosis in patients with CHC and nonviral CLD was 0.92 vs. 0.88 with LSM, 0.81 vs. 0.72 with hyaluronic acid, 0.87 vs. 0.81 with type 4 collagen, 0.85 vs. 0.67 with platelet count, 0.85 vs. 0.81 with prothrombin index, and 0.92 vs. 0.77 with APRI. Although all the indices had higher AUROC curves in patients with CHC than in those with nonviral CLD, LSM consistently had the highest AUROC curve value in comparison with the surrogate serum markers. Ganne-Carrié et al. had reported that alcoholic steatohepatitis and nonalcoholic steatohepatitis (NASH) exhibited higher cutoff values than CHC of 21.5 vs. 10.4 kPa for the diagnosis of cirrhosis.[13] Although our study grouped PBC, NAFLD, and AIH as nonviral CLD, a similar tendency was also observed; nonviral CLD had higher median LSM values than CHC in F = 3 with 27.2 vs. 13.7 kPa (P = 0.019) and F = 4 57.1 vs. 21.9 kPa (P = 0.003), respectively; and the cutoff values of predicting F ≥ 3 (15.8 vs. 10.3 kPa) and F = 4 (21.8 vs. 17.2) were also higher in patients with nonviral CLD than in those with CHC. In addition, the AUROC curve predicting F ≥ 3 (0.99 vs. 0.85) and F = 4 (0.99 vs. 0.90) was also higher in nonviral CLD than in CHC.

Although many surrogate serum markers have been developed to predict liver fibrosis (e.g., APRI, Forns' index, and Fibrotest), the majority of them were invented for patients with CHC infection, whereas few reliable surrogate markers were developed for nonviral CLD.[9,19,22–24] A strong association between liver stiffness and the degree of liver fibrosis has been demonstrated in patients with chronic viral hepatitis.[9,11,13]

However, in patients with nonviral CLD, few studies have been performed concerning the utility of transient elastography for the assessment of liver fibrosis in comparison with patients with viral hepatitis.[10,12,13,15] The diagnosis of CHC and CHB infection is commonly based on the presence of serological HCV-RNA or HBV-DNA. LB is performed to assess the extent of fibrosis (staging) and the activity. On the other hand, despite the invasiveness and expense of the procedure,[6,7] the diagnosis of NASH is essentially based on pathology,[25] and it is more dependent on LB than other types of CLD. In addition, PBC and AIH are difficult to be ruled out without histological diagnosis.[20,26,27] Furthermore, NAFLD has become the most common cause of CLD worldwide.[28,29] However, simple bland steatosis often remains stable for a number of years and will probably never progress in many patients,[30,31] whereas a subset of patients, particularly those with more advanced fibrosis, are at higher risk for progressing to decompensated cirrhosis, portal hypertension, and hepatocellular carcinoma (HCC).[32–34] Therefore, we need to perform LB for the patients with significant liver fibrosis who are at a high risk of progressing liver fibrosis and, eventually, end-stage liver disease. In spite of the lower morbidity of PBC and AIH compared with NAFLD, it is equally necessary for us to evaluate the staging of the progression of liver diseases. Especially, as liver transplantation is the only lifesaving therapy for those with

progressive liver disease, determining of disease stage is critical. In contrast, in patients with nonsignificant liver fibrosis, it may be better to observe them without performing LB.[35]

Transient elastography is a simple, safe, and reproducible method that measures a quantitative physical parameter directly on the liver with no possible interference from extrahepatic disorders.[14] It is able to determine the stiffness of a volume of the liver parenchyma that is approximately 100 times larger than that of a needle biopsy specimen and can be performed in about 95% of patients. Although it is difficult to assess LSM in patients with ascites, narrow intercostal spaces, or a body mass index above 28 kg/m^2,[36] LSM can be employed for most patients with a variety of liver diseases in daily practice.

Although differing from surrogate serum markers in that not all patients can be evaluated for liver stiffness by Fibroscan, our study indicated that it is more efficient for predicting liver fibrosis than the simple biomarkers and extracellular matrix markers used in routine laboratory tests. In the patients with nonviral CLD, advanced liver fibrosis ($F \geq 3$) tended to be accompanied by higher LSM values than CHC. We believe the heterogeneous components of the etiology of liver diseases are accountable for this observation. In patients with NAFLD, the pathological fibrosis initiates from zone 3 perisinusoidal fibrosis with possible additional portal/periportal fibrosis, leading to architectural remodeling.[37] A study comparing METAVIR portal-based scoring originally developed for CHC with a published system for nonalcoholic steatohepatitis demonstrated 47% agreement of fibrosis score; the differences were attributed to lack of detection of zone 3 perisinusoidal fibrosis by METAVIR.[38] Moreover, it is well known that patients with PBC sometimes develop esophageal varices before developing cirrhosis.[39] AIH has not been estimated for liver fibrosis quantitatively such as METAVIR score. Although more weight is given to histological activity than fibrosis,[20] the existence of significant liver fibrosis similarly influences prognosis. In either case, LSM could be different as a result of the etiology leading to hepatic fibrosis. Thus, it seems feasible to accumulate more data of LSM to perform better prediction of liver fibrosis in each etiology.

In conclusion, transient elastography demonstrated a better diagnostic accuracy and correlation with fibrosis stage than the existing serum surrogate markers, suggesting that transient elastography could be used as an alternative to LB for assessment of the liver fibrosis stage not in only patients with CHC but also those with nonviral CLD.

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Analysis of the Entire Nucleotide Sequence of Hepatitis B Causing Consecutive Cases of Fatal Fulminant Hepatitis in Miyagi Prefecture Japan

Futoshi Nagasaki,[1] Yoshiyuki Ueno,[1]* Hirofumi Niitsuma,[1] Jun Inoue,[1] Takayuki Kogure,[1] Koji Fukushima,[1] Koju Kobayashi,[2] and Tooru Shimosegawa[1]

[1]*Division of Gastroenterology, Tohoku University Graduate School of Medicine, Sendai, Japan*
[2]*Tohoku University School of Health Sciences, Comprehensive Research and Education Center for Planning of Drug Development and Clinical Evaluation, Sendai, Japan*

We encountered five consecutive patients with fulminant hepatitis induced by acute hepatitis B virus (HBV) infection in 2000–2001 in Japan. They had not had previous contact each other, and were referred to us from different hospitals. Although a 69-year-old woman could be rescued by intensive internal treatment, the four patients died. We analyzed the partial (nt 278–646) and entire nucleotide sequences of the HBV obtained from them, and their divergences were 0–0.3% and 0–0.2%, respectively. The results suggested that they had been infected with the same HBV isolates. The isolates belonged to genotype B and subgenotype B2 on the phylogenetic tree analysis (AB302942–AB302946). As for the nucleotides sequences of them, previously reported mutations of G1896A, A1762T, and G1764A were present. Amino acid analysis revealed that previously reported Ile97-Leu and Pro130Non-Pro in the core region and Trp28Stop in the precore region were present. As for the entire nucleotide sequences among B2, AB302942 showed low divergences with AF121245 and AB073834 (1.7%), and X97850 from patients with fulminant hepatitis (3.2%). We compared the two consensus nucleotides derived from AB302942 and X97850 (fulminant hepatitis) versus AY121245 and AB073834 (non-fulminant hepatitis), which revealed a difference in nt 1,504 located in the P and X region. Nucleotide 1,504 was C for isolates from fulminant hepatitis and G for non-fulminant hepatitis, and it was recognized among most of the isolates belonging to B2 registered on GenBank. Further studies could disclose the mechanism of severe inflammation of liver that finally leads to fulminant hepatitis. ***J. Med. Virol. 80:967–973, 2008.*** © 2008 Wiley-Liss, Inc.

KEY WORDS: hepatitis B virus; genotype B; subgenotype B2; fulminant hepatitis

INTRODUCTION

Hepatitis B virus (HBV) infection has a wide spectrum of clinical presentations, including self-limited acute hepatitis, fulminant hepatitis, liver cirrhosis and hepatocellular carcinoma [Lee, 1997]. The clinical manifestations of HBV infection are related to interactions between the virus and host immune responses to HBV antigens [Chisari and Ferrari, 1995].

HBV is each characterized according to the genotype based on the comparison of entire genomes, with intergroup divergences of more than 8% [Okamoto et al., 1988]. The distribution of genotypes throughout the world includes eight different genotypes of HBV, named A to H, that have been determined to date [Okamoto et al., 1988; Norder et al., 1992]. Recently, HBV strains have been shown to be composed of subgenotypes [Norder et al., 2004]. For example, genotype B (HBV/B) is divided into five subgroups, B1–B5, according to the countries where they are found [Norder et al., 2004; Nagasaki et al., 2006]. Thus, HBV needs to be examined with regards not only to its genotype but also to its subgenotype.

Clinically, there have been some reports that the outcomes vary according to the HBV genotype or subgenotype. For example, as for HBV/B and genotype C (HBV/C), which are commonly found in Asia, HBV/B has been found to cause HBe-seroconversion more frequently than HBV/C, and chronic infected patients with HBV/B appear to have better prognoses [Kikuchi

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*Correspondence to: Yoshiyuki Ueno, MD, PhD, Division of Gastroenterology, Tohoku University Graduate School of Medicine, Sendai, Japan E-mail: yueno@mail.tains.tohoku.ac.jp

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et al., 2000]. Furthermore, as for HBV/B, B1 is known to show a good clinical prognosis compared with B2–B5.

Fulminant hepatitis is an often fatal complication of acute HBV infection or acute exacerbation of chronic HBV infection. The pathogenesis is not completely understood and it shows high mortality without liver transplantation. Virologically, the association between of the precore stop mutation (G1896A), the core promoter mutations (A1762T and G1764A) and fulminant hepatitis induced by HBV infection has been recognized [Carman et al., 1989; Aritomi et al., 1998; Friedt et al., 1999]. Similarly, an association between the amino acid Ile97Leu and Pro130Non-Pro in the core region and Trp28Stop in the precore region was previously reported [Kosaka et al., 1991; Aye et al., 1994].

We encountered five consecutive patients with fulminant hepatitis induced by acute HBV infection in 2000–2001. They were all residents of Miyagi prefecture in Japan. We investigated entire nucleotide sequences of the HBV detected in their serum samples and compared them with those of previous reports.

METHODS

Patients

The clinical characters of five patients are shown in Table I. The criteria for fulminant hepatitis induced by HBV infection were the development of hepatic encephalopathy, prolongation of the prothrombin time during the course of hepatitis, and immunoglobulin M antibody to hepatitis B core antigen [Perrillo and Aach, 1981]. They were all referred to our hospital from other clinics or hospitals. They were Japanese and had not had contact with known each other. They had never been abroad, had not met nor had with sexual contacts with foreigners, nor were they intravenous drug abusers. None of them had had prior hepatitis. The 66-year-old male patient (FH-3) had been administered medication for hyperlipidemia and hypertension, and the 71-year-old female patient (FH-4) had been administered medication for hypertension.

Thereafter, all but one patient (FH-4) died despite treatment in the intensive care unit due to multiple organ failure, including liver. We obtained serum samples from them and analyzed their HBV-DNA.

None of these five patients were positive for anti-hepatitis C virus antibody or for serum HCV-RNA (RT-PCR assay, Amplicor qualification assay, Roche Japan, Tokyo, Japan), the presence of which could worse the outcome of hepatitis B virus infection [Feray et al., 1993; Sagnelli et al., 2002; Liaw et al., 2004]. In addition, other serum markers related to acute infection of hepatitis A virus, EB virus, and Cytomegalovirus were all negative.

Assays of HBV Related Markers

HBeAg, anti-HBe, and anti-HBc were detected by immunoassays (Abbott Laboratories, N. Chicago, IL).

TABLE I. Clinical Characters and Data of Present Five Patients With Fulminant HBV Infection

Patients	Age	Sex	Onset	Symptom	Admission	Therapy	Outcome (hospital day)	T-Bil (mg/dl)	ALT (IU/L)	PT (%)	HBsAg	anti-HBs	HBeAg (COI)	anti-HBe (%)	anti-HBc (%)	IgM anti-HBc (COI)	HBV-DNA (LGE/ml)
FH-1	69	F	May 29, 2000	Fever, anorexia	June 2, 2000	—	Died (2)	10.2	3,945	8.0	+	−	− (0.57)	+ (98.5)	+ (98.5)	+ (2.81)	5.6
FH-2	71	M	July 26, 2000	Fever	August 3, 2000	PE	Died (44)	16.8	2,715	38.0	+	−	− (0.43)	+ (97.3)	+ (99.0)	+ (3.04)	7.5
FH-3	66	M	October 24, 2000	Fever	November 6, 2000	PE, CHDF, mPLS	Died (2)	13.9	6,950	15.0	+	+	− (0.62)	+ (97.1)	+ (98.9)	+ (2.50)	6.2
FH-4	71	F	September 1, 2000	Epigastralgia	January 4, 2001	—	rescued (50)	9.4	3,380	27.0	+	−	− (0.53)	+ (97.0)	+ (82.7)	+ (2.95)	4.9
FH-5	60	M	December 12, 2000	Fever, malaise	December 6, 2000	PE	Died (4)	4.4	7,884	24.0	+	−	− (0.71)	+ (70.0)	n.t.	+ (2.32)	6.2

HBeAg, anti-HBe, and anti-HBc were detected by immunoassays (positive range, HBeAg ≥1.0; anti-HBe and anti-HBc ≥70.0). IgM anti-HBc was detected by CORE-M-IMx (positive range, COI (cut off index) ≥1.0). Serum HBV-DNA levels were measured by transcription-mediated amplification-hybridization protection assay (TMA-HPA; Chugai Diagnostics, Ltd., Tokyo, Japan). Percent in each parenthesis meant for inhibition percent. For details, please see method section in the text.
F, female; M, male; PE, plasma exchange; CHDF, continuous hemodiafiltration; mPSL, methyl-prednisolone pulse administration; T-Bil, total bilirubin; ALT, alanine aminotransferase; PT, prothrombin time; HBsAg, hepatitis B s antigen; anti-HBs, antibody to hepatitis B s antigen; HBeAg, hepatitis B e antigen; anti-HBe, antibody to hepatitis e antigen; anti-HBc, antibody to hepatitis B c antigen; IgM anti-HBc, immunoglobulin M antibody to hepatitis c antigen; n.t., not tested; COI, cut off index; LGE, logarithm of the genome equivalent.

IgM anti-HBc was detected by CORE-M-IMx (Abbott Laboratories). Serum HBV-DNA levels were measured by transcription-mediated amplification-hybridization protection assay (TMA-HPA; Chugai Diagnostics, Ltd., Tokyo, Japan) with frozen stocked sera as described previously [Sakugawa et al., 2001; Kobayashi et al., 2007]. The results of this TMA-HPA assay were indicated as logarithm of the genome equivalent (LGE)/ml and its measurable range was 3.7–8.7 LGE/ml (equivalent to HBV-DNA $10^{3.7}$–$10^{8.7}$ copies/ml).

Amplification of HBV DNA by Polymerase Chain Reaction (PCR)

Nucleic acids were extracted from 200 μl of serum as described previously [Niitsuma et al., 1995]. For analysis of the entire nucleotide sequence, we divided the entire HBV genome into six overlapping segments and amplified each segment. Extracted DNA was subjected to the first round of PCR with each set of primers. PCR was performed with TaKaRa Ex Taq™ (TaKaRa Co. Ltd., Shiga, Japan) for 35 cycles (consisting of denaturation for 1 min at 93°C, annealing for 1 min at 55°C, and extension for 1 min at 74°C), followed by an extension cycle at 74°C for 8 min. The second round of PCR was carried out for 30 cycles consisting of the same protocol as in the first round.

The primers for the first and the second PCR rounds were as previously reported [Shan et al., 2002].

We used the standard numbering system method in this report, with the numbering of the bases commencing at the cleavage site for the restriction enzyme *Eco*RI in the preS2 region and counting of the full lengths of the 3,215 base pairs.

Nucleotide Sequences of HBV Isolates

We used each set of sequencing primers previously described [Shan et al., 2002]. Direct sequencing of the PCR products was carried out by a fluorescence autosequencer (model 377, PE Japan Applied Biosystems, Chiba, Japan) using a Big Dye Terminator Sequencing Kit (PE Japan Applied Biosystems) according to the manufacturer's instructions.

Phylogenetic Analysis of the Isolated HBV Clones

Six overlapping segments were joined and phylogenetic determination of the sequences of the HBV clone was performed by the neighbor-joining method with the aid of ClustalW (DNA Data Bank of Japan; DDBJ, http://www.ddbj.nig.ac.jp/search/clustalw-j.html).

We compared the present isolated clones with the eight reported HBV clones and confirmed their genotype by phylogenetic analysis. The accession numbers of the clones and genotypes of these HBV sequences used in the analysis were as follows: AB014370 (genotype A); X97850 (genotype B); X75665 (genotype C); J02203 (genotype D); X75664 (genotype E); X75663 (genotype F); AF160501 (genotype G); and AY090457 (genotype H).

RESULTS

Serum Test Findings

All of the patients were IgM class anti-HBc positive, as shown in Table I, and showed severe liver dysfunction, and coagulopathy compatible with fulminant hepatitis.

Entire Genome Sequences Detected From the Present Patients

We determined the entire nucleotide sequences except in one isolate (FH-5). The divergences among the four isolates were 0–0.2% (Table II). As for the partial nucleotide analysis in the HBs region (nt 278–646), all but one isolate were completely matched. Only one isolate (FH-4) demonstrated single different nucleotide.

Phylogenetic Analysis With HBV Entire Genome

We constructed a phylogenetic tree using the present four entire nucleotide sequences and other HBV isolates retrieved from the DNA database (DDBJ/GenBank) as representative genotypes (A, B, C, D, E, F, G, and H) (Fig. 1).

The genotype was B. The HBV/B isolates detected worldwide are divided into five subgenotypes: B1, B2, B3, B4, and B5; the present subgenotype was B2.

Comparison of the Nucleotides and Amino Acids of AB302942 With the Isolates From Previous Reports

Some nucleotide and amino acid mutations related to HBV fulminant hepatitis were previously reported [Carman et al., 1989; Aritomi et al., 1998; Sterneck et al., 1998; Friedt et al., 1999; Yuasa et al., 2000]. As for the nucleotides sequences of AB302942, the mutations of

TABLE II. Percentage Divergences of Entire HBV Nucleotide Sequences Among the Five Isolates From the Present Patients and Other HBV Isolates Registered on GenBank

		Accession number (country)						
Patient	Accession number	AB302942 (Japan)	AB302942 (Japan)	AB302942 (Japan)	AB302942 (Japan)	AF121245 (Vietnam)	AB073834 (Vietnam)	X97850 (China)
FH-1	AB302942	—	0.1	0	0.2	1.7	1.7	3.2
FH-2	AB302943		—	0.1	0.2	1.7	1.7	3.1
FH-3	AB302944			—	0.2	1.7	1.7	3.2
FH-4	AB302945				—	1.7	1.7	3.2